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Biochemistry

In vivo expression of the *lacY* gene in two segments leads to functional *lac* permease

(membranes/bioenergetics/transport/protein–protein interactions)

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ABSTRACT **The *lacY* gene of *Escherichia coli* was cut into two approximately equal-size fragments with *Afl* II and subcloned individually or together under separate *lac* operator/promoters in plasmid pT7-5. Under these conditions, *lac* permease is expressed in two portions: (*i*) the N-terminal portion (the N terminus, the first six putative transmembrane helices, and most of putative loop 7) and (*ii*) the C-terminal portion (the last six putative transmembrane helices and the C terminus). Cells harboring pT7-5 encoding both fragments transport lactose at about 30% the rate of cells expressing intact permease to a comparable steady-state level of accumulation. In contrast, cells expressing either half of the permease independently do not transport lactose. As judged by [^{35}S]methionine labeling and immunoblotting, intact permease is completely absent from the membrane of cells expressing *lacY* fragments either individually or together. Thus, transport activity must result from an association between independently synthesized pieces of *lac* permease. When the gene fragments are expressed individually, the N-terminal portion of the permease is observed inconsistently, and the C-terminal portion is not observed. When the gene fragments are expressed together, polypeptides identified as the N- and C-terminal moieties of the permease are found in the membrane. It is concluded 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.**

The *lac* permease of *Escherichia coli* is a polytopic cytoplasmic membrane protein that catalyzes the coupled transport of β-galactosides and H^+ with a stoichiometry of 1 (i.e., β-galactoside/H^+ symport or cotransport; see refs. 1 and 2 for reviews). The permease has been solubilized from the membrane, purified to homogeneity, and reconstituted into phospholipid vesicles (3, 4); it is functional as a monomer (5). The *lacY* gene has been cloned and sequenced, and the amino acid sequence of the permease has been deduced from the DNA sequence (6). Based on circular dichroism and hydropathy analysis of the primary sequence, a secondary-structure model was proposed (7) in which the polypeptide has 12 hydrophobic domains in α-helical conformation that traverse the membrane in a zigzag fashion connected by hydrophilic loops, with the N and C termini on the cytoplasmic face of the membrane (see Fig. 2). The model is consistent with other spectroscopic measurements (8),† chemical modification (9), limited proteolysis (10, 11), and immunological studies (12–18), but none of these approaches differentiates the 12-helix structure from another model (8) containing 14 helices. However, recent analyses of *lacY–phoA* fusions (19) have provided strong, exclusive support for the 12-helix motif.

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Many proteins maintain tertiary structure when the peptide backbone is cleaved, and substrate binding and/or catalytic activity may be retained. Among many examples, bacteriorhodopsin can be split into two fragments that reconstitute to form an active complex (20). In a similar vein, *lac* permease binds ligand after proteolysis, although transport activity is abolished (10). Thus, forces between different domains within a protein are able to maintain three-dimensional structure when the peptide backbone is not intact. Furthermore, studies with the β-adrenergic receptor (21) and the sodium channel (22) indicate that functional complexes are formed even when the mRNAs encoding these proteins are expressed as discontinuous fragments.

In this paper, we describe the *in vivo* synthesis of two polypeptide fragments from independently cloned portions of the *lacY* gene and their apparent association to form functional *lac* permease in the membrane.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose and L-[^{35}S]methionine were purchased from the Amersham, and ^{125}I-labeled protein A was purchased from ICN. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. *E. coli* HB101 [*hsdS20*(r_B^-, m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(Sm^r), *xyl-5*, *mtl-1*, *supE44*, λ^-/F$^-$] (23) was used as carrier for the plasmids described and for detection of *lac* permease activity on MacConkey plates (Difco) containing 25 mM lactose. *E. coli* T184 [$lacI^+O^+Z^-Y^-(A)$, *rpsL*, *met*$^-$, *thr*$^-$, *recA*, *hsdM*, *hsdR*/F′, $lacI^qO^+Z^{U118}(Y^+A^+)$] (24) was used for overexpression of *lac* permease, [^{35}S]methionine labeling, and lactose transport. Cloned DNA was overexpressed using the T7 RNA polymerase system (25–27); *E. coli* T184 was transformed with two plasmids, pGP1-2 and pT7-5 [kindly provided by S. Tabor and C. C. Richardson (Harvard Medical School)], or one of the pT7-5-derived plasmids described (see *Results*). pACYC184 (New England Laboratories) was the source of the tetracycline-resistance gene. A cassette *lacY* gene containing the *lac* operator/promoter [p/o(*lac*)] (J. C. Pastore, J. D. Larigan, and H.R.K., unpublished work) was cloned into pT7-5 and used for all *lacY* gene manipulations.

Growth of Cells and Overexpression of *lacY*. *E. coli* T184 harboring pGP1-2 and pT7-5(lacY) or a derivative (i.e., pTN6, pTC6, or pTN6/C6; see Fig. 1) was grown at 30°C in M9CA broth (GIBCO) supplemented with thiamine (1 μg/ml), streptomycin (10 μg/ml), ampicillin (50 μg/ml), kanamycin (10 μg/ml), and 0.2% glycerol. T184 harboring pTN6/

Abbreviations: p/o(*lac*), *lac* promoter/operator; N6, 5′ portion of *lacY*; C6, 3′ portion of *lacY*.

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†In addition to circular dichroic and laser Raman spectroscopy, Fourier-transform infrared studies also show that purified *lac* permease is largely helical (P. D. Roepe, H.R.K., and K. J. Rothschild, unpublished work).

C6 also contained tetracycline (10 μg/ml). Overnight cultures were diluted 1:10 with fresh medium at 30°C, and growth was continued for 3 hr. Isopropyl β-D-thiogalactoside (0.2 mM) was added, and the cultures were heat shocked at 42°C for 40 min, transferred to 30°C, and grown for 90 min.

Transport Assays. Transport of [1-^{14}C]lactose (10 mCi/mmol; 1 Ci = 37 GBq) at a final concentration of 0.4 mM was assayed by rapid filtration as described (28).

[^{35}S]Methionine Labeling and Membrane Preparation. [^{35}S]Methionine labeling in the presence of rifampicin was carried out for 60 min with [^{35}S]methionine (1000 Ci/mmol) at a final concentration of 25 pM, and membranes were prepared as described (26, 27).

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography were performed as described (3, 4, 26).

Immunoblots. Immunoblotting was carried out with monoclonal antibody 4A10R (12), which is directed primarily against the C terminus of *lac* permease (29).

Protein. Protein was assayed as described (30).

RESULTS

Construction of Plasmids. The strategy behind the plasmid constructs was to cut *lacY* in approximately the middle with *Afl* II and subclone each fragment into pT7-5, either individually (pTN6 or pTC6) or together (pTN6/C6) under separate p/o(*lac*)s (Fig. 1). By this means, *lac* permease is expressed in two segments, one containing the N-terminal half of the permease and the other containing the C-terminal half (Fig. 2). Constructions were carried out as follows (Fig. 1).

A cassette gene of *lacY* (J. C. Pastore, D. L. Larigan, and H.R.K., unpublished work), which contains an intact p/o(*lac*), was cloned into pT7-5 digested with *Eco*RI and *Hin*dIII (Fig. 1*A*). The orientation of *lacY* is such that it is under control of both the T7 RNA polymerase promoter [p(T7)] and p/o(*lac*); note that the β-lactamase gene is in the opposite orientation.

In the recombinant plasmid [pT7-5(lacY)], there are two *Afl* II sites, both in *lacY*. One site is in the middle of the coding region and the other is in the 3′ end and contains the

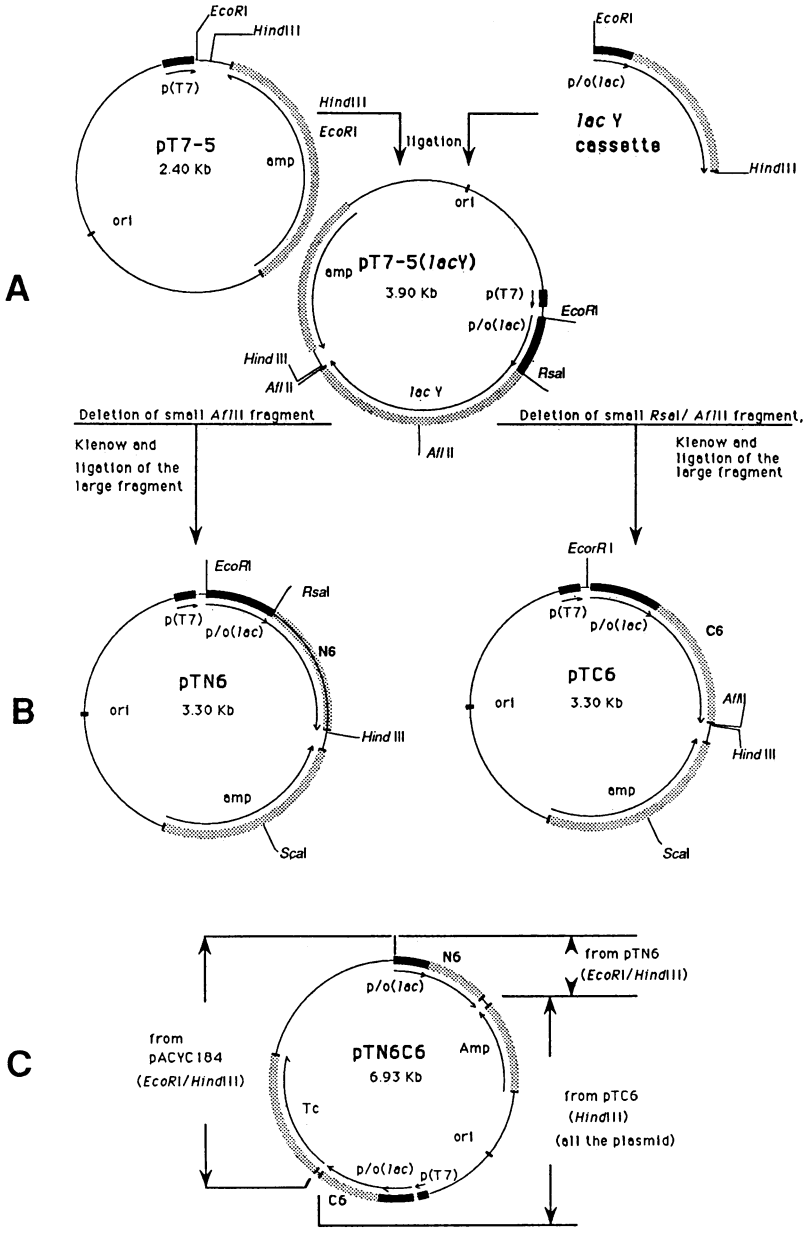

FIG. 1. Construction of plasmids pT7-5(lacY), pTN6, pTC6, and pTN6/C6. See the text for a detailed explanation of the operations shown. amp, β-lactamase gene; ori, origin of replication; Tc, tetracycline-resistance gene.

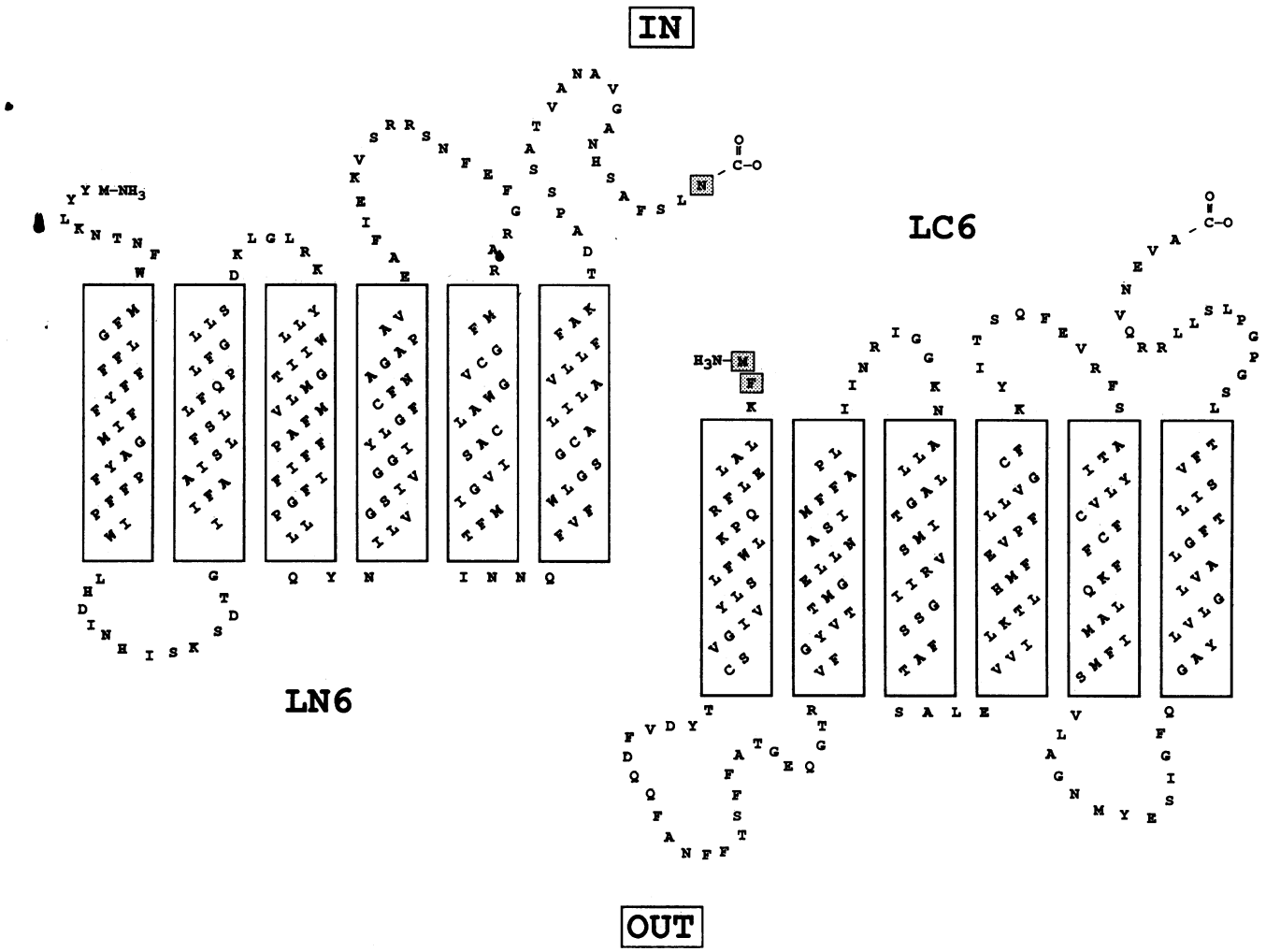

FIG. 2. Secondary structure of *lac* permease showing portions encoded by the *lacY* gene fragments described. The single-letter amino acid code is used, hydrophobic transmembrane helices are shown in boxes, and new amino acid residues inserted are shown in shaded boxes.

stop codon TAA. Construction of pTN6, which contains the 5′ portion of *lacY* (N6), was accomplished by deleting the small *Afl* II fragment (Fig. 1*B*). The large *Afl* II fragment was then treated with DNA polymerase (Klenow fragment) and ligated to itself. The original stop codon of *lacY* is in frame with the 5′ portion of the gene.

Construction of plasmid pTC6, which contains the 3′ portion of *lacY* (C6), was more complicated (Fig. 1*B*). There are eight *Rsa* I sites in pT7-5(lacY), one of which is at a codon next to the ATG start codon of *lacY*. Therefore pT7-5(lacY) was partially digested with *Rsa* I, and a 3.9-kilobase (kb) linear fragment was isolated. The fragment was partially digested with *Afl* II to form a 3.3-kb fragment containing the 3′ end of *lacY*, which was isolated, treated with the Klenow fragment of DNA polymerase, and ligated to itself. The construct, pTC6, contains the 3′ half of *lacY* in frame with the start codon (ATG). As a result of the manipulations, there is a new codon in the 3′ end of N6 (AAT encoding asparagine) and two new codons in the 5′ end of C6 (ATG encoding methionine and TTT encoding phenylalanine).

The two recombinant plasmids were then used to construct a third plasmid (Fig. 1*C*; pTN6/C6), which contains both halves of *lacY*, each under control of an independent p/o(*lac*) and both under control of the T7 RNA polymerase promoter. The plasmid was also constructed so as to prevent recombination between homologous direct repeats, an important consideration, since both N6 and C6 were cloned with the same p/o(*lac*) (0.297-kb). The strategy was to introduce a β-lactamase gene or a tetracycline-resistance gene, respectively, between N6 and C6 on either side. In the first step, a DNA fragment containing N6 and p/o(*lac*) was cloned into pACYC184 by ligating the small *Eco*RI–*Sca* I fragment from pTN6 with the large *Eco*RI–*Pvu* II fragment from pACYC-184. By this means, the chloramphenicol-resistance gene was interrupted, but the tetracycline-resistance gene remained intact. In the next step, the origin of replication of pACYC184 was removed with *Hin*dIII and ligated with the linear form of pTC6 (obtained by digestion with *Hin*dIII). Finally, the orientation of N6 and C6 was ascertained by using *Eco*RI digestion analysis.

Active Transport of Lactose. *E. coli* T184 harboring pGP1-2 and pT7-5(lacY) transports lactose rapidly, and within about 5 min, a steady-state level of accumulation is achieved and maintained for up to 80 min (Fig. 3). The same cells harboring pGP1-2 and either pT7-5, pTN6, or pTC6, which encode no *lac* permease, the N-terminal portion of *lac* permease, or the C-terminal portion, respectively, do not transport the disaccharide. Strikingly, T184 harboring pGP1-2 and pTN6/C6, which encodes both portions of *lac* permease in separate *lacY* gene fragments, transports lactose at about 30% the rate of T184 expressing wild-type permease to a comparable steady-state.

[^{35}S]Methionine Labeling. When intact *lacY* is expressed from pT7-5 in the presence of [^{35}S]methionine and membranes are subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography, two heavily labeled bands

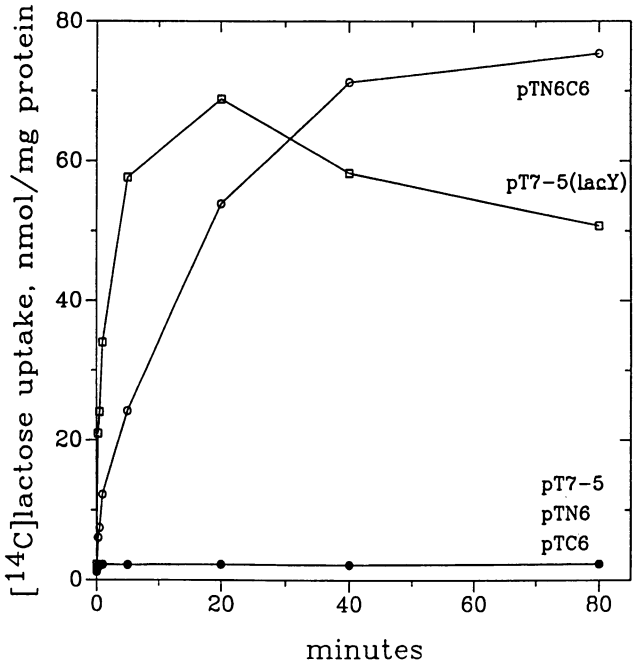

FIG. 3. Lactose transport by *E. coli* T184 harboring pGP1-2 and either pT7-5 (●), pT7-5(lacY) (□), pTN6 (●), pTC6 (●), or pTN6/C6 (○). Cells were induced by heat shock in the presence of isopropyl β-D-thiogalactoside. Aliquots of washed cells in 50 mM potassium phosphate, pH 7.5/10 mM magnesium sulfate were assayed for [1-^{14}C]lactose (10 mCi/mmol) uptake at a final concentration of 0.4 mM as described (28). The same symbol (●) is used for pT7-5, pTN6, and pTC6 for reasons of clarity; there was no significant difference between these samples.

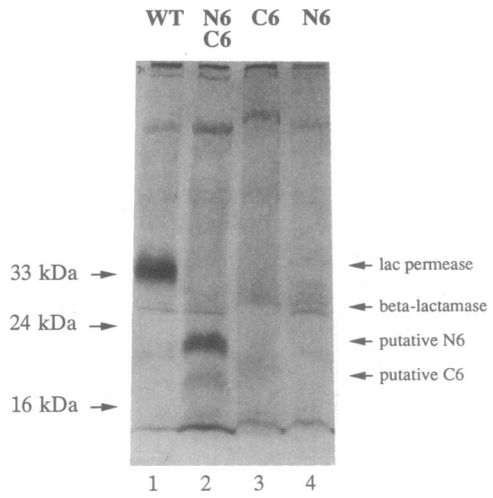

FIG. 4. Autoradiograph of membranes prepared from cells labeled with [^{35}S]methionine after NaDodSO$_4$/polyacrylamide gel electrophoresis. WT (lane 1), N6/C6 (lane 2), C6 (lane 3), and N6 (lane 4) correspond to samples of membranes prepared from *E. coli* T184 harboring pGP1-2 and either pT7-5(lacY), pTN6/C6, pTC6, or pTN6, respectively. Aliquots containing the same amount of membrane protein (30 μg) were applied to each lane, and prestained molecular size markers (Bio-Rad) were used as indicated by the arrows at the left.

are observed, a diffuse band with a molecular mass of about 33 kDa that corresponds to intact permease (3, 4, 12)‡ and another sharp band migrating at about 30 kDa that is probably β-lactamase precursor (31) (Fig. 4, lane 1). When the C- or N-terminal portions are expressed individually, only the pre-β-lactamase band is observed (Fig. 4, lanes 3 and 4, respectively).§ Finally, when the N- and C-terminal moieties are expressed together, three bands are observed, one corresponding to pre-β-lactamase, another diffuse band migrating below pre-β-lactamase that is probably the N-terminal portion of *lac* permease, and a third, more rapidly migrating, diffuse band (putative C6 polypeptide; Fig. 4, lane 2) (see below). Importantly, no intact permease is apparent when the fragments are expressed either individually or together (compare lane 1 to lanes 2, 3, and 4 in Fig. 4).

Immunoblotting. Monoclonal antibody 4A10R is directed primarily against the C terminus of *lac* permease (29). As shown in lane 1 of the autoradiograph presented in Fig. 5, which was purposely overexposed, antibody binds to the diffuse band at about 33 kDa, which corresponds to intact permease,‡ to various slower migrating species that represent incompletely disaggregated permease, and to a band at about 20 kDa, which is a proteolytic fragment of the permease observed sporadically (10). When the N- or C-terminal fragments are expressed individually, no uniquely identifiable bands are observed (Fig. 5, lanes 2 and 3). However, when the N- and C-terminal fragments are expressed together (Fig. 5, lane 4), a unique band is observed that corresponds to the lower diffuse band in lane 2 of Fig. 4. Antibody binding is not observed at the position corresponding to intact permease

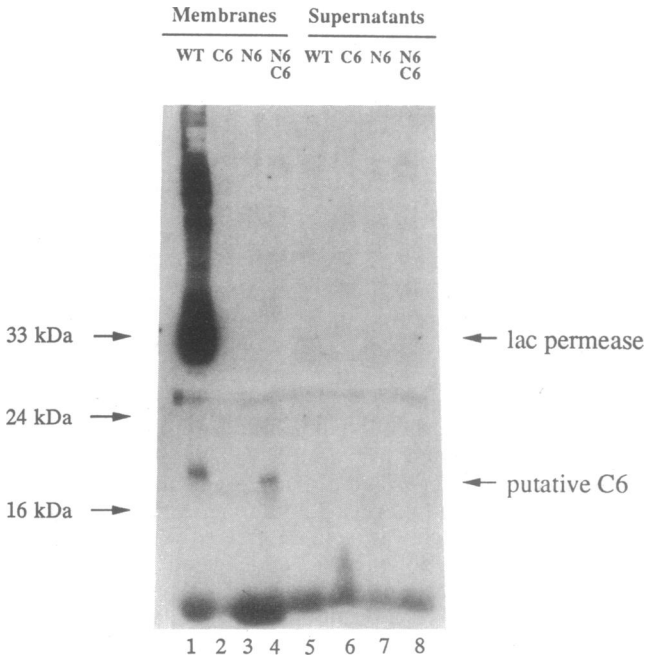

FIG. 5. Immunoblot of membrane and soluble fractions from *E. coli* T184 harboring pGP1-2 and either pT7-5(lacY) (WT; lanes 1 and 5), pTC6 (C6; lanes 2 and 6), pTN6 (N6; lanes 3 and 7), or pTN6/C6 (N6/C6; lanes 4 and 8) after NaDodSO$_4$/polyacrylamide gel electrophoresis. After electroblotting of a NaDodSO$_4$/polyacrylamide gel similar to that shown in Fig. 4, the nitrocellulose paper was blocked, incubated with monoclonal antibody 4A10R, washed, labeled with ^{125}I-labeled protein A, and autoradiographed as described (29). Lanes 1–4 are membrane fractions and lanes 5–8 are soluble fractions from the cells.

‡Although *lac* permease is 46.5 kDa, as determined from the DNA sequence of *lacY* (6) and from the amino acid composition of purified permease (3, 4), the protein migrates with an apparent molecular mass of *ca.* 33 kDa on electrophoresis in NaDodSO$_4$/12% polyacrylamide gels, and its mobility varies with polyacrylamide concentration (1, 2).

§In some [^{35}S]methionine-labeling experiments, a fragment corresponding to N6 (see Fig. 4, lane 2) is observed in membranes from cells harboring pTN6. The appearance of the band is variable, however, and may be related to the specific labeling conditions.

(i.e., 33 kDa) in Fig. 5, lanes 2, 3, or 4, or in the supernatants resulting from high-speed centrifugation (Fig. 5, lanes 5–8).

DISCUSSION

These data demonstrate that expression of the *lacY* gene as two independently cloned fragments in the same plasmid leads to membrane insertion of functional *lac* permease and synthesis of polypeptides that correspond to the N- and C-terminal portions of the protein. In contradistinction, when the two fragments are expressed individually, transport activity is not observed, and although the N-terminal fragment is sometimes observed,[§] the C-terminal fragment is not. Therefore, the N- and C-terminal polypeptides are probably unstable and are proteolyzed either before or after membrane insertion when expressed individually. When the polypeptides are synthesized together, however, they apparently form a more stable, catalytically active complex in the membrane.

The obvious caveat of the experiments is that intact permease is synthesized and inserted into the membrane when the two pieces of *lacY* are expressed together. This consideration is highly relevant because there is only a qualitative relationship between the amount of permease and the initial rate of transport in intact cells. However, given the nature of the plasmid construct and the results presented in Figs. 4 and 5, it is unlikely that intact permease is present. First, pTN6/C6 was constructed with a β-lactamase gene between the 3′ end of the N6 fragment and the 5′ end of the C6 fragment and a tetracycline-resistance gene between the 3′ end of the C6 fragment and the 5′ end of the N6 fragment. By this means, homologous recombination is prevented because recombinants would lose one of the antibiotic-resistance markers. Second, as judged by [^{35}S]methionine labeling and immunoblotting, no intact permease is detected.

As for the polypeptides expressed from pTN6/C6, since intact permease migrates with an aberrantly high mobility that is sensitive to polyacrylamide concentration,[‡] it is difficult to predict where the N6 and C6 polypeptides should migrate, particularly since the distribution of charged amino acid residues is asymmetric. In any event, as judged by [^{35}S]methionine labeling and immunoblotting with a monoclonal antibody directed primarily against the C terminus, two unique polypeptides tentatively identified as N6 and C6 are apparent when the two gene fragments are expressed from pTN6/C6.

The observation that an N-terminal fragment of the permease is not observed consistently when N6 is expressed alone[§] is unexpected. Stochaj *et al.* (32) demonstrated that polypeptides expressed from 5′ *lacY* fragments encoding as few as 170 amino acid residues are detected in the membrane following overexpression and concluded that a "helical hairpin" at the N terminus is required for insertion. Similarly, Roepe and Kaback (27) showed that site-directed truncation mutants in the C-terminal third of the permease are detected in the membrane after overexpression, although certain truncated molecules exhibit markedly shortened lifetimes. Possibly, the stability of N-terminal polypeptides depends specifically on the site at which the permease is truncated (19). On the other hand, overexpression of *lacY* gene constructs devoid of 5′ sequences encoding segments of the permease corresponding to the N terminus and/or putative helices I and II does not yield identifiable polypeptides (unpublished work). Therefore, it is not surprising that a C6 polypeptide is absent from cells harboring pTC6. In any case, it is clear that both polypeptides appear in the membrane when the gene fragments are expressed together. Thus, it seems likely that an association between the complementing polypeptides before, during, or after membrane insertion stabilizes the complex to proteolysis.

1. Kaback, H. R. (1986) in *Physiology of Membrane Disorders*, Andreoli, T. E., Hoffman, J. F., Fanestil, D. D. & Schultz, S. G. (Plenum, New York), pp. 387–408.
2. Kaback, H. R. (1989) *Harvey Lect.* **83,** 77–103.
3. Newman, M. J., Foster, D., Wilson, T. H. & Kaback, H. R. (1981) *J. Biol. Chem.* **256,** 11804–11808.
4. Viitanen, P., Newman, M. J., Foster, D. L., Wilson, T. H. & Kaback, H. R. (1985) *Methods Enzymol.* **125,** 370–377.
5. Costello, M. J., Escaig, J., Matsushita, K., Viitanen, P. V., Menick, D. R. & Kaback, H. R. (1987) *J. Biol. Chem.* **262,** 17072–17082.
6. Büchel, D. E., Groneborn, B. & Müller-Hill, B. (1980) *Nature (London)* **283,** 541–545.
7. Foster, D. L., Boublik, M. & Kaback, H. R. (1983) *J. Biol. Chem.* **258,** 31–34.
8. Vogel, H., Wright, J. K. & Jähnig, F. (1985) *EMBO J.* **4,** 3625–3631.
9. Page, M. G. P. & Rosenbusch, J. P. (1988) *J. Biol. Chem.* **263,** 15906–15914.
10. Goldkorn, T., Rimon, G. & Kaback, H. R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3322–3326.
11. Stochaj, V., Bieseler, B. & Ehring, R. (1986) *Eur. J. Biochem.* **158,** 423–428.
12. Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T. & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6894–6898.
13. Seckler, R., Wright, J. K. & Overath, P. (1983) *J. Biol. Chem.* **258,** 10817–10820.
14. Seckler, R. & Wright, J. K. (1984) *Eur. J. Biochem.* **142,** 269–279.
15. Carrasco, N., Viitanen, P., Herzlinger, D. & Kaback, H. R. (1984) *Biochemistry* **23,** 3681–3687.
16. Herzlinger, D., Viitanen, P., Carrasco, N. & Kaback, H. R. (1984) *Biochemistry* **23,** 3688–3693.
17. Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F. & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 4672–4676.
18. Seckler, R., Möröy, T., Wright, J. K. & Overath, P. (1986) *Biochemistry* **25,** 2403–2409.
19. Calamia, J. & Manoil, C. (1990) *Proc. Natl. Acad. Sci. USA* **87,** in press.
20. Liao, M. J., Huang, K. S. & Khorana, G. (1984) *J. Biol. Chem.* **259,** 4200–4204.
21. Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. & Lefkowitz, R. J. (1988) *Science* **240,** 1310–1316.
22. Stühmer, W., Conti, F., Suzuki, H., Wang, X., Noda, M., Yahagi, N., Kubo, H. & Numa, S. (1989) *Nature (London)* **339,** 597–603.
23. Boyer, H. W. & Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41,** 459–472.
24. Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Fürst, M., Aichele, G., Wilhelm, V. & Overath, P. (1980) *Eur. J. Biochem.* **108,** 223–231.
25. Tabor, S. & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1074–1078.
26. Roepe, P. D., Zbar, R., Sarkar, H. K. & Kaback, H. R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3992–3996.
27. Roepe, P. D. & Kaback, H. R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6087–6091.
28. Trumble, W. R., Viitanen, P. V., Sarkar, H. K., Poonian, M. S. & Kaback, H. R. (1984) *Biochem. Biophys. Res. Commun.* **119,** 860–867.
29. Herzlinger, D., Carrasco, N. & Kaback, H. R. (1985) *Biochemistry* **24,** 221–229.
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
31. Pollitt, S. & Zalkin, H. (1983) *J. Bacteriol.* **153,** 27–32.
32. Stochaj, U., Fritz, H.-J., Heibach, C., Markgraf, M., Schaewen, A. V., Sonnewald, U. & Ehring, R. (1988) *J. Bacteriol.* **170,** 2639–2645.