JOURNAL OF BACTERIOLOGY, Feb. 1990, p. 1035–1042
0021-9193/90/021035-08$02.00/0

Vol. 172, No. 2

Analysis of Protein Localization by Use of Gene Fusions with Complementary Properties

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Received 31 July 1989/Accepted 10 November 1989

This report describes a new transposon designed to facilitate the combined use of β-galactosidase and alkaline phosphatase gene fusions in the analysis of protein localization. The transposon, called Tn*lacZ*, is a Tn*5* derivative that permits the generation of gene fusions encoding hybrid proteins carrying β-galactosidase at their C termini. In tests with plasmids, Tn*lacZ* insertions that led to high cellular β-galactosidase activity were restricted to sequences encoding either cytoplasmic proteins or cytoplasmic segments of a membrane protein. The fusion characteristics of Tn*lacZ* are thus complementary to those of Tn*phoA*, a transposon able to generate alkaline phosphatase fusions whose high-activity insertion sites generally correspond to periplasmic sequences. The structure of Tn*lacZ* allows the conversion of a Tn*lacZ* fusion into the corresponding Tn*phoA* fusion (and vice versa) through recombination or in vitro manipulation in a process called fusion switching. Fusion switching was used to generate the following two types of fusions with unusual properties: a low-specific-activity β-galactosidase–alkaline phosphatase gene fusion and two toxic periplasmic-domain serine chemoreceptor–β-galactosidase gene fusions. The generation of both β-galactosidase and alkaline phosphatase fusions at exactly the same site in a protein permits a comparison of the two enzyme activities in evaluating the subcellular location of the site, such as in studies of membrane protein topology. In addition, fusion switching makes it possible to generate gene fusions whose properties should facilitate the isolation of mutants defective in the export or membrane anchoring of different cell envelope proteins.

Experimental strategies based on the use of gene fusions have been of central importance in the genetic analysis of protein export from the cytoplasm. Gene fusions leading to hybrid proteins with β-galactosidase or alkaline phosphatase at their carboxy termini have most frequently been used for such studies in bacteria (15, 23, 31). These two hybrid protein enzymatic activities respond to export from the cytoplasm in reciprocal ways: alkaline phosphatase hybrids require export for activity, whereas β-galactosidase hybrids lose activity if the cell attempts to export them. The two types of hybrids thus function as complementary sensors of the normal subcellular location of a protein or site in a protein (15, 23, 31). These properties have led to the use of such gene fusions to analyze the normal transmembrane topology of cytoplasmic membrane proteins (12, 24, 25).

This paper describes a new transposon for generating *lacZ* translational fusions and presents a method using the transposon for interconverting β-galactosidase and alkaline phosphatase gene fusions. The ability to analyze fusions with β-galactosidase or alkaline phosphatase attached at exactly the same site in a protein should assist in the identification of the normal subcellular location or membrane topology of a protein. Furthermore, this method makes it possible to produce low-activity or potentially toxic fusions that are difficult to generate in one step by transposon insertion. Such gene fusions may be particularly useful in isolating mutants with altered protein localization.

MATERIALS AND METHODS

Bacteria and plasmids. The following *Escherichia coli* K-12 strains were used in this study: CC117 [Δ(*ara leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am)], CC118 [CC117 *recA1*], CC130 [MPh44 (26) *mutD5*], DHB4 [Δ(*ara leu*)*7697* Δ*lacX74* Δ*phoA Pvu*II Δ*malF3 phoR galE galK thi rpsL* (F′ *lacI*^{q} *pro*) (6)], DHB36 [DHB4 F$^-$ *pcnB zad*::Tn*10*], CGSC 6630 [*lacI22* Δ*lacZ58 rpsL135 malA1 xyl-7 mtl-2 thi-1*], X90 [(F′ *lacI*^{q} *pro*) *ara* Δ(*lac pro*) *nalA argE*(Am) *rpoB thi*], CC170 [CC118 containing a chromosomal insertion of Tn*lacZ*], CC311 [(pOxgen::Tn*lacZ*) (17) Δ(*ara leu*)*7697* Δ*lacX74 galE galK rpsL recA1 thi*], LS11 [CC117 Δ*phoA Pvu*II Δ(*phoB phoR*)]. Bacteriophage λ431 (*b221 c*I857 *rex*::Tn*5*) was the gift of R. Isberg. The structures of the following plasmids have been described previously: pBR322 (1), pBR325 (29), pACYC184 (30), pRI122 (16) pJFG5 (14), pI^q, (6), and pUC118 and pUC119 (34). Descriptions of other plasmids are presented in Table 1.

Media and cell growth. The media used have been described previously (27). TYE agar is L agar with 8 g of NaCl per liter. Medium supplements were used at the following concentrations: kanamycin (30 μg/ml), tetracycline (10 to 15 μg/ml), ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), XG (5-bromo-4-chloro-3-indolyl galactoside) (40 μg/ml), and XP (5-bromo-4-chloro-3-indolyl phosphate toluidine salt) (40 μg/ml).

DNA manipulations. Standard DNA preparations and manipulations were used (22). DNA was sequenced by the dideoxynucleotide termination method by using a modified phage T7 DNA polymerase with double-stranded plasmid DNA templates and appropriate oligonucleotide primers (33).

Assays. The alkaline phosphatase and β-galactosidase activities of permeabilized cells were determined as described previously (7, 27). For both assays, cultures grown overnight at ambient temperature in LB were diluted 1/100 into fresh medium and were generally grown further for 2 h at 37°C to bring them into exponential-phase growth at the time of assay. Strains derived from DHB36 were assayed after 4 to 5 h of growth (at an optical density at 600 nm of 0.5 to 1.0).

Construction of Tn*lacZ*. In summary, Tn*lacZ* was made from a derivative of Tn*5* carrying a unique *Xba*I restriction site 52 base pairs (bp) from its left end (Tn*5*$_x$) by inserting *lacZ* sequences at that site and eliminating two nonsense

TABLE 1. Plasmids

Plasmid	Description
pJFG5	pBR322 derivative carrying *tsr* (14)
pCM117	Tn*5*$_x$ inserted in the *bla* gene of pBR322 with IS*50*$_R$ promoter proximal
pCM119	pBR322 *tet*::Tn*lacZ*(Op Am) (aa 17)[a]
pCM130	pACYC184 *cat*::Tn*phoA* (aa 36)[a] deleted of DNA between the first *Xho*I site in Tn*phoA* and the *Sac*I site in pACYC184
pCM134	Made by insertion of Tn*lacZ* into pCM130 approximately 100 bp distal to the *Xho*I-*Sca*I junction followed by deletion of sequences between the *Sal*I sites of Tn*lacZ* and *tet*
pCM137	pCM134 which has recombined between the Tn*phoA* and Tn*lacZ* left ends to generate a *cat-lacZ* fusion
pCM139	pUC118 deleted of sequences between *Eco*O109 and *Hin*dIII sites
pCM140	pUC119 deleted of sequences between *Eco*O109 and *Eco*RI sites
pCM163	pCM140 *lacZ*::Tn*lacZ* (aa9[a] in the polylinker sequence) deleted of DNA between the *Bam*HI site immediately distal to *lacZ* in Tn*lacZ* and the *Bam*HI site in the pCM140 polylinker sequence
pCM163-bl	pCM163 carrying a *bla*::Tn*phoA* fusion, with Tn*phoA* situated promoter distal to the *bla Sca*I site
pCM176	pCM163-bl which has recombined between the left ends of Tn*phoA* and Tn*lacZ* to generate a *lacZ-phoA* fusion
pCM178	Cointegrate recombinant between pCM130 and pCM197 encoding a *cat-lacZ* fusion
pCM179	Cointegrate recombinant between pCM130 and pCM198 encoding a *cat-lacZ* fusion
pCM197	pCM192 (Table 2) deleted of DNA between its two *Xho*I sites
pCM198	pCM193 (Table 2) deleted of DNA between its two *Xho*I sites
pCM255	pJFG5 deleted of DNA between its two *Nde*I sites (3) (an *Nde*I site was not regenerated in this construction)
pCM309	Derived from pCM201 (18) encoding a periplasmic-domain *tsr-phoA* fusion with downstream Tn*lacZ* sequences situated distal to the first *Bst*EII site of Tn*phoA*
pCM310	Analogous in structure to pCM309 but derived from a plasmid with a different periplasmic-domain *tsr-phoA* fusion (pCM203)
pCM321	pCM309 which has recombined between the left ends of Tn*phoA* and Tn*lacZ* to generate a periplasmic-domain *tsr-lacZ* fusion
pCM322	Same as pCM321 but derived from pCM310

[a] C-terminal-most amino acid (aa) encoded by the target gene before Tn*lacZ*- or Tn*phoA*-encoded sequences in gene fusions.

codon sequences in frame with *lacZ* in the Tn*5* left-end sequence. Initially, Tn*5*$_x$ was made by recombination between a plasmid carrying IS*50* with an *Xba*I site near its outside end (pRI122) and λ::Tn*5* (λ431) followed by transposition in a manner analogous to that used in the construction of Tn*phoA*(Op) (see Fig. 1 of reference 23). A plasmid (pCM117) was generated by transposition of Tn*5*$_x$ into the pBR322 *bla* gene with IS*50*$_R$ proximal to the *bla* promoter. A DNA fragment carrying most of *lacZ* derived from pMC1871 (8) by digestion with *Sma*I and *Sal*I was then inserted between the *Xba*I and *Xho*I sites of the IS*50*$_L$ of Tn*5*$_x$ after the *Xba*I end was made blunt by DNA polymerase I Klenow fragment treatment. The *lacZ* insertion step was performed after the right end of Tn*5*$_x$ (from the *Sal*I site to a *Sal*I site in pBR322) had been deleted to eliminate redundant *Xho*I sites; the deleted fragment was replaced after the *lacZ* sequences had been inserted. The resulting transposon carried opal and amber codons in frame with *lacZ* derived from the left end of Tn*5* (Fig. 1). To eliminate these nonsense codons, an insert of the transposon into plasmid pBR325 that generated a *tet-lacZ* fusion was isolated. This gene fusion required opal and amber suppressors to be present in host cells to show maximal β-galactosidase activity. The plasmid was mutagenized by growth in a *mutD* host and screened for derivatives that had lost their dependence on an opal suppressor to show β-galactosidase activity. One such mutant was mutagenized a second time by further growth in a *mutD* strain followed by a screen for derivatives that had lost their requirement for both opal and amber nonsense suppression for high β-galactosidase activity. The sequence of one such derivative showed that both nonsense codons had been eliminated (Fig. 1). Unmutagenized DNA fragments encoding *lacZ* (*Bam*HI-*Bam*HI) and the right end of Tn*5* (from the unique *Sph*I site to the right end) (18) were inserted in place of the corresponding mutagenized fragments to generate Tn*lacZ* (Fig. 1).

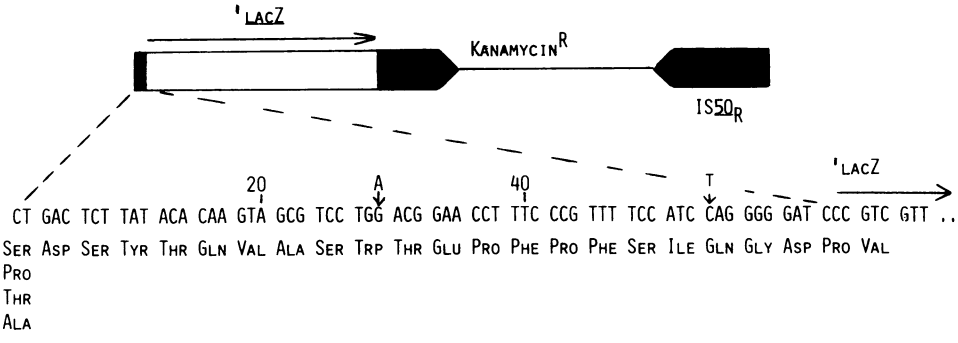

FIG. 1. Structure of Tn*lacZ*. Shown are the nucleotide sequence of the Tn*lacZ* left end and the corresponding amino acid sequence. The two changes eliminating nonsense codon sequences in the transposon end are indicated.

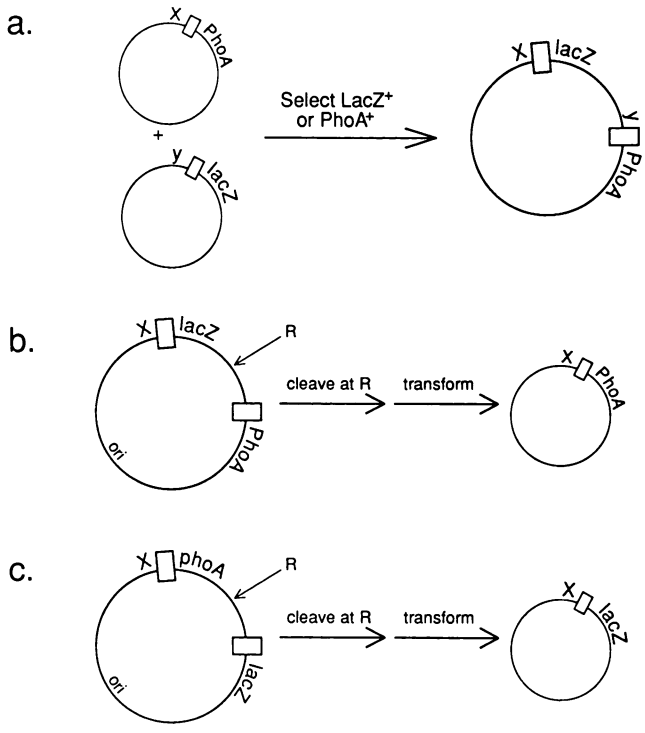

FIG. 2. Methods for isolating recombinant plasmids that have undergone fusion switching. (a) Recombination at the left-end sequences of Tn*lacZ* and Tn*phoA* on different plasmids to yield a cointegrate plasmid in which the two fusions have been interconverted (X-*phoA* → X-*lacZ* and Y-*lacZ* → Y-*phoA*). (b and c) Intramolecular recombination to convert an X-*lacZ* fusion into an X-*phoA* fusion (b) or an X-*phoA* fusion into an X-*lacZ* fusion (c).

Isolation of plasmids carrying Tn*lacZ* insertions. To isolate Tn*lacZ* insertions into a high-copy-number plasmid, the plasmid was first transformed into CC170 or CC311. Single transformant colonies were suspended in 1 ml of LB and plated, either directly or after dilution, onto TYE agar containing antibiotic selective for the plasmid and a high concentration of kanamycin (300 μg/ml) to favor growth of cells carrying plasmids with transposon insertions. Plasmid DNA was isolated by alkaline-sodium dodecyl sulfate extraction of pooled colonies that had grown after 1 or 2 days of incubation at 37°C. The pooled plasmid DNA was transformed into recipient cells (usually CC118 cells), and selection for growth was done on TYE agar containing kanamycin and the β-galactosidase-chromogenic substrate XG. Colonies that grew generally contained plasmids carrying Tn*lacZ* insertions, and the intensity of blue color resulting from XG cleavage reflected the level of β-galactosidase activity due to such plasmids.

Fusion switching. The methods used to isolate plasmids that had undergone the recombination event that leads to fusion switching are diagrammed in Fig. 2.

(i) Method 1. A *cat-phoA* fusion was converted into a *cat-lacZ* fusion by recombination between two plasmids (Fig. 2a). A plasmid carrying the low-activity *cat*::Tn*phoA* fusion (pCM130) and a second plasmid carrying an out-of-frame inactive Tn*lacZ* insert into *tsr* (pCM197 or pCM198) were introduced into strain CGSC 6630. Cells were then streaked onto MacConkey lactose agar medium containing tetracycline and kanamycin. Secondary colonies (papillae) that had grown over the background of cells after 2 days of incubation at 37°C were purified on TYE agar containing tetracycline and kanamycin. Plasmid DNA was isolated from these cells and used to transform CC118 with selection for both tetracycline resistance and kanamycin resistance on TYE agar containing XG. Transformant cells containing cointegrate plasmids of the appropriate structure (pCM178 and pCM179) formed small, dark blue colonies after incubation for 1 day at 37°C.

(ii) Method 2. A second method was used to generate a *cat-lacZ* fusion (Fig. 2c), a *lacZ-phoA* fusion (Fig. 2b), and two periplasmic-domain *tsr-lacZ* fusions (Fig. 2c). To generate the *cat-lacZ* fusion, we first constructed a plasmid (pCM134) carrying a *cat-phoA* fusion and downstream Tn*lacZ* sequences. Plasmid DNA was treated with *Dra*I to cleave the DNA once in the *phoA* gene and then transformed into CC117, with selection for growth on TYE agar containing kanamycin and XG. Approximately 1% of the transformant colonies were blue, and three blue colonies analyzed all contained plasmids which had undergone the *cat-phoA* → *cat-lacZ* conversion. Six white transformant colonies contained plasmids indistinguishable from their parents. These may have resulted from incomplete cleavage by *Dra*I before transformation or ligation of the linear ends after transformation. To generate the *lacZ-phoA* fusion, we constructed a plasmid (pCM163-b1) carrying a *lacZ-lacZ* fusion and a downstream active *bla-phoA* fusion in the same orientation. Plasmid DNA was cleaved at a unique *Sca*I site between the Tn*lacZ* and the Tn*phoA* sequences and transformed into DHB4(pI^q) or X90, with selection for growth on TYE agar containing kanamycin and XP. Cells derived from white transformant colonies were used as a source of plasmid DNA. Of four such plasmids analyzed by restriction mapping, two showed the structure expected for the derivatives that had undergone the desired recombination event. It was determined that the structure of one of these plasmids (pCM176) was appropriate by DNA sequence analysis of the fusion junction. To generate the in-frame periplasmic-domain *tsr-lacZ* fusions, we constructed plasmids (pCM309 and pCM310) carrying periplasmic-domain *tsr-phoA* fusions and downstream Tn*lacZ* sequences with *lacZ* and *phoA* in the same orientation. These plasmids had been deleted of sequences encoding *Bst*EII restriction sites so that they contained single *Bst*EII sites between their *phoA* and *lacZ* sequences. Linear plasmid DNA generated by cleavage with *Bst*EII was transformed into LS11 cells, with selection for growth on TYE agar containing ampicillin and XG. Most transformant colonies were large and white or pale blue, but a few (1 to 5%) tiny blue colonies were also observed. Restriction analysis of representative transformant plasmid DNA showed that the large colonies contained plasmids indistinguishable from the parent, while the tiny blue colonies contained DNA that had apparently undergone the recombination event that leads to fusion switching (Fig. 2c). DNA sequence analysis of two such plasmids (pCM321 and pCM322) confirmed that their fusion junction sequences corresponded to those expected from this recombination event.

RESULTS

Construction of Tn*lacZ*. We constructed a derivative of transposon Tn*5* that can generate gene fusions encoding hybrid proteins with β-galactosidase at their C termini (see Materials and Methods). A DNA fragment encoding all of β-galactosidase except its first eight amino acids was inserted 51 bp from the left end of Tn*5*, and two nonsense

TABLE 2. Properties of Tn*lacZ* gene fusions

Plasmid[a]	Fusion	Position[b]	β-Galactosidase activity (U)
In-frame fusion			
pCM311	*cat-lacZ*	34	6,100
pCM312	*cat-lacZ*	81	4,220
pCM313	*tet-lacZ*	17	4,210
pCM314	*tet-lacZ*	27	199
pCM315	*tet-lacZ*	74	437
pCM191	*tsr-lacZ*	3	2,039
pCM196	*tsr-lacZ*	247	1,487
pCM189	*tsr-lacZ*	261	1,248
pCM187	*tsr-lacZ*	342	1,981
pCM188	*tsr-lacZ*	476	1,781
Out-of-frame fusion			
pCM316	*cat-lacZ*	31 (−1)	10
pCM317	*cat-lacZ*	106 (+1)	4
pCM318	*bla-lacZ*	75 (−1)	22
pCM319	*bla-lacZ*	169 (+1)	13
pCM192	*tsr-lacZ*	57 (+1)	6
pCM193	*tsr-lacZ*	87 (+1)	14
pCM194	*tsr-lacZ*	144 (+1)	4
pCM190	*tsr-lacZ*	178 (+1)	228
pCM195	*tsr-lacZ*	242 (+1)	23

[a] Plasmids consist of Tn*lacZ* insertions into either pBR325 (pCM311 through pCM319), pJFG5 (pCM187 through pCM189), or pCM255 (pCM190, pCM191, pCM192 through pCM194, pCM195, and pCM196).

[b] Most C-terminal amino acid residue encoded by the gene carrying the Tn*lacZ* insert. The relative reading frames of *lacZ* (+1 or −1) in the out-of-frame insertions are also indicated.

codons in frame with *lacZ* in the transposon end were sequentially eliminated. The structure of Tn*lacZ*, including the DNA sequence of the end region with the mutations changing the nonsense codons, is shown in Fig. 1. The leftmost 48-bp sequence of Tn*lacZ* is the same as that of Tn*phoA*, a transposon for generating fusions to alkaline phosphatase (23). Furthermore, hybrid proteins generated by using either of the two transposons are translated by using the same reading frame of this 48-bp sequence.

Fusion of *lacZ* to plasmid pBR325 genes (*cat*, *tet*, and *bla*). The ability of Tn*lacZ* to generate β-galactosidase fusions to proteins directed to different cellular compartments was characterized by using plasmid pBR325. Plasmid pBR325 encodes a cytoplasmic protein (chloramphenicol transacetylase, the *cat* product), a periplasmic protein (β-lactamase, the *bla* product), and a cytoplasmic membrane protein (the tetracycline resistance protein, the *tet* product). We isolated 27 independently derived Tn*lacZ* insertions into the plasmid that led to detectable β-galactosidase activity and loss of one of the three antibiotic resistance activities (see Materials and Methods). This collection consisted of 19 Tn*lacZ* insertions into *cat*, 6 insertions into *tet*, and 2 insertions into *bla*. The *cat* insertion plasmids fell into two classes defined by the level of β-galactosidase activity of the cells carrying them. Cells carrying high-activity *cat-lacZ* insertions (10 plasmids) showed at least 100-times-greater activity than cells carrying low-activity insertions (9 plasmids) (Table 2 and data not shown). Two high-activity insertions analyzed by DNA sequencing had *cat* and *lacZ* positioned in the same translational reading frame, whereas two low-activity insertions had *cat* and *lacZ* positioned out of frame. The β-galactosidase activities of six *tet-lacZ* fusions extended over about a 10-fold range, but each of four of the fusions analyzed by DNA sequencing carried *tet* and *lacZ* sequences in frame (Table 2 and data not shown). Both Tn*lacZ* insertions into *bla* expressed low β-galactosidase activity and had *bla* and *lacZ* sequences out of frame (Table 2). These results show that active β-galactosidase translational fusions to both a cytoplasmic protein and a cytoplasmic membrane protein can be readily generated by in-frame insertion of Tn*lacZ*. However, the results also show that out-of-frame Tn*lacZ* insertion can lead to significant β-galactosidase activity.

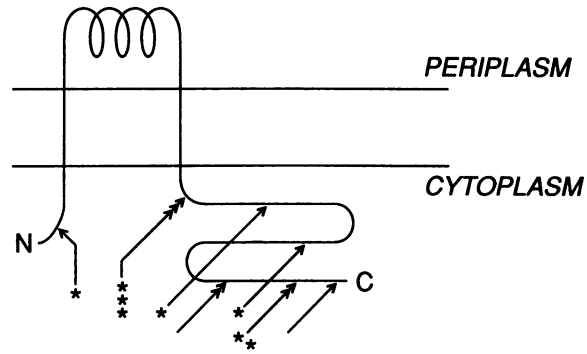

FIG. 3. Serine chemoreceptor–β-galactosidase fusions. The positions of Tn*lacZ* insertions into *tsr* leading to high β-galactosidase activity are shown relative to the presumptive Tsr protein transmembrane topology. Fusion junctions positioned by DNA sequencing are indicated (*); others were positioned by restriction mapping. The first Tsr protein membrane-spanning segment corresponds to amino acids 7 to 25, and the second corresponds to amino acids 192 to 214 (5).

To test further whether in-frame *bla-lacZ* fusions could be generated by Tn*lacZ* insertion, we constructed plasmids (pCM139 and pCM140) that contained only *bla* and the very beginning of *lacZ* as targets for gene fusion (see Materials and Methods). Twelve independently derived insertions of Tn*lacZ* into pCM139 and pCM140 leading to increased β-galactosidase activity in transformant colonies were isolated. Seven of these insertions were situated in the *bla* gene with *bla* and *lacZ* sequences in the same orientation. None of the seven *bla*::Tn*lacZ* insertion plasmids were found to direct the synthesis of detectable hybrid protein in screens of whole-cell protein labeled after brief exposure to [^{35}S]methionine (not shown). This result suggests that the *bla* and *lacZ* sequences were out of frame in these inserts. DNA sequence analysis of one of these Tn*lacZ* inserts (pCM153) confirmed that *bla* and *lacZ* were out of frame (not shown). The inability to isolate in-frame *bla-lacZ* fusions may be due to either toxicity or very low β-galactosidase activity of the corresponding hybrid proteins (31).

Serine chemoreceptor–β-galactosidase (*tsr-lacZ*) fusions. To determine whether the positions of β-galactosidase fusions to cytoplasmic membrane proteins generated by Tn*lacZ* insertion would be correlated with the topology of the membrane protein, we analyzed insertions into a plasmid encoding the serine chemoreceptor (the *tsr* gene product). Biochemical and genetic studies have indicated that this protein contains two transmembrane segments separating a large periplasmic domain from short and long cytoplasmic domains (Fig. 3) (5, 11, 24). Tn*lacZ* insertions were isolated in plasmids carrying the wild-type gene (pJFG5) or a gene deleted of sequences encoding most (277 of 322 residues) of the cytoplasmic C-terminal domain (pCM255). The deleted *tsr* gene of pCM255 encodes a protein with a smaller cytoplasmic region for β-galactosidase fusion. Eleven independently derived high-activity Tn*lacZ* insertions into the plasmids (seven into pJFG5 and four into pCM255) were all situated in sequences corresponding to cytoplasmic regions

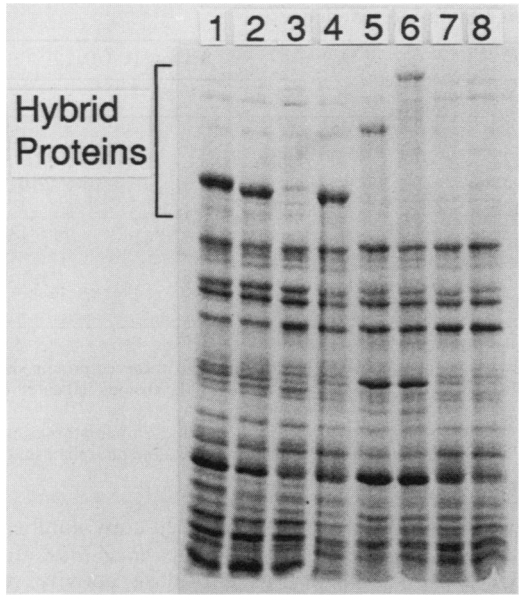

FIG. 4. Hybrid proteins encoded by Tn*lacZ* gene fusions. Whole-cell protein of strain CC118 carrying different fusion plasmids was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: 1, pCM311 (*cat-lacZ*); 2, pCM313 (*tet-lacZ*); 3, pCM314 (*tet-lacZ*); 4, pCM191 (*tsr-lacZ*); 5, pCM196 (*tsr-lacZ*); 6, pCM188 (*tsr-lacZ*); 7, no plasmid; 8, pCM190 (*tsr-lacZ*, out of frame). Cell protein was radioactively labeled by exposure of exponential-phase cultures to [^{35}S]methionine for 2 min, followed by acid precipitation and electrophoresis (23).

of the *tsr* protein, with *tsr* and *lacZ* in the same orientation (Fig. 2 and Table 2). Eight of these insertions were analyzed by DNA sequencing, and each had the *tsr* and *lacZ* sequences in frame. Thirteen lower-activity insertions with junctions situated in either periplasmic (seven isolates) or cytoplasmic (six isolates) domains with *tsr* and *lacZ* in the same orientation were also isolated. Seven of these low-activity insertions (five periplasmic and two cytoplasmic) were analyzed by DNA sequencing, and all had *tsr* and *lacZ* sequences out of frame (Table 2 and data not shown). Thus, although out-of-frame Tn*lacZ* insertions again could show significant β-galactosidase activity, there was a strong correlation between the sites of in-frame Tn*lacZ* insertion and the regions of *tsr* encoding cytoplasmic segments of the protein.

Hybrid protein production (*cat-lacZ*, *tet-lacZ*, and *tsr-lacZ* fusions). Cells carrying representative Tn*lacZ* fusion plasmids directed synthesis of high-molecular-weight hybrid proteins which were easily detectable in whole-cell extracts (Fig. 4). The sizes of the proteins corresponded to those expected from the positions of the corresponding (in-frame) Tn*lacZ* insertions (Table 2), and the hybrid proteins encoded by two of the plasmids tested (pCM191 and pCM192) were precipitated with antibody to β-galactosidase (not shown). Cells carrying a plasmid with an out-of-frame insert of Tn*lacZ* into *tsr*(pCM190) synthesized a small amount of a protein only slightly larger than β-galactosidase itself.

We were surprised to observe a much higher level of hybrid protein production by the shortest *tet-lacZ* fusion (pCM313) than by a somewhat longer fusion (pCM314) (Fig. 4). The increased amount of hybrid protein synthesized by the short fusion corresponds to an unusually high β-galactosidase activity of cells carrying the fusion (Table 2). The increased synthesis of the short *tet-lacZ* fusion is reminiscent of the overproduction of a short *malF-lacZ* hybrid observed previously (12) and may result from the disruption of some structural or regulatory feature limiting expression of the *tet* gene.

Fusion junctions of out-of-frame Tn*lacZ* insertions. DNA sequence analysis of 12 out-of-frame Tn*lacZ* insertions leading to detectable β-galactosidase activity revealed that in most cases (9 of 12), a potential translation initiation codon sequence (ATG, GTG, or TTG) (32) lay within two codons of the site of insertion (Table 2 and data not shown). The β-galactosidase activity observed for such insertions may thus result from translational reinitiation at such sites after termination at a nonsense codon in the transposon end. The finding that cells carrying an out-of-frame *tsr-lacZ* fusion synthesized a protein only slightly larger than β-galactosidase itself (Fig. 4, lane 8) is in accord with this interpretation.

Fusion switching (*cat-phoA* → *cat-lacZ*). The fact that Tn*lacZ* and Tn*phoA* both have the same 48-bp left-end sequence derived from Tn*5* makes it possible to interconvert gene fusions generated by the two transposons through recombination. We have developed two methods to facilitate this process, which is called fusion switching (Fig. 2).

The first method relies on the in vivo selection of recombinants in cells carrying Tn*phoA* and Tn*lacZ* in separate replicons (Fig. 2a). This approach was used to convert a *cat-phoA* fusion (showing low alkaline phosphatase activity) into a *cat-lacZ* fusion (showing high β-galactosidase activity). We first introduced compatible plasmids, one carrying a low-activity *cat-phoA* fusion (pCM130) and the other carrying an inactive insert of Tn*lacZ* (pCM197 or pCM198), into a recombination-proficient *lacZ* mutant (CGSC6630). Lac$^+$ derivatives of such cells were selected as clonal outgrowths on MacConkey lactose agar. In six cases examined, such cells harbored cointegrate plasmids in which recombination at the shared 48-bp transposon sequence had converted the *cat-phoA* fusion into a *cat-lacZ* fusion as determined by restriction mapping (see Materials and Methods). That the structures of two such plasmids (pCM178 and pCM179) were appropriate was further verified by DNA sequence analysis of both fusion junctions of each (not shown).

This first method for fusion switching is limited by the fact that the resultant gene fusion (e.g., *cat-lacZ*) must express sufficient enzymatic activity to permit the selective growth of cells carrying it. Many desirable gene fusions are expected to lead to the synthesis of hybrid proteins that are toxic or show low enzymatic activity and may be difficult to isolate by such a method. We therefore developed a second method for fusion switching which does not require that the switched fusion leads to detectable enzyme activity (Fig. 2b and c). This method takes advantage of the fact that a plasmid rendered linear, such as by restriction enzyme cleavage, is not stably maintained after introduction into cells unless it has been recircularized by a recombination event (10). If the Tn*lacZ* and Tn*phoA* left-end sequences are on each side of the cleavage site of such a plasmid and are in the same orientation, recombination at the end leads to fusion switching. As a test of this method, we constructed a plasmid (pCM134) carrying Tn*lacZ* sequences downstream of a *cat-phoA* fusion with *lacZ* and *phoA* in the same orientation. The structure of the plasmid was such that the 48 bp from the left ends of the transposons was the only known extended sequence identity present on each side of the cleavage site. Plasmid DNA was cleaved at a unique restriction site between the Tn*phoA* and Tn*lacZ* left ends and was trans-

TABLE 3. Properties of gene fusions generated by fusion switching

Fusion	Amino acid position[a]	Protein size (kilodaltons)[b]		Activity (U/OD_{600})[c]	
		Predicted	Observed	β-Gal	AP
cat-phoA	36	52	52		13
cat-lacZ	36	122	124	7,886	
lacZ-phoA	9	49	47.5		9.2 (74)
lacZ-lacZ	9	119	120	1,097 (5,102)	
tsr-phoA	89	58	56		130
tsr-lacZ	89	128	124	<0.5	
tsr-phoA	167	66	68		133
tsr-lacZ	167	136	132	<0.5	

[a] C-terminal-most amino acid of the target gene product before Tn*lacZ*- or Tn*phoA*-encoded residues.

[b] Protein sizes were predicted from the DNA sequence of each fusion junction and the known sequences of the fused proteins. The observed protein size was determined for protein precipitated by antibody to β-galactosidase or alkaline phosphatase from cells carrying plasmids encoding the fusions indicated or, for *tsr-lacZ* hybrids, from whole-cell protein gels.

[c] Assay values shown are for plasmids in DHB36, except for the *cat-phoA* and *cat-lacZ* plasmids, which were in CC118. The values in parentheses are also for plasmids in CC118. DHB36 maintains the plasmids at a lower copy number than does CC118 (21). β-Gal, β-Galactosidase; AP, alkaline phosphatase; OD_{600}, optical density at 600 nm.

formed into recombination-proficient cells (see Materials and Methods). About 1% of the transformant cells expressed high β-galactosidase activity, and each of three plasmids isolated from such transformants carried *cat-lacZ* fusions of the appropriate structure as determined by restriction mapping. One of these plasmids (pCM137) directed synthesis of a hybrid protein of the appropriate size (Table 3) and had the expected fusion junction sequence (not shown).

Other fusion switches (*lacZ-lacZ* → *lacZ-phoA* and *tsr-phoA* → *tsr-lacZ*). We sought to demonstrate the utility of fusion switching by using the method to generate two types of fusions difficult to isolate in one step by transposition. We first attempted to generate a *lacZ-phoA* fusion, a type of fusion found in earlier studies to encode a hybrid protein with low cellular alkaline phosphatase activity (23), presumably due to its cytoplasmic localization. To generate such a fusion by switching, we first isolated a Tn*lacZ* insertion into a plasmid (pCM140) encoding 5 amino acids of β-galactosidase followed by 20 amino acids encoded by a restriction site linker sequence. One insert carried Tn*lacZ* situated in the linker region and regenerated an active β-galactosidase gene (a *lacZ-lacZ* fusion). This *lacZ-lacZ* fusion was converted into the corresponding *lacZ-phoA* fusion after downstream insertion of Tn*phoA* by the method shown in Fig. 2b (see Materials and Methods). Similarly, two different periplasmic-domain *tsr-lacZ* fusions were generated from plasmids carrying periplasmic *tsr-phoA* fusions with a downstream Tn*lacZ* (Fig. 2c) (see Materials and Methods). Cells carrying these periplasmic-domain *tsr-lacZ* fusions formed very small colonies, indicating that production of the corresponding hybrid proteins was toxic. It was verified that the structures of these three gene fusions generated by fusion switching were appropriate by restriction mapping and DNA sequence analysis of each (not shown) and by analysis of hybrid protein production (Table 3).

Other properties of fusions generated by fusion switching. The alkaline phosphatase and β-galactosidase activities of cells carrying gene fusions generated by fusion switching were determined (Table 3). Cells expressing the *cat-lacZ* hybrid protein of pCM137 showed very high β-galactosidase activity, whereas those expressing the corresponding *cat-phoA* hybrid protein (pCM130) showed low alkaline phosphatase activity. This pattern of enzymatic activity for the pair of fusions reflects the cytoplasmic location of chloramphenicol acetyltransferase itself. The relative β-galactosidase and alkaline phosphatase activities of cells carrying *lacZ-phoA* and *lacZ-lacZ* fusion plasmids depended on plasmid copy number (Table 3). At a reduced copy number in a *pcnB* strain (21), cells carrying the *lacZ-lacZ* fusion (pCM163) expressed high β-galactosidase activity, while cells carrying the corresponding *lacZ-phoA* fusion (pCM176) expressed relatively low alkaline phosphatase activity. At high copy number, the *lacZ-phoA* fusion plasmid expressed unexpectedly high alkaline phosphatase activity. We suspect that this alkaline phosphatase activity may be largely a consequence of cell lysis (M. Lee and C. Manoil, unpublished results).

Cells carrying periplasmic-domain serine chemoreceptor fusions exhibited properties that were the reciprocal of those of cells carrying fusions to the two cytoplasmic proteins, i.e., relatively high alkaline phosphatase activities and low β-galactosidase activities. Enzyme assays were done with cells that maintain these plasmids at a reduced copy number; at high copy number, cultures of cells carrying the periplasmic-domain *tsr-lacZ* fusion plasmids were extremely slow growing and were commonly overgrown by faster-growing $LacZ^-$ variants.

DISCUSSION

This paper describes the construction and properties of Tn*lacZ*, a transposon Tn*5* derivative that functions to generate gene fusions encoding hybrid proteins with C-terminal β-galactosidase. Tn*lacZ* was designed to be used in conjunction with Tn*phoA*, a transposon for generating hybrid proteins with alkaline phosphatase at their C termini (23). The two transposons show reciprocal fusion characteristics. High-activity Tn*phoA* insertions correspond to exported domains of cell envelope proteins (6, 24), whereas high-activity Tn*lacZ* insertions correspond to cytoplasmic sites. In addition, the structure of Tn*lacZ* makes it possible to interconvert *lacZ* and *phoA* fusions so that the activities of β-galactosidase and alkaline phosphatase individually fused at a particular site can be compared. The use of Tn*lacZ* should aid in the evaluation of whether a protein or segment of a protein is normally situated in the cytoplasm. The combined use of Tn*lacZ* and Tn*phoA* fusions should also facilitate the isolation of mutants exhibiting altered subcellular localization of exported proteins.

We characterized the gene fusion behavior of Tn*lacZ* by using insertions into multicopy plasmids encoding proteins directed to different subcellular compartments. These studies found that hybrid proteins containing β-galactosidase attached to cytoplasmic proteins (chloramphenicol acetyl-

transferase and β-galactosidase) and cytoplasmic membrane proteins (the tetracycline resistance protein and the *tsr* serine chemoreceptor) could be readily generated by Tn*lacZ* insertion. Cells producing the hybrid proteins showed high β-galactosidase activity, and the hybrid proteins were detected by electrophoresis of whole-cell protein extracts and after precipitation with antibody to β-galactosidase. In contrast, it was not possible to generate gene fusions encoding hybrid proteins containing β-galactosidase attached to a periplasmic protein (β-lactamase). The inability to generate such fusions is presumably due to either toxicity or low enzymatic specific activity of β-galactosidase that the cell attempts to export through the cytoplasmic membrane (31).

A detailed analysis of β-galactosidase fusions to a cytoplasmic membrane protein of well-established topology (the *tsr* protein) showed that highly active fusion sites were situated in the cytoplasmic regions of the protein. A correspondence between β-galactosidase hybrid protein activity and membrane protein topology was first observed for a set of *malF-lacZ* fusions generated by a combination of in vitro and genetic methods (12) and was also observed for λp*lac*Mu-generated fusions to *tsr* (14). Our results imply that the analysis of Tn*lacZ* fusions may also help to identify cytoplasmic regions in studies of membrane protein topology.

In addition to in-frame high-activity Tn*lacZ* inserts leading to hybrid proteins, a number of out-of-frame insertions showing detectable β-galactosidase activity were obtained in these studies. Such insertions showed as much as 10% of the activity of in-frame insertions into the same genes (Table 2). In most cases, potential translation initiation codons were found in frame with *lacZ* in the target gene close to the point of Tn*lacZ* insertion. This finding suggests that the β-galactosidase activity observed for these insertions results from translation reinitiation at such codons. In one case that was analyzed, it appeared that a hybrid protein of the size expected from such translational reinitiation was detected. Out-of-frame *lacZ* fusions showing detectable β-galactosidase activity have been previously observed with other fusion systems (8). Out-of-frame Tn*lacZ* insertions can generally be avoided by analyzing only fusions exhibiting substantial β-galactosidase activity.

The most novel feature of Tn*lacZ* as a gene fusion transposon is that its structure allows the conversion of a *lacZ* fusion into the corresponding *phoA* fusion (and vice versa) through recombination in a process called fusion switching. I developed two methods to recover plasmids which had undergone the appropriate homologous recombination event in vivo and recently constructed a Tn*phoA* derivative to allow fusion switching by using in vitro methods (unpublished results). I used fusion switching to isolate two types of fusions with unusual properties. One of these types, a *lacZ-phoA* fusion, encoded a hybrid protein with relatively low alkaline phosphatase specific activity, presumably due to lack of export. The other type, periplasmic-domain *tsr-lacZ* fusions, expressed hybrid proteins with low β-galactosidase specific activities that were extremely toxic when made in large amounts. These constructions illustrate the fact that fusion switching can be used to generate fusions with properties (such as low enzymatic activity and cellular toxicity) that would render them difficult to isolate in a single step by transposition.

I envision a number of uses for Tn*lacZ*. Tn*lacZ* should be a valuable supplement to existing transposons (3) for generating β-galactosidase hybrid proteins in the study of biological processes that do not necessarily involve exported proteins (31). Tn*lacZ* may be especially useful in the analysis of bacteria other than *E. coli* and its close relatives, since, being a Tn*5* derivative, it should transpose in a broad range of gram-negative bacteria (4). A Tn*5* derivative for generating *lacZ* translational fusions was independently constructed by Krebs and Reznikoff (19). This derivative differs from Tn*lacZ* in that it carries *lacY* as well as *lacZ*, has a shorter left-end sequence derived from Tn*5*, and encodes resistance to tetracycline rather than to kanamycin.

Tn*lacZ* was designed primarily to facilitate the combined use of β-galactosidase and alkaline phosphatase fusions in the study of proteins exported from the cytoplasm. Alkaline phosphatase fusions generated by Tn*phoA* insertion have been used to analyze cytoplasmic membrane protein topology, since the sites of high-activity fusions usually correspond to the periplasmic domains of such proteins (24, 25). In such studies, cytoplasmic sites of fusion generally lead to low alkaline phosphatase activity and can be difficult to isolate by transposon insertion. Fusions at cytoplasmic sites must therefore often be constructed in vitro after their positions in the protein sequence have been inferred (6). The use of Tn*lacZ* fusions provides a simple alternative means to identify such cytoplasmic sites, since the sites of highly active β-galactosidase fusions generated by using Tn*lacZ* appear to correspond to cytoplasmic domains of cytoplasmic membrane proteins (12, 25). One advantage to using Tn*lacZ* rather than other transposons that generate *lacZ* fusions in such studies is that the Tn*lacZ* and Tn*phoA* fusion junction sequences are nearly identical, largely eliminating complications that different junction sequences might introduce in comparisons of the two types of fusions. Furthermore, the technique of fusion switching makes it possible to analyze the activities of both alkaline phosphatase and β-galactosidase fused at a particular site in a membrane protein. This comparison of the two fusion activities may be particularly valuable in helping to characterize exceptional sites at which fusion protein activity is not directly correlated with topology, such as when the fusion junction falls within and disrupts a sequence acting as a topogenic determinant (6).

The comparison of the enzymatic activities of alkaline phosphatase and β-galactosidase fused at a particular site of a protein should also help in evaluating the normal subcellular location of the product of a gene cloned into a plasmid. For a site in a cytoplasmic protein, fusion of β-galactosidase should normally generate a protein with a high cellular specific activity, whereas fusion of alkaline phosphatase should normally yield a hybrid protein with a low cellular specific activity. Fusions to periplasmic proteins should normally show the opposite properties. The comparison of the two fusion types should thus help distinguish cases in which the high-level production of a cytoplasmic alkaline phosphatase hybrid gives significant alkaline phosphatase activity (23) (Table 3).

The combined use of Tn*lacZ* and Tn*phoA* fusions also provides systematic methods to isolate mutants in which the localization of exported proteins is changed. To isolate mutants blocked in the export of a periplasmic protein or periplasmic domain of a cytoplasmic membrane protein, a high-activity alkaline phosphatase fusion to the protein or domain can first be isolated by Tn*phoA* insertion. The fusion can then be converted into the corresponding *lacZ* fusion by fusion switching. β-Galactosidase hybrids that cells attempt to export generally show low β-galactosidase enzymatic activity and are toxic if made in large amounts. Either of these properties can allow the selection of export-deficient mutants (28, 31). Conversely, the cytoplasmic domains of

cytoplasmic membrane proteins can be fused to β-galactosidase by using Tn*lacZ* to yield high-activity hybrid proteins. Such fusions can be converted into the corresponding *phoA* fusions, which are expected to show low activity because of the sequestering of their alkaline phosphatase moieties in the cytoplasm. Selection of cells showing increased alkaline phosphatase activity should yield mutants with decreased cytoplasmic anchoring of such domains. The analysis of mutants isolated by using either of these two approaches may help identify sequences in exported proteins that direct their localization as well as to identify cell components required for the functioning of such sequences.

A growing variety of gene fusions generated by transposition and in vitro methods can be used to characterize and manipulate genes (3). For example, there exist Tn*5* derivatives for generating transcriptional fusions to the genes for β-galactosidase (20) and neomycin phosphotransferase (2), for activating transcription of DNA adjacent to insertion sites (9), for simplified cloning of DNA adjacent to insertion sites (13), and for mobilizing conjugational transfer from the site of insertion (35). The ability to interconvert different gene fusions should, in general, greatly increase the versatility of experimental approaches that make use of gene fusions to analyze biological processes.

ACKNOWLEDGMENTS

I acknowledge helpful discussions with C. Lee, S. Lory, and L. Seligman.

This work was supported by Public Health Service grant GM36896 from the National Institutes of Health and by grant DMB-8911278 from the National Science Foundation.

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