Expression of Lactose Permease in Contiguous Fragments as a Probe for Membrane-Spanning Domains

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ABSTRACT: The lactose permease of *Escherichia coli* is a membrane transport protein containing 12 transmembrane hydrophobic domains connected by hydrophilic loops. Coexpression of *lacY* gene fragments encoding contiguous polypeptides corresponding to the first and second halves of the permease [Bibi, E., & Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 4325–4329] or the first two transmembrane domains and the remainder of the molecule [Wrubel, W., Stochaj, U., Sonnewald, U., Theres, C., & Ehring, R. (1990) *J. Bacteriol. 172,* 5374–5381] leads to active lactose transport. It is shown here that contiguous permease fragments with discontinuities in loop 1 (periplasmic), loop 6 (cytoplasmic), or loop 7 (periplasmic) exhibit transport activity; however, fragments with discontinuities in transmembrane domains III or VII fail to do so. The results are consistent with the interpretation that contiguous permease fragments with discontinuities in hydrophilic loops form functional duplexes, while fragments with discontinuities in transmembrane α-helical domains do not. On the basis of this notion, a series of contiguous, nonoverlapping permease fragments with discontinuities at various positions in loop 6, putative helix VII, and loop 7 were coexpressed to approximate the boundaries of putative transmembrane domain VII. Contiguous fragments with a discontinuity between Leu222 and Trp223 or between Gly254 and Glu255 are functional, but fragments with a discontinuity between Cys234 and Thr235, between Gln241 and Gln242, or between Phe247 and Thr248 are inactive. Therefore, it is likely that Leu222 and Gly254 are located in hydrophilic loops 6 and 7, respectively, while Cys234, Gln241, and Phe247 are probably located within transmembrane domain VII. These and other results are consistent with a modified secondary-structure model of lactose permease in which Asp237 and Asp240 are contained within transmembrane domain VII rather than periplasmic loop 7.

The lactose (lac)[1] permease of *Escherichia coli* catalyzes the coupled stoichiometric translocation of β-galactosides with H^+ [i.e., lactose/H^+ symport; reviewed in Kaback (1989, 1992), Kaback et al. (1993), and Poolman and Konings (1993)]. Encoded by the *lacY* gene which has been cloned and sequenced (Teather et al., 1978; Büchel et al., 1980), this hydrophobic polytopic cytoplasmic membrane protein has been solubilized from the membrane, purified to homogeneity (Newman et al., 1981; Foster et al., 1982; Viitanen et al., 1986; Wright et al., 1986), reconstituted into proteoliposomes (Newman & Wilson, 1980), and shown to catalyze β-galactoside translocation with turnover numbers comparable to those observed *in vivo* (Matsushita et al., 1983; Viitanen et al., 1984) and is probably functional as a monomer (Vogel et al., 1985; Costello et al., 1987; Sahin-Toth et al., 1994). On the basis of circular dichroic measurements and hydropathy analysis of the primary amino acid sequence (Foster et al., 1983), a secondary structure with 12 hydrophobic domains in α-helical conformation that span the membrane in zigzag fashion connected by short hydrophilic segments ("loops") was postulated (Figure 1). Evidence consistent with the general features of the model and demonstrating that the N and C termini (hydrophilic domains 1 and 13, respectively), as well as hydrophilic domains 5 and 7, are on the cytoplasmic surface of the membrane has been derived from other spectroscopic measurements (Dornmair et al., 1985), chemical modification (Page & Rosenbusch, 1988), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), and immunological studies (Carrasco et al., 1982, 1984a,b; Seckler et al., 1983, 1986; Seckler & Wright, 1984; Herzlinger et al., 1984, 1985; Danho et al., 1985). Moreover, Calamia and Manoil (1990) have provided unequivocal support for the topological predictions of the 12-helix model by analyzing an extensive series of lac permease–alkaline phosphatase (*lacY–phoA*) fusion proteins and demonstrated that approximately half a transmembrane domain is required to translocate alkaline phosphatase across the membrane.

Bibi and Kaback (1990) restricted the *lacY* gene into two approximately equal-size fragments that were subcloned individually or together under separate *lac* operator/promoters. Under these conditions, lac permease is expressed in two portions (Figure 1): (i) the N terminus, the first six putative transmembrane helices and most of putative loop 6 (N_6) and (ii) the last six transmembrane helices and the C terminus (C_6). Cells expressing both fragments catalyze active lactose transport, while cells expressing either half of the permease independently do not. Since intact permease is completely absent from the membrane of cells expressing *lacY* fragments either individually or together, transport activity must result from association between independently synthesized pieces of lac permease. When the gene fragments are expressed individually, the N-terminal portion of the permease is observed sporadically and the C-terminal portion is not observed. When

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[1] Abbreviations: lac, lactose; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP_i, potassium phosphate; RE, restriction endonuclease; ECL, enhanced chemiluminescence; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase.

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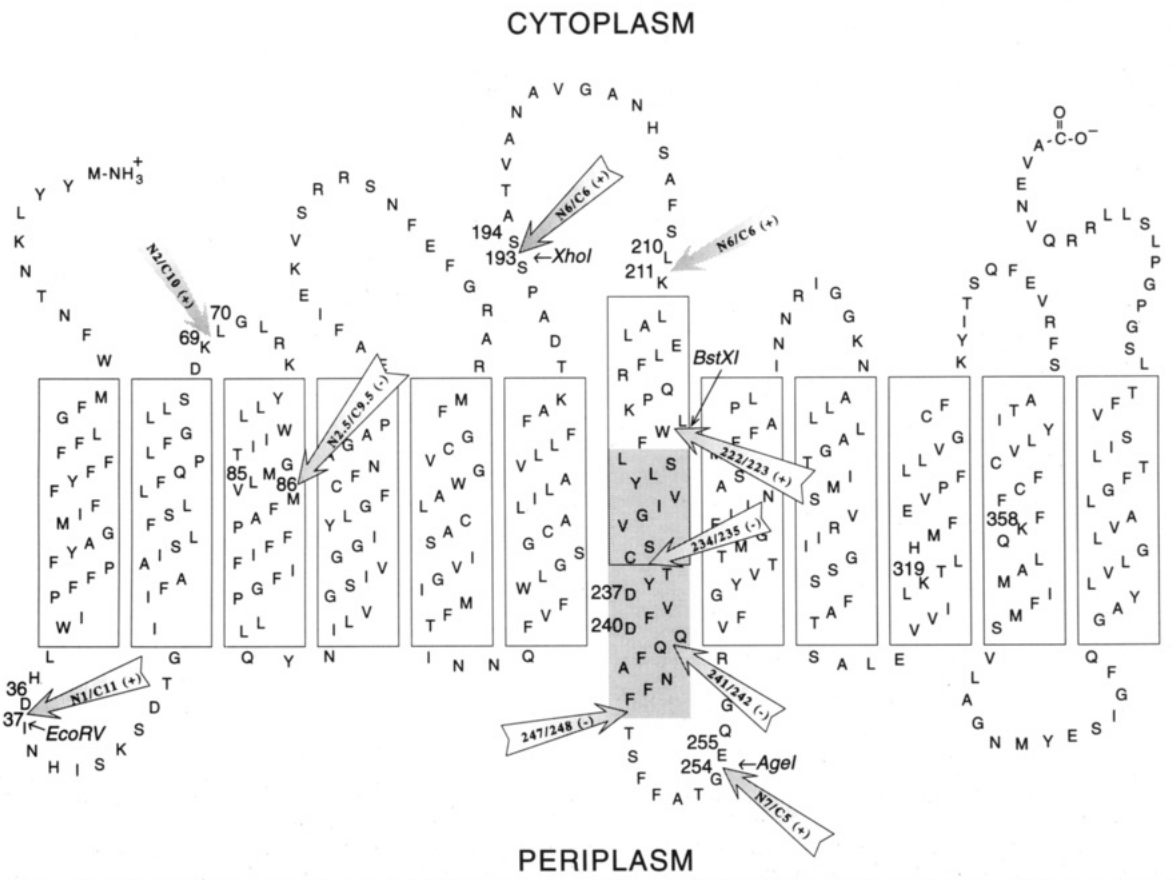

FIGURE 1: Putative secondary-structure model of lactose permease showing nonoverlapping contiguous permease fragments and the transport activity of coexpressed fragments. The model is based on the hydropathy plot of the primary sequence of the permease (Foster et al., 1983) and the modification suggested by King et al. (1991). The single-letter amino acid code is used, 12 hydrophobic putative transmembrane helices are shown in boxes, and the shaded box represents the modified model. Shaded arrows with outlines indicate sites of discontinuity, and the plus or minus signs within the arrows indicate transport activity when the two fragments are coexpressed. Constructs denoted by shaded arrows without outlines (N_2/C_{10} and N_6/C_6) were described previously [Wrubel et al. (1990) and Bibi and Kaback (1990), respectively].

the gene fragments are expressed together, however, polypeptides identified as the N- and C-terminal moieties of the permease are found in the membrane. The results are consistent with the conclusion that the N- or C-terminal halves of lac permease are proteolyzed when synthesized independently and that association between the two complementing polypeptides leads to a more stable, catalytically active complex. At about the same time, Wrubel et al. (1990) demonstrated that coexpression of independently cloned fragments of the *lacY* gene encoding N_2 and C_{10} also form stable duplexes in the membrane which interact to form functional permease, while expression of the fragments by themselves yields polypeptides that are relatively unstable and exhibit no transport activity. Other transmembrane proteins that also exhibit functional complementation from association of nonoverlapping but contiguous inactive fragments are bacteriorhodopsin (Liao et al., 1984; Popot et al., 1987), the β_2-adrenergic receptor (Kobilka et al., 1988), a voltage-dependent Na^+ channel (Stuhmer et al., 1989), the yeast a-factor transporter STE6 (Berkower & Michaelis, 1991), adenylate cyclase from brain (Tang et al., 1991), and the erythroid glucose transporter (G. D. Holman, personal communication).[2]

In this paper, we provide evidence consistent with the idea that contiguous, nonoverlapping permease fragments with discontinuities in either cytoplasmic or periplasmic loops catalyze active transport, while fragments with discontinuities in transmembrane domains do not. Evidence is also presented demonstrating that the coexpressed permease fragments stabilize each other against proteolysis. The approach is then used to obtain evidence indicating that transmembrane domain VII encompasses Asp237 and Asp240 (King et al., 1991; Sahin-Toth et al., 1992; Lee et al., 1992; Dunten et al., 1993; Sahin-Toth & Kaback, 1993).

MATERIALS AND METHODS

Materials [1-^{14}C]Lactose, L-[^{35}S]methionine, [^{35}S]dATPαS and protein A-conjugated horseradish peroxidase, enhanced chemiluminescence (ECL) detection kits were purchased from Amersham (Arlington Heights, IL). Deoxyoligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Site-directed rabbit antiserum against C-terminal dodecapeptide of lac permease (Carrasco et al., 1984a) was prepared by BabCo (Richmond, CA). Restriction endonucleases (REs), Klenow DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs (Beverly, MA). Isopropyl 1-thio-β-D-galactopyranoside (IPTG), calf intestine alkaline phosphatase, *Thermus aquaticus* (Taq) polymerase, and associated polymerase chain reaction (PCR) reagents were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Sequenase (modified T7 DNA polymerase) and Sequenase reaction kits were from United States Biochemical (Cleveland, OH). GeneClean glassmilk DNA purification kits from Bio101 (La Jolla, CA) were used to remove DNA from agarose. Magic Mini and Maxi Prep kits from Promega (Madison, WI) were used to prepare plasmid DNA. Poly(vinylidene difluoride) (PVDF)

[2] Cope, D. L., Holman, G. D., & Wolstenholme, A. J. (1993) *2nd IUBMB Conference on Biochemistry of Cell Membranes Abs.*, p 337.

Table 1: DNA Sequences of Oligodeoxynucleotides Used for Constructing Fragmented *LacY* Genes

construct	oligo name	oligo sequence[a]
N_1/C_{11}	sense +0	TAGCACCAGGCGTTTAAGGGCACCAAT
N_6/N_6	sense +1	GTAGCACCAGGCGTTTAAGGGCACCAAT
	sense +2	CGTAGCACCAGGCGTTTAAGGGCACCAAT
	antisense +0	GCATTATTCCTTTCCTAGGTAC
	antisense +1	GCATTATTCCTTTCCTAGGTACA
	antisense +2	GCATTATTCCTTTCCTAGGTACAT
$N_{2.5}/C_{9.5}$	H3-SD antisense	TAATGGCCGTACAATCACATTCGAAATTCCTTTCCTAGGTAC
	H3-SD sense	TAAGCTTTAAGGAAAGGATCCATGTTTGCGCCGTTCTTT
	outside sense	ATCAATCATATCAGC
	outside antisense	GCCTACAAACCGTCACAACCGACC
Leu222/Trp223	*Bst*XI sense	GTAACGCGTAAGGAAATGGATTTATGAAACT
	*Bst*XI antisense	TTGACATTGCGCATTCCTTTACCTAATACT
Cys234/Thr235	sense	TAAAAGCTTGGATCCAGGATATATACATATGACCTACGATGTTTTTGACCAAC
	antisense	CATACAATAACCGCAAAGGACGATTTTCGAACCTAGGTCCTATATATGTATAC
Gln241/Gln242	sense	TGAAGCTTCCATGGATCCAGGATATATACATATGCAGTTTGCTAATTTCTTTACTTC
	antisense	GTGGATGCTACAAAAACTGGTTACTTCGAAGGTACCTAGGTCCTATATATGTATAC
Phe247/Thr248	sense	TGAAGCTTCCATGGATCCAGGATATATACATATGACTTCGTTCTTTGCTACCGGTG
	antisense	GTTGTCAAACGATTAAAGAAAACTTCGAAGGTACCTAGGTCCTATATATGTATAC
N_7/C_5	sense	GCTAATACCGGTTAAGCTTAGGAATCCATTATGGAACAGGGTACCCGCGTATTTGGCTAC
	antisense	CAAGTGCGAACAGCTGGGCCCGGGCGAAAGG

[a] Sequences of mutagenic primers are presented in 5′→3′ order.

membranes were from Schleicher and Schuell (Keene, NH), and 0.7 μM glass microfiber filters (GF/F) were from Whatman (Maidstone, England). Prestained molecular weight markers were obtained from Bio-Rad (Richmond, CA). All other materials were of reagent grade and obtained from commercial sources.

Bacterial Strains. The following strains of *E. coli* K-12 were used: T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*),*rpsL*,*met*$^-$,*thr*$^-$,*recA*, *hsdM*,*hsdR*/*F*′,*lacI*^{q}*O*$^+$*Z*^{U118}(*Y*$^+$*A*$^+$)] (Teather et al., 1980) and HB101 [*hsdS20* (r^-_B, m^-_B), *recA13*,*ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(*Sm*^{r}), *xyl-5*, *mtl-1*, *supE44*, λ$^-$/F$^-$] (Boyer & Roulland-Dussoix, 1969).

Construction of Split LacY Genes. Three procedures were used to obtain split *lacY* mutants: insertion of the chloramphenicol acetyltransferase (CAT) gene, linker insertion, or PCR mutagenesis. Table 1 shows eight mutant constructs and the sequences of the sense and antisense mutagenic oligonucleotides.

(1) Asp36/Ile37 (N_1/C_{11}) and Ser193/Ser194 (N_6/C_6). A unique insert (Figure 2) containing the CAT gene as a selection marker was created and termed the "CAT sandwich". It has a stop codon (TAG) that can be placed in frame with the selection of the appropriate nucleotide overhang, the CAT gene in the reverse orientation relative to the rest of the insert, a T7 promoter, the *lac* promoter/operator, a ribosome binding (Shine–Delgarno) sequence (Keng et al., 1982), and a start codon (ATG) that can be placed in frame with the selection of the appropriate nucleotide overhang. The CAT sandwich was obtained by PCR amplification of plasmid pT7-5/MCAT (Figure 2). The plasmid pT7-5/MCAT was derived from pT7-5/cassette *lacY* (EMBL-X56095). The CAT gene from pACYC184 was placed immediately proximal to the T7 promoter in reverse orientation relative to the *lacY* gene. PCR amplification of the CAT sandwich was performed in 100 μL total volume with a light mineral oil overlay. A three-temperature protocol was used that included the following: 94 °C for 1 min, a 1.5-min ramp to 37 °C, 37 °C for 1 min, and a rapid step to 72 °C for 1 min. The protocol was repeated 30–50 times. The oligonucleotide primers were phosphorylated using T4 polynucleotide kinase. The template DNA (pT7-5/MCAT) concentration was 1 ng/μL, and 50 pmol of each phosphorylated primer was used. A set of six oligonucleotide (Figure 2), three antisense and three sense primers, with 0, +1, and +2 nucleotide overhangs were synthesized to make all of the possible framing permutations. DNA encoding N_1/C_{11} was prepared by linearizing the pT7-5/cassette *lacY* with *Eco*RV (which leaves blunt ends and requires no nucleotide overhangs for framing). For N_6/C_6, a unique *Xho*I site followed by Klenow treatment to create blunt ends (+2 and +0 nucleotides overhangs, respectively) was used. After the linearized pT7-5/cassette *lacY* was treated with calf intestine alkaline phosphatase to prevent religation of the DNA, the CAT sandwich insert was ligated into the plasmid.

(2) Gly254/Glu255 (N_7/C_5), Val85/Met86 ($N_{2.5}/C_{9.5}$), Cys234/Thr235, Gln241/Gln242, and Phe247/Thr248. N_7/C_5 was created by PCR mutagenesis using a sense mutagenic primer containing *Age*I, termination codon, a Shine–Delgarno sequence, a start codon with the cassette *lacY* sequence after *age*I, and an antisense primer complementary to the downstream portion of *lacY* gene (Table 1). A two-stage PCR protocol was used to create $N_{2.5}/C_{9.5}$, Cys234/Thr235, Gln241/Gln242, and Phe247/Thr248. Two mutagenic oligonucleotides with a complementary region encoding the termination codon, the ribosome binding sequence, initiation codon, and distinct sequences complementary to the insertion site of the cassette *lacY* gene were synthesized (Table 1). Two independent PCR protocols were carried out using a modified pT7-5/cassette *lacY* plasmid (template DNA) that was devoid of a ribosome binding sequence and *Bam*HI site to prevent possible annealing of the mutagenic oligonucleotides to this region during amplification. Each mutagenic oligonucleotide was paired with a second oligonucleotide (outside primer) designed to anneal upstream or downstream depending upon the orientation of the mutagenic oligonucleotide (sense or antisense). Roughly 150 bp of DNA was amplified in each reaction, and the resulting DNA fragments each contained a unique RE site for convenient digestion and ligation. The two PCR products were extracted with phenol/chloroform

and precipitated with ethanol. Each product was combined to form the template DNA for a second PCR amplification using the two outside primers. The resulting PCR product (≈300 bp) was extracted and precipitated as described and then digested with the two unique REs. The mutant DNA fragment was ligated into pT7-5/cassette *lacY* devoid of this DNA fragment created by digestion with the same two REs.

(3) Leu222/Trp223. A linker adaptor containing appropriate ends for annealing, a stop codon (TAA), a ribosome binding sequence, and a start codon (ATG) was ligated into the *Bst*XI-linearized pT7-5/cassette *lacY* which had been dephosphorylated with alkaline phosphatase. Both antisense and sense oligonucleotide strands (Table 1) were phosphorylated with T4 polynucleotide kinase and allowed to anneal to create the linker adaptor for ligation into the linearized plasmid.

Characterization of Constructs. Plasmids encoding discontinuous lac permeases were transformed into *E. coli* HB101 (Z^+Y^-), and the transformants were plated on MacConkey indicator medium containing 25 mM lactose. For the CAT sandwich constructs (N_1/C_{11} and N_6/C_6), colonies harboring plasmid with the appropriate insert were identified by growth in the presence of chloramphenicol (50 μg/mL). Selected colonies were grown overnight in Luria broth, and plasmid DNA was isolated by alkaline lysis or Magic Minipreps for restriction endonuclease digestion and sequence analysis. The DNA was screened for the proper orientation of the CAT sandwich by *Bam*HI digestion, and constructs with correctly oriented inserts were verified by sequence analysis. $N_{2.5}/C_{9.5}$ or Leu222/Trp223 was detected initially by the appearance of a new *Bam*HI or *Afl*III RE site, respectively, and confirmed by DNA sequencing. For Cys234/Thr235, Gln241/Gln242, Phe247/Thr248, and N_7/C_5, plasmid DNA was screened for a new *Hin*dIII RE site, and the constructs with a *Hin*dIII fragment of the correct size were verified by DNA sequencing.

DNA Sequencing. All mutations were verified by dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978). The entire region ligated into the cassette from PCR amplification or linker adaptor and the ligation junctions were sequenced in double-stranded DNA after alkaline denaturation (Hattori & Sakaki, 1986). For the CAT sandwich constructs, the position of the stop codon and the region with the T7 promoter, *lac* promoter/operator, Shine–Delgarno sequence, and the start codon position were sequenced. The remainder of the insert was not sequenced because this region is superfluous aside from carrying the selection marker.

Lactose Transport Assays. Transport of [1-^{14}C]lactose (2.5 mCi/mmol; 1 Ci = 37 GBq) at a final concentration of 0.4 mM was measured by rapid filtration in *E. coli* T184 (Z^-Y^-) harboring plasmid pGP1-2 and transformed with each of the plasmids (McKenna et al., 1991). All transport assays with cells expressing split lac permeases were carried out in parallel with cells harboring pT7-5 (the plasmid expression vector with no *lacY* insert) and with cells harboring pT7-5/cassette *lacY* (pT7-5 containing wild-type *lacY*) as negative and positive controls, respectively.

Membrane Preparation and Immunological Analyses. Membranes were prepared as described (McKenna et al., 1991). Western blots using polyclonal antibodies directed against the C terminus of the lac permease were performed as described (McKenna et al., 1992a).

[^{35}S]*Methionine Pulse-Chase Labeling.* *LacY* fragments were overexpressed either individually or together, as indicted, and selectively labeled with [^{35}S]methionine using the T7 RNA polymerase system as described (McKenna et al., 1991).

Table 2: DNA Sequences around the Splice Junctions of Eight Fragmented *LacY* Constructs[a]

N_1C_{11}	D36/I37	CAT GAT TAG --"CAT GENE"-- AGGAAAGGATCC ATG ATC AAC
$N_{2.5}C_{9.5}$	V85/M86	TTA GTG TAA GCTTTAAGGAAAGGATCC ATG TTT GCG
N_6C_6	S193/S194	TCG ACG TAG --"CAT GENE"-- AGGAAAGGATCC ATG TCG AGT
N_6C_6	L222/W223	AAA CTG TAA CGCGTAAGGAAATGGATTT ATG AAA CTG TGG
	C234/T235	TCC TGC TAA AAGCTTGGATCCAGGATATATACAT ATG ACC TAC
	Q241/Q242	GAC CAA TGA AGCTTCCATGGATCCAGGATATATACAT ATG CAG TTT
	F247/T248	TTC TTT TGA AGCTTCCATGGATCCAGGATATATACAT ATG ACT TCG
N_7C_5	G254/E255	ACG GGT TAA GCTTAGGAATCCATT ATG GAA CAG

[a] Translational termination codons (TAA, TGA, or TAG) and the initiation codon (ATG) are indicated by boldface letters, and the underlined AGGA denotes the Shine–Delgarno ribosome binding sequence.

Throughout growth, appropriate antibiotics were included in the media.

Protein Determinations. Protein was assayed either according to the method of Schaffner and Weissmann (1973) or according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

RESULTS

Construction and Verification of Split LacY Genes. Many of the split *lacY* gene constructs (Table 2) were made by inserting a translational stop codon (TAA, TGA, or TAG) at a given position within *lacY*, followed by a ribosome binding sequence (AGGA) and a restart codon (ATG). All inserts and ligation junctions were verified by dideoxynucleotide sequencing of the double-stranded plasmid DNA after alkali denaturation. For Asp36/Ile37 (N_1/C_{11}) and Ser193/Ser194 (N_6/C_6), which contain a CAT sandwich (Figure 2), a *Bam*HI diagnostic digestion was performed to determine the orientation of the CAT sandwich within the *lacY* gene. Initially, the CAT gene was included after the termination codon to prevent homologous recombination and to aid in selection (Bibi & Kaback, 1990). However, because of difficulties with blunt-end ligation of the CAT sandwich insert, a linker adaptor strategy was used to make Leu222/Trp223 by using the *Bst*XI site. Gly254/Glu255 (N_7/C_5) was created by PCR using the *Age*I site of cassette *lacY* to insert a termination codon, a ribosome binding site, and an initiation codon. Construction of Val85/Met86 ($N_{2.5}/C_{9.5}$), Cys234/Thr235, Gln241/Gln242, and Phe247/Thr248 required two-stage PCR for insertion of the stop codon, ribosome binding sequence, and restart codon.

Colony Morphology. As a preliminary, qualitative test of permease activity, *E. coli* HB101 ($lacZ^+Y^-$) was transformed with pT7-5 encoding each *lacY* split gene construct. HB101 is a "cryptic" strain that produces an active β-galactosidase but carries a defective *lacY* gene. Cells expressing functional lac permease hydrolyze the imported lactose, and subsequent metabolism of monosaccharides causes acidification and the appearance of red colonies on indicator plates. Cells impermeable to lactose appear as white colonies, and permesae mutants with low activity grow as red colonies with a white halo. HB101 expressing Asp36/Ile37 (N_1/C_{11}), Ser193/Ser194 (N_6/C_6), Leu222/Trp223, or Gly254/Glu255 (N_7/C_5) permease grow as red colonies with a white halo. In contrast, cells harboring Val85/Met86 ($N_{2.5}/C_{9.5}$), Cys234/Thr235, Gln241/Gln242, or Phe247/Thr248 yield white colonies indistinguishable from *E. coli* HB101 harboring pT7-5 with no *lacY* insert.

Lactose Transport Activity of N_1/C_{11}, N_6/C_6, N_7/C_5, and $N_{2.5}/C_{9.5}$. Previous experiments demonstrate that coexpression

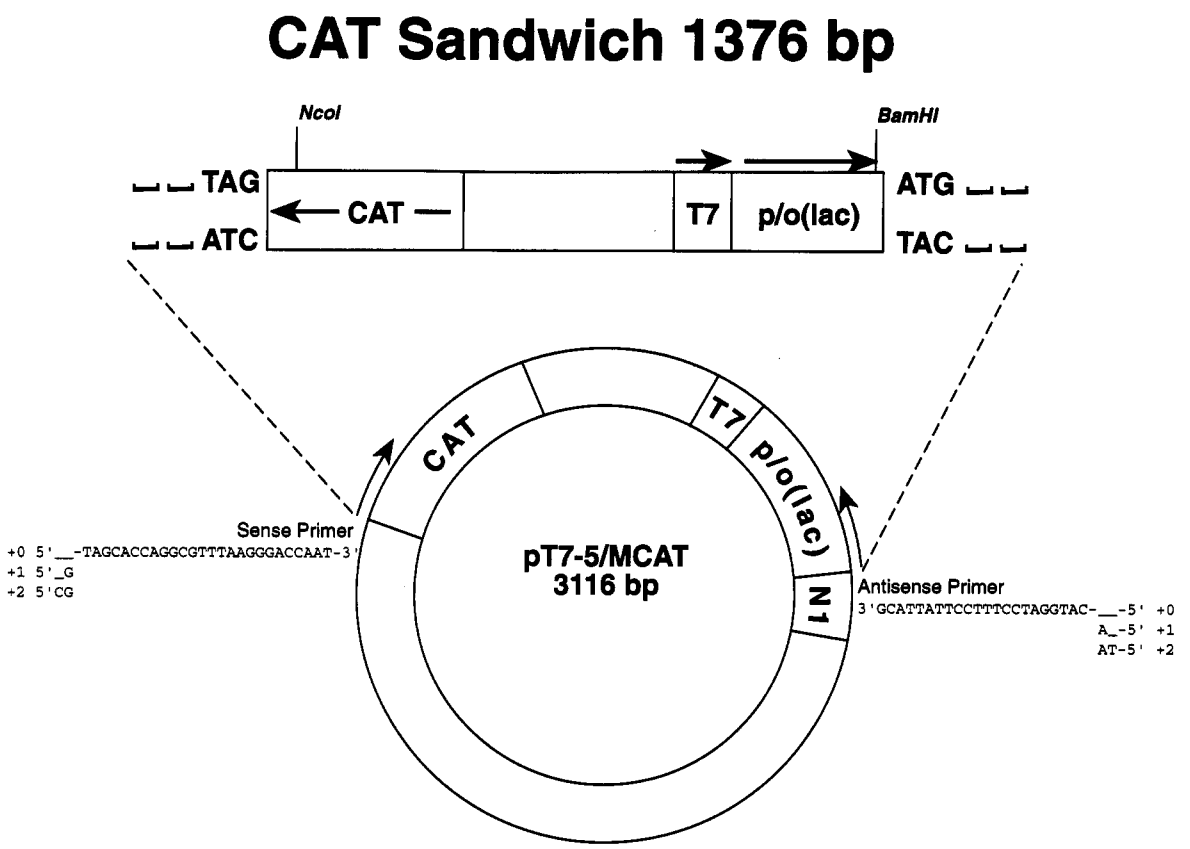

FIGURE 2: CAT sandwich insert. The insert is comprised of a stop codon (TAG), the CAT gene in reverse orientation relative to the rest of the insert, a T7 promoter, the *lac* promoter/operator plus Shine–Delgarno ribosomal binding sequence, and a start codon (ATG). The CAT sandwich was amplified from pT7-5/MCAT by the polymerase chain reaction (PCR). Proper framing into the *lacY* gene was achieved by selection of an appropriate primer (+0, +1, or +2). The locations of two useful restriction endonuclease sites (*Nco*I and *Bam*HI) for creating and expressing individual *lacY* gene fragments are shown.

of *lacY* fragments encoding contiguous polypeptides corresponding to the first and second halves of the permease (Bibi & Kaback, 1990) or the first two transmembrane domains and the remainder of the molecule (Wrubel et al., 1990) leads to increased stability of the polypeptides and functional complementation. In each instance, the discontinuity is in a cytoplasmic loop. Here we examine the transport activity of permease constructs split in cytoplasmic as well as periplasmic loops and also in hydrophobic transmembrane domains. Initially, four regions were selected for study (Figure 1): between Asp36 and Ile37 in loop 1, the periplasmic loop connecting helices I and II (N_1/C_{11}); between Ser193 and Ser194 in loop 6, the cytoplasmic loop connecting helices VI and VII (N_6/C_6); between Gly254 and Glu255 in loop 7, the periplasmic loop connecting helices VII and VIII (N_7/C_5); and between Val85 and Met86 in helix III ($N_{2.5}/C_{9.5}$), a transmembrane domain studied in detail by Calamia and Manoil (1990). The N_6/C_6 (Ser193/Ser194) construct described here is different from the previous N_6/C_6 construct (Leu210/Lys211) described by Bibi and Kaback (1990).

Time courses of [1-^{14}C]lactose transport in *E. coli* T184 (Z^-Y^-) harboring pGP1-2 and pT7-5 (negative control), pT7-5/cassette *lacY* (positive control), Asp36/Ile37 (N_1/C_{11}), Ser193/Ser194 (N_6/C_6), Gly254/Glu255 (N_7/C_5), or Val85/Met86 ($N_{2.5}/C_{9.5}$) are shown in Figure 3. Clearly, N_1/C_{11}, N_6/C_6, or N_7/C_5 exhibits highly significant transport activity. In marked contrast, $N_{2.5}/C_{9.5}$ fails to transport lactose, and uptake is indistinguishable from the negative control. Although data are not presented, expression of each gene fragment individually does not lead to lactose transport, as cells expressing the fragments alone exhibit the same transport activity as pT7-5 which has no *lacY* insert. The results are consistent with the idea that coexpression of contiguous, nonoverlapping permease fragments with discontinuities in either periplasmic or cytoplasmic loops associate to form functional duplexes, while coexpression of fragments with discontinuities in transmembrane domains does not lead to functional complementation.

Immunological Analyses. Expression of permease fragments in the membrane was tested by Western blotting with anti-C-terminal antibody (Figure 4). Most importantly, in no case does coexpression of complementary fragments exhibit a band corresponding to intact lac permease (M_r ca. 33 000). Thus, the transport activity observed with N_1/C_{11}, N_6/C_6, and N_7/C_5 must be due to an association between fragments and cannot be due to intact lac permease [see Bibi and Kaback (1990) and Wrubël et al. (1990) in addition]. As expected, individual expression of N-terminal fragments yields no bands that react with anti-C-terminal antibody. When the C-terminal fragments are expressed individually, no immunoreactive material is observed with C_6 (lane 9) or $C_{9.5}$ (lane 6); however, when C_{11} is expressed alone, an immunoreactive band is observed at ca. 30 kDa with a proteolytic fragment at ca. 22 kDa (lane 3). In contrast, when the N- and C-terminal fragments are coexpressed, unique immunoreactive bands are observed at ca. 30 (lane 2), 27 (lane 5), 21 (lane 8), and 18 kDa (lane 10), respectively, with N_1/C_{11}, $N_{2.5}/C_{9.5}$, N_6/C_6, and N_7/C_5. Detection of an immunoreactive C-terminal fragment from $N_{2.5}/C_{9.5}$ suggests that the lack of transport activity of this construct does not result from limited insertion of the fragments or instability following insertion.

Stability of Membrane-Inserted Fragments. When intact *lacY* is expressed from pT7-5 in the presence of [^{35}S]-methionine and membranes are subjected to NaDodSO$_4$/

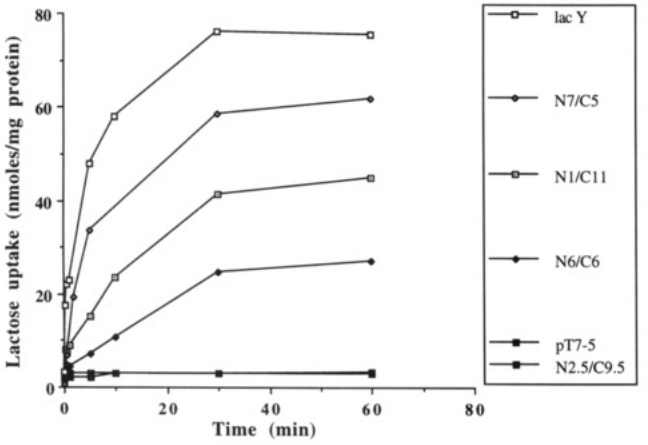

FIGURE 3: Transport of [1-^{14}C]lactose by *E. coli* T184 harboring plasmids pGP1-2 and pT7-5 with no insert (pT7-5), wild-type *lacY*, or *lacY* encoding N_1/C_{11}, $N_{2.5}/C_{9.5}$, N_6/C_6, or N_7/C_5. Plasmid pGP1-2 contains the T7 polymerase gene which is activated by heat shock at 42 °C from the λ_{PL} promoter. *E. coli* T184 cells from overnight cultures were diluted 10-fold into fresh Luria broth supplemented with 10 μg/mL streptomycin, 20 μg/mL kanamycin, and 50 μg/mL ampicillin and were grown at 30 °C to an OD_{600} of 0.5 (3–4 h). After a 20-min incubation at 42 °C, IPTG was added to a final concentration of 0.2 mM, and growth was continued at 30 °C for 90 min. Cells were harvested by centrifugation, washed in 100 mM potassium phosphate (KP_i, pH 7.5)/10 mM $MgSO_4$, concentrated by centrifugation, diluted to an OD_{420} of 10 in the same solution (about 0.7 mg of protein/mL), and kept on ice until use. Aliquots (50 μL) of the cell suspension were used for each time point, and the reactions were carried out at room temperature in plastic tubes. The assay was initiated by addition of [1-^{14}C]lactose to a final concentration of 0.4 mM and was terminated at a given time by addition of 3.0 mL of 100 mM KP_i, pH 5.5/100 mM LiCl, followed by rapid filtration through filter (Whatman GF/F). The filters were washed once with stop solution, and the radioactivity trapped on the filters was determined by liquid scintillation spectrometry. Nonspecific adsorption of radioactivity was determined by adding stop solution to a cell suspension prior to addition of radioactive lactose. The results represent the average of three independent experiments.

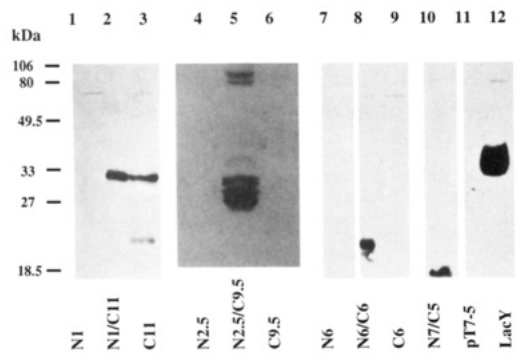

FIGURE 4: Immunoblots of membranes from *E. coli* T184 harboring plasmids pGP1-2 and pT7-5 encoding wild-type *lacY*, N_1/C_{11}, $N_{2.5}/C_{9.5}$, N_6/C_6, or N_7/C_5. Transformed T184 cells were obtained as described in the legend of Figure 3. An aliquot (100 μL) of the suspension was centrifuged, resuspended in 100 μL of water, and sonicated to clarity in a bath-type sonifier. Cell debris was removed by low-speed centrifugation, and the membranes were recovered by centrifugation at 150000g_{max} for 30 min. Samples were dissolved in sample buffer at room temperature and subjected to electrophoresis using 12% and 4.8% polyacrylamide, respectively, in the running and stacking gels, followed by electroblotting onto Immobilon P membranes. The blot was probed with a polyclonal antibody directed against the C terminus, and bound immunoglobulin was visualized using the horseradish peroxidase–protein A and ECL detection system. The positions of prestained molecular weight markers are indicated on the left side of each panel.

polyacrylamide electrophoresis and autoradiography, essentially two labeled bands are observed in the 30–35-kDa range: a heavily labeled diffuse band migrating at about 33 kDa that

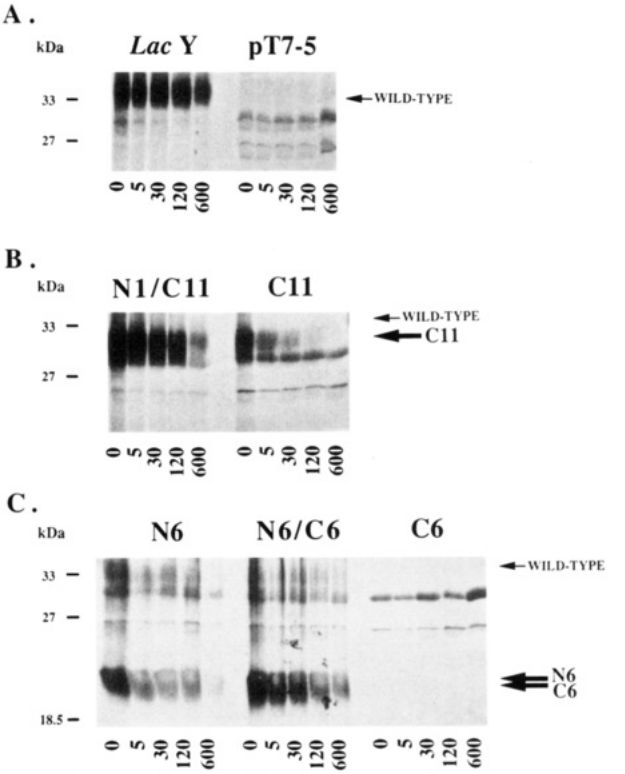

FIGURE 5: Pulse-chase labeling of cells expressing various permease fragments. *E. coli* T184 cells harboring pGP1-2 and pT7-5/*lacY*, pT7-5 (no insert), pT7-5/N_1/C_{11}, pT7-5/C_{11}, pT7-5/N_6, pT7-5/N_6/C_6, or pT7-5/C_6 were grown at 30 °C in Luria broth supplemented with streptomycin (10 μg/mL), kanamycin (20 μg/mL), and ampicillin (50 μg/mL). Overnight cultures were diluted 1:7.5 with fresh medium at 30 °C, and the growth was continued for 4 h. Cells were washed three times in M9 salts/1.0 mM $MgSO_4$/$FeSO_4$ (0.55 μg/mL) prewarmed to 30 °C and resuspended in the same medium containing 0.5% glycerol, 0.02% thiamin, and 0.005% amino acids except methionine and cysteine. The cells were grown under these sulfur-starved conditions for 1 h at 30 °C and then heat-shocked at 42 °C for 15 min. Rifampicin (10 mg/mL in methanol) was added to a final concentration of 0.2 mM to inhibit *E. coli* RNA polymerase, IPTG was added to a final concentration of 0.2 mM, and incubation at 42 °C was continued for an additional 15 min. Labeling was initiated by adding [^{35}S]methionine (1000 Ci/mmol) to a final concentration of 25 pM, and the cells were returned to 30 °C for 10 min. Phenylmethanesulfonyl fluoride (0.4 mM, final concentration) was added to an aliquot of cells which was then quick-frozen and used as the zero time point. Excess unlabeled methionine (200 μM) was added to the remainder of the suspension, and at a given time (5, 30, 120, 600 min), aliquots were removed and rapidly frozen. Membranes prepared from cell aliquots were subjected to SDS–polyacrylamide electrophoresis and autoradiographed. The positions of prestained molecular weight markers are indicated on the left side of each panel.

corresponds to intact permease and another less intense, sharper band at about 30 kDa that is probably β-lactamase precursor [Figure 5A; see Bibi and Kaback (1990) for a complete gel]. When N_1 is expressed by itself, no radioactive band is observed, presumably because the fragment expressed is too small to be detected under the conditions used (data not shown). However, when C_{11} is expressed independently, a labeled fragment of the size expected is observed, which exhibits a half-life of about 5 min after addition of unlabeled methionine (Figure 5B). Moreover, when N_1 is coexpressed with C_{11}, it is apparent that the lifetime of C_{11} is significantly increased. In contrast, as reported previously for a different N_6/C_6 construct (Bibi & Kaback, 1990), a labeled band corresponding to N_6 is observed when the fragment is expressed independently, while nothing corresponding to C_6 is detectable (Figure 5C). When both fragments are coexpressed, however, a diffuse band that overlaps the M_rs of both polypeptides is

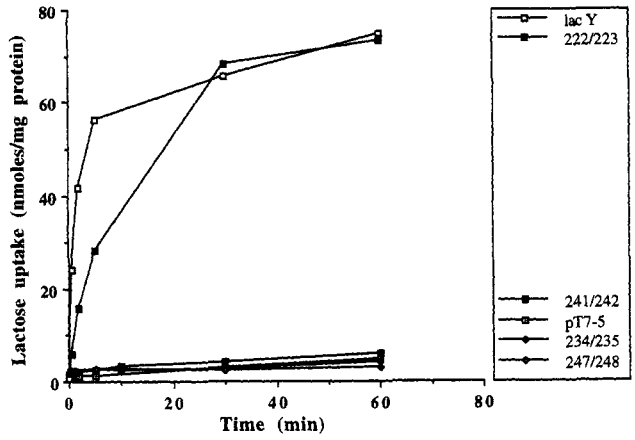

FIGURE 6: Lactose transport by *E. coli* T184 cells harboring plasmids pGP1-2 and pT7-5 with no insert (pT7-5), wild-type *lacY*, or *lacY* encoding Leu222/Trp223, Cys234/Thr235, Gln241/Gln242, or Phe247/Thr248. Experiments were carried out as described in Figure 3. The data represent average values from three independent experiments.

observed. Thus, it is not clear from this experiment whether or not C_6 stabilizes N_6 since the M_rs of both fragments are similar. On the other hand, C_6 is detected immunologically by coexpression with N_6 (Figure 4, lane 8), indicating that N_6 stabilizes C_6.

Use of Split Permeases To Approximate the Boundaries of Helix VII. Second-site suppressor analysis (King et al., 1991; Lee et al., 1992) and site-directed mutagenesis on a functional permease devoid of Cys residues (Sahin-Toth et al., 1992; Dunten et al., 1993; Sahin-Toth & Kaback, 1993) have provided strong evidence that Asp237 and Asp240, which were placed originally in the periplasmic loop between helices VII and VIII (Foster et al., 1983), interact with Lys358 (helix XI) and Lys 319 (helix X), respectively. Thus, King et al. (1991) proposed that Asp237 and Asp240 are contained within helix VII rather than in the periplasmic loop between helices VII and VIII because the putative salt bridges would be more stable in the low dielectric of the membrane (Figure 1). Since the data presented above suggest that contiguous permease fragments with discontinuities in hydrophilic loops exhibit functional complementation, while fragments discontinuous within transmembrane domains do not, a series of discontinuous *lacY* genes was constructed that encode nonoverlapping contiguous permease fragments with discontinuities in loop 6 (cytoplasmic), transmembrane domain VII, or loop 7 (periplasmic). As shown previously, cells coexpressing contiguous fragments with discontinuities between Lys69 and Leu70 (Wrubel et al., 1990) or between Leu210 and Lys211 (Bibi & Kaback, 1990) catalyze active lactose transport (Figure 1). As shown in Figure 6, cells expressing contiguous fragments with discontinuities between Leu222 and Trp223, like the Gly254/Glu255 construct (N_7/C_5; see Figure 3), transport lactose at about 50% the rate of cells expressing wild-type permease to comparable steady states, while cells expressing fragments with discontinuities between Cys234 and Thr235, between Gln241 and Gln242, or between Phe247 and Thr248 exhibit no transport activity whatsoever. The findings are consistent with the interpretation that the boundaries of transmembrane domain VII are between Leu222 and Gly254 and that Asp237 and Asp240 are contained within the transmembrane domain (Figure 1).

DISCUSSION

Initial reports (Bibi & Kaback, 1990; Wrubel et al., 1990) on expression of the *lacY* gene in fragments demonstrate that individual expression of either the N- or C-terminal portions of lac permease leads to neither transport activity nor the presence of identifiable C-terminal polypeptides in the membrane. Remarkably, however, when the gene fragments are coexpressed, active transport is observed, and appropriate N- and C-terminal polypeptides are observed in the membrane. Importantly, intact permease is completely absent from the membrane of cells expressing *lacY* fragments either individually or together. Thus, the transport activity observed must result from an association between independently synthesized moieties of the permease which stabilize each other to form a catalytically active complex. In both of these instances, the discontinuities in the permease are located in cytoplasmic loops. Although functional complementation of fragments has important implications for the mechanism of membrane insertion and assembly of membrane proteins [see Popot and Engelman (1990)], it has not been recognized that the approach may have potential for obtaining more detailed information about secondary structure, more specifically, the boundaries of transmembrane domains. Here we provide evidence consistent with the notion that contiguous lac permease fragments discontinuous in either cytoplasmic or periplasmic domains are able to associate to form catalytically active duplexes, while permease fragments discontinuous in transmembrane domains do not exhibit this property. The approach is then used to test the prediction (King et al., 1991) that Asp237 and Asp240 are contained within transmembrane domain VII, rather than in periplasmic loop 7 (Foster et al., 1983). The results obtained are clearly consistent with this prediction.

The observation that permease fragments with discontinuities in hydrophilic loops can complement functionally is consistent with studies (McKenna et al., 1992b) demonstrating that two or six His residues can be inserted into most of the hydrophilic domains in the permease without abolishing activity. In this respect, it is particularly interesting that His insertions between Ala244 and Asn245 abolish activity [see Figure 5 in Kaback (1992)], while insertions between Gly254 and Glu255 hardly affect activity. The observations are consistent with the results presented here where contiguous permease fragments discontinuous between Gln241 and Gln242 or between Phe247 and Thr248 are inactive, while a discontinuity between Gly254 and Glu255 does not abolish activity.

The finding that functional complementation and increased stability are observed with contiguous, nonoverlapping permease fragments discontinuous in hydrophilic domains on either the cytoplasmic or periplasmic surfaces of the membrane is particularly interesting with respect to membrane insertion. Calamia and Manoil (1992) have demonstrated that each transmembrane domain that spans the membrane with the N terminus on the cytoplasmic surface acts as an independent topological determinant. Thus, it is not difficult to visualize how the N terminus of a C-terminal fragment beginning from a cytoplasmic loop may insert into the membrane. On the other hand, it is not apparent how the N termini of C-terminal fragments beginning from periplasmic loops are inserted through the membrane. In most cases, the C-terminal fragments are not detected in the membrane when expressed individually, suggesting that membrane insertion does not occur. However, when C_{II} is expressed by itself, an identifiable fragment is identified either immunologically or by methionine labeling, although the fragment is clearly stabilized by coexpression of N_I. Possibly, the first transmembrane domain in C_{II} (helix II) is not inserted into the membrane, and the

process of insertion begins with transmembrane domain III. However, when N_I and C_{II} are coexpressed, functional complementation is observed, implying that the loop between N_I and C_{II} is "reconstituted" on the periplasmic surface, despite the discontinuity. Whether such an event occurs before, during, or after the polypeptides are inserted into the membrane cannot be ascertained at the present time. However, if is hard to imagine that the two fragments assemble in a functional manner if the fragments are inserted independently and then assemble. Rather, it seems more likely that interaction occurs before or during insertion. On the other hand, Ehrmann and Beckwith (1991) have shown that the malF protein, a polytopic membrane protein with eight putative transmembrane domains, is inserted into the membrane in a functional manner even when the first transmembrane domain is deleted. Thus, the C-terminal seven transmembrane domains of the malF deletion apparently insert normally although the N terminus is now periplasmic. Since C_{II} lac permease is nonfunctional and relatively unstable, it is difficult to resolve the alternatives.

Regarding the contention that contiguous permease fragments with discontinuities in transmembrane domains do not exhibit functional complementation, evidence has been presented (McKenna et al., 1991, 1992a) that the last turn of putative helix XII must be intact for the permease to insert into the membrane in a form that is stable to proteolysis. However, it should be emphasized that proteolytic degradation cannot explain the lack of transport activity observed with permease duplexes containing discontinuities in transmembrane domains. Thus, an immunoreactive C-terminal fragment is observed with $N_{2.5}/C_{9.5}$. Moreover, permease having the first 22 amino acid residues (the N terminus and half of the first transmembrane domain) deleted is stable and functional when expressed at a high rate (Bibi et al., 1992). Therefore, it seems reasonable to suggest that the lack of transport activity observed with duplexes split in transmembrane domains may be due to an alteration in the transfer of conformational information from one side of a transmembrane domain to the other.

Part of the rationale for initiating these experiments was to approximate the boundaries of transmembrane domain VII in lac permease because it has been postulated (King et al., 1991) that Asp237 and Asp240 are located in the middle of helix VII, rather than in loop 7 on the periplasmic surface of the membrane [see Sahin-Toth et al. (1992), Lee et al. (1992), Dunten et al. (1993), and Sahin-Toth and Kaback et al. (1993) in addition]. However, site-directed spin and fluorescent labeling of permease molecules with single Cys residues at position 237 or 240 indicates that these residues are in an amphipathic environment at a membrane–water interface, suggesting that these positions in the molecule may be close to the C terminus of helix VII rather than in the middle.[3] Since the findings presented here indicate that the approximate cytoplasmic and periplasmic boundaries of transmembrane domain VII are Leu222 and Gly254, respectively (Figure 1), they favor the interpretation that Asp237 and Asp240 are disposed toward the middle of helix VII. One possible explanation for the apparent amphiphilic nature of the protein at these positions is that the hydrophilic face of helix VII may form part of the solvent-filled cleft within the permease (Costello et al., 1984, 1987; Li & Tooth, 1987).

Finally, it is noteworthy that the approach described here should be applicable to other polytopic membrane proteins where the use of gene fusions (Traxler et al., 1993) is difficult or prohibitive for studying topology.

[3] Jung, H., Lopez, R., Altenbach, C., Hubbell, W. L., & Kaback, H. R. (1993) *Biophys. J. 64*, A14 (Abstr. MPMH6).

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