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Genetics

lac permease of *Escherichia coli:* Topology and sequence elements promoting membrane insertion

(membrane-spanning segment/gene fusion/alkaline phosphatase)

JUSTINA CALAMIA AND COLIN MANOIL

Department of Genetics, University of Washington, Seattle, WA 98195

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ABSTRACT **The membrane topology of *Escherichia coli lac* permease was analyzed using a set of 36 *lac* permease–alkaline phosphatase (*lacY–phoA*) gene fusions. The level of enzymatic activity of alkaline phosphatase fused to a cytoplasmic membrane protein appears to reflect whether the fusion junction site normally faces the cytoplasm or periplasm. The alkaline phosphatase activities of cells expressing the *lacY–phoA* fusions distinguish between models previously proposed for the topology of *lac* permease and favor one with 12 transmembrane segments. This model is fully compatible with the results of earlier biochemical and immunological studies. The properties of fusions with junctions spanning two of the transmembrane segments at 2- or 3-amino acid intervals indicate that approximately half of the residues of either segment (9–11 amino acids) suffices to promote alkaline phosphatase translocation across the membrane. The additional transmembrane segment amino acids that are not required for this membrane insertion process may normally be needed in unfused *lac* permease after insertion for stable association with the membrane.**

The membrane insertion of proteins with multiple membrane-spanning segments is usually pictured as a process in which individual or pairs of membrane-spanning segments are inserted independently into the lipid bilayer, followed by interaction among the segments without major topological changes to generate the fully folded protein. To help assess the validity of this picture, we have begun an analysis of the biogenesis of *Escherichia coli lac* permease.

lac permease has been the subject of extensive biochemical and genetic studies aimed at revealing the precise mechanism of solute transport across a membrane (1–3). Two models for the membrane topology of *lac* permease have been proposed from its amino acid sequence, one with 12 membrane-spanning helical segments (1, 4) and the other with 14 membrane-spanning helices (5). Although spectroscopic evidence indicates that *lac* permease is indeed largely helical in conformation (4, 5), more detailed immunological and biochemical studies have not distinguished between the two topology models. However, these studies have established that the N and C termini and two internal segments of the protein are cytoplasmically disposed (1, 6, 7).

To distinguish between the two topology models, we have analyzed cells producing different *lac* permease–alkaline phosphatase (LacY–PhoA) hybrid proteins. Earlier studies have indicated that the alkaline phosphatase activities of such hybrid proteins generally correlate with the normal topology of the membrane protein (8–10). The results of this analysis strongly support the 12-helix model for *lac* permease. In addition, these studies indicate that only about half of the amino acid residues of an outwardly oriented (i.e., amino-end cytoplasmic) transmembrane segment are needed to promote alkaline phosphatase export from the cytoplasm.

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MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. *E. coli* strains used were CC159 [F128 *lacI^q/Δ(ara,leu)7697 ΔlacX74 phoAΔ20 galE galK thi rpsE rpoB argE*(am) *recA1*], CC181 [CC159 *lacY328*(am)], CC192 [CC159 *mutS215*::Tn*10 recA*::*cat*]. Plasmids used were pTE18, a pBR322 derivative carrying *lacY* (11), and pCM472, a pTE18 derivative carrying the M13 IG sequence (12) and the left end of Tn*phoA* (up to its leftmost *Xho* I site) inserted 3′ to *lacY* (in *lacA* sequences) with *lacY* and *phoA* in the same orientation.

Media and Enzymes. Media were made according to Miller (13). Enzymes were purchased from New England Biolabs.

Assay. Alkaline phosphatase activity was assayed by measuring the rate of *p*-nitrophenyl phosphate hydrolysis by permeabilized cells (14). Cells growing exponentially in LB at 37°C were exposed to 2 mM isopropyl thiogalactoside for 60 min prior to assay.

Isolation of *lacY–phoA* Fusions. Fusions were isolated using *in vivo* and *in vitro* methods. Fusions were generated *in vivo* by insertion of Tn*phoA* into pTE18 using λTn*phoA* (15), and *in vitro* using the oligonucleotide deletion method (9) with pCM472 grown in CC159 and transformed into CC192 after mutagenesis. In both approaches, fusion plasmids were identified among transformant colonies after replica plating onto L agar containing ampicillin (200 μg/ml), isopropyl thiogalactoside (1 mM), and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (40 μg/ml). DNA from fusion plasmids was analyzed by restriction mapping followed by double-strand DNA sequencing (16) to identify fusion junction sites.

Antibody Precipitation and Electrophoresis. Protein was immunoprecipitated from cell extracts after [^{35}S]methionine incorporation according to the protocol of Ito *et al.* (17) except that heat treatments in the presence of $NaDodSO_4$ were at 50°C. Electrophoresis and quantitation of protein after electrophoresis were as described (18).

RESULTS

Isolation of *lac* Permease–Alkaline Phosphatase (*lacY–phoA*) Gene Fusions. We used a combination of *in vivo* and *in vitro* techniques to isolate gene fusions encoding hybrid proteins with different lengths of *lac* permease at their N termini joined to C-terminal alkaline phosphatase. We first generated *lacY–phoA* fusions *in vivo* by transposition of Tn*phoA* (18) into the *lacY* gene carried on a plasmid. Tn*phoA* insertions that led to increased alkaline phosphatase activity were detected among transformants as pale to dark blue colonies after replica plating onto nutrient agar containing the alkaline phosphatase indicator 5-bromo-4-chloro-3-indolyl phosphate. Sixty-five such independently derived Tn*phoA* insertions were found by restriction endonuclease and DNA sequence analyses to generate in-frame *lacY–phoA* fusions

and to be situated at 27 sites in *lacY* (Table 1). Approximately one-third of the fusions were at two sites in *lacY*, apparent hot spots for Tn*phoA* insertion.

We then constructed five additional fusions with predefined endpoints using an *in vitro* oligonucleotide-directed method (9). This method allows the construction of fusions with exactly the same junction sequence as fusions generated by Tn*phoA* insertion, so that the two types of fusions can be directly compared. The junction points of the five fusions were chosen to allow maximal discrimination between models for *lac* permease topology (1, 4, 5). Four additional fusions were constructed to help identify the shortest sequences promoting alkaline phosphatase activity for two of the transmembrane segments (see below). The junction site locations for the full set of 36 *lacY–phoA* fusions are shown in Table 1.

Alkaline Phosphatase Activities of LacY–PhoA Hybrid Proteins. We measured the alkaline phosphatase activities of cells expressing the various *lacY–phoA* fusions (Table 1). The fusions and their activities are shown relative to the 12-helix model for *lac* permease in Fig. 1 *Upper*. The pattern of activities correlates well with the model, with cells carrying fusions with junctions in or near predicted periplasmic segments generally expressing 20–100 times greater alkaline phosphatase activity than those with junctions in or near cytoplasmic segments. The fusions and their activities are also shown relative to the 14-helix model for *lac* permease in the central region where it differs significantly from the 12-helix model (Fig. 1 *Lower*). Four of the fusions expressed alkaline phosphatase activities incompatible with the 14-helix model: two with junction sites predicted to be periplasmic showed low activities (fusions 21 and 22) and two with junction sites predicted to be cytoplasmic showed high activities (fusions 24 and 25). This analysis thus strongly favors the 12-helix model over the 14-helix model for the topology of *lac* permease.

Fusions Spanning Two Transmembrane Segments. To determine the minimum number of transmembrane segment

Table 1. Properties of LacY–PhoA hybrid proteins

Fusion	Isolates, no.	Junction	AP activity, units/OD_{600}	Colony size	Stability, min
1	1	25,p	635	3	
2	4	38,p	376	2.5	143*
3	11	71,c	15	4	3
4	1	75,c	4	4	
5	2	76,c	3	3.5	
6	—	79,c	<1	4	
7	—	82,c	11	3.5	
8	—	85,p	621	2	
9	1	88,p	722	2	
10	1	108,p	456	2.5	62*
11	2	113,p	372	2	
12	1	124,c	65	3	
13	2	126,c	99	3	11*
14	2	147,c	4	4	7
15	3	150,c	5	3.5	
16	1	152,c	4	3.5	
17	—	155,p	191	3	
18	1	177,p	400	3	88*
19	1	195,c	13	3.5	
20	1	204,c	12	3	
21	1	211,c	12	3	
22	—	218,c	5	3.5	
23	1	231,p	500	2	
24	1	237,p	482	1.5	
25	—	244,p	507	2	20*
26	10	255,p	272	2	
27	3	259,p	399	2	
28	—	289,c	3	3.5	
29	4	312,p	90	2	6, 24
30	—	344,c	4	3.5	7
31	2	367,p	242	1	15*
32	1	368,p	46	2.5	5, 24
33	2	373,p	247	1	16*
34	1	382,p	244	1	
35	3	404,c	32	3	
36	—	417,c	23	3	43

Number of isolates corresponds to the number of independent isolates of fusions generated *in vivo* by Tn*phoA* insertion. Fusions without values were constructed *in vitro*. Results under the heading Junction correspond to the number of the last amino acid residue of *lac* permease in the hybrid protein and assignment of the site as facing the periplasm (p) or cytoplasm (c) based on the 12-helix model. Colony size was scored on a scale of 1–4 with 4 the largest for colonies grown ≈16 hr at 37°C on TYE agar containing isopropyl thiogalactoside (1 mM) and 5-bromo-4-chloro-3-indolyl phosphate (40 μg/ml). Colonies carrying the parent plasmid (pTE18) scored 4 on this scale. The approximate correspondence between the size score and colony diameter is as follows: 1, 0.25 mm; 2, 0.5 mm; 3, 0.75 mm; 4, 1.0 mm. Stability values correspond to the half-life of the hybrid protein in minutes derived from pulse–chase experiments. Hybrid proteins 29 and 32 showed biphasic loss kinetics. AP, alkaline phosphatase.

*Hybrid protein gave rise to alkaline phosphatase-sized fragment in chase period.

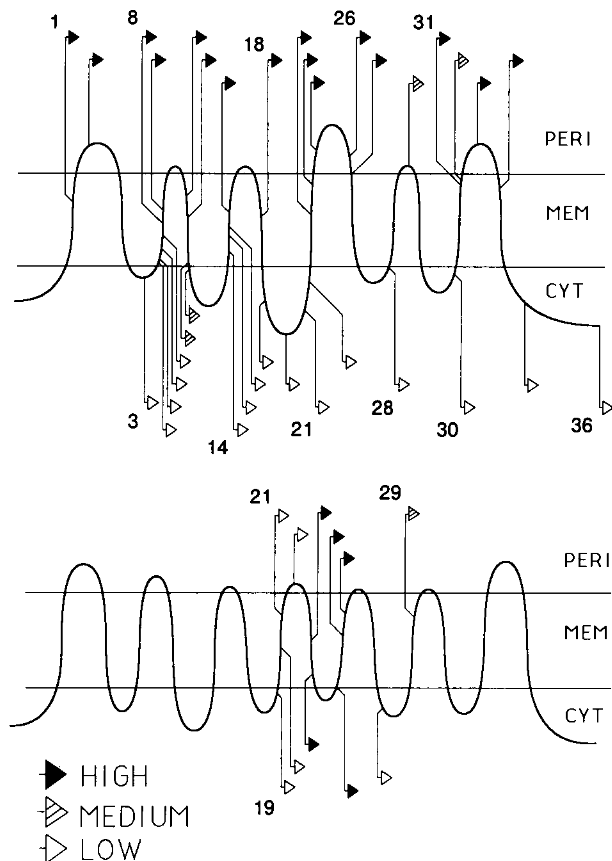

FIG. 1. Comparison of 12-helix and 14-helix models for *lac* permease. The positions and alkaline phosphatase activities of LacY–PhoA fusions are shown relative to the entire 12-helix model for *lac* permease (*Upper*) and to the 14-helix model in the central region where it differs significantly from the 12-helix model (*Lower*). Solid arrows correspond to fusions with high alkaline phosphatase activity (>190 units), hatched arrows to intermediate activity fusions (46–99 units), and open arrows to low activity fusions (<35 units). Fusions are numbered sequentially from the N terminus, and a few fusions are identified for orientation. The 12-helix model shown is a modification of those proposed by Kaback's laboratory (1, 4) with membrane-spanning segments corresponding to *lac* permease residues 11–34, 47–66, 75–99, 103–125, 145–164, 168–187, 222–239, 260–283, 291–309, 315–334, 347–369, and 381–400 (19). The model is essentially that of Kaback (1) except that transmembrane segment 7 corresponds more closely to that of Foster *et al.* (4), following a suggestion of T. H. Wilson (personal communication), and the ends of some transmembrane segments have been adjusted to maximize their hydrophobicity. The 14-helix model corresponds to that proposed by Vogel *et al.* (5). Peri, periplasmic space; Mem, membrane; Cyt, cytoplasm.

amino acids required for alkaline phosphatase activity, we constructed fusions spanning transmembrane segments 3 and 5 spaced at 2- or 3-residue intervals (Fig. 2). Remarkably, in each case, there was a dramatic increase in alkaline phosphatase activity (to essentially periplasmic fusion levels) over an increase in length from 8 to 11 transmembrane segment residues. The amino acid sequence encoded by Tn*phoA* at the fusion junction is generally hydrophilic (Fig. 2) and is not expected to contribute significantly to promoting export. Thus, it appears that 9–11 apolar membrane-spanning segment residues can suffice to promote efficient alkaline phosphatase translocation across the membrane.

Hybrid Protein Production and Stability. Hybrid proteins produced by cells carrying representative *lacY–phoA* fusion plasmids were identified by precipitation with antibody to alkaline phosphatase (Fig. 3). The apparent molecular weights of the hybrid proteins based on their electrophoretic

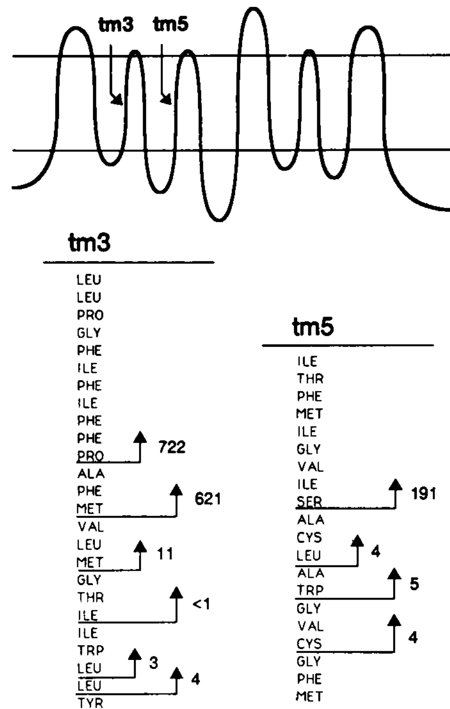

FIG. 2. Alkaline phosphatase gene fusions spanning two *lac* permease transmembrane segments. The positions and alkaline phosphatase activities of LacY–PhoA fusions with junctions falling within the putative transmembrane segment 3 (fusions 4–9) and transmembrane segment 5 (fusions 14–17) are shown relative to the *lac* permease amino acid sequence. The amino acid sequence at each junction encoded by Tn*phoA* is (Ser,Pro,Thr,Ala)-Asp-Ser-Tyr-Thr-Gln-Val-Ala-Ser-Trp-Thr-Glu . . . (18). tm, Transmembrane segment.

mobilities were consistently smaller than that predicted from the amino acid sequence, as is observed for *lac* permease itself (19). Three of the hybrid proteins (corresponding to fusions 18, 21, and 25) migrated as smears, presumably due to aggregation or heterogeneity in conformation or detergent binding.

We examined the stabilities of 13 LacY–PhoA hybrid proteins in pulse–chase experiments (Fig. 4 and Table 1). Hybrid proteins with putative periplasmic junction sites exhibited decreased stability with increased length, from a half-life of 143 min for the shortest hybrid examined (fusion 2) to 15 min for one of the longest periplasmic segment fusions (fusion 31). Most periplasmic segment hybrids were degraded

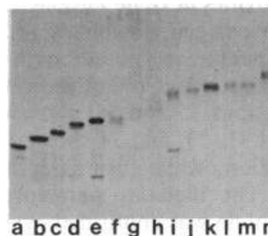

FIG. 3. LacY–PhoA hybrid proteins. Representative hybrid proteins identified by alkaline phosphatase antibody precipitation are shown as follows. Lanes: a, fusion 2; b, fusion 3; c, fusion 10; d, fusion 13; e, fusion 14; f, fusion 18; g, fusion 21; h, fusion 25; i, fusion 28; j, fusion 29; k, fusion 30; l, fusion 31; m, fusion 32; n, fusion 36. Cells carrying fusion plasmids were exposed to [^{35}S]methionine for 1 min at 37°C prior to antibody precipitation. In addition to the hybrid protein bands, fusions 2 and 28 (lanes a and i) show alkaline phosphatase-sized bands, and fusion 14 (lane e) shows a band smaller than alkaline phosphatase.

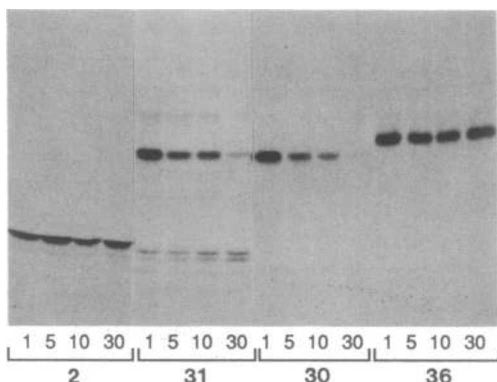

FIG. 4. Stability of LacY–PhoA hybrid proteins. Representative hybrid proteins (fusions 2, 31, 30, and 36) were immunoprecipitated after labeling for 2 min followed by chase periods of 1, 5, 10, or 30 min. Fusion 2 encodes a short periplasmic segment hybrid protein; fusion 31 encodes a long periplasmic segment hybrid; fusion 30 encodes a cytoplasmic segment hybrid, and fusion 36 encodes the hybrid containing the full-length *lac* permease. For fusions 2 and 31, protein bands about the size of alkaline phosphatase itself accumulated during the chase period.

in part to alkaline phosphatase-sized fragments (Fig. 4 and Table 1).

The cytoplasmic segment LacY–PhoA hybrid proteins were, with one exception, very unstable, and most did not yield detectable breakdown products. The exception, the hybrid that contains all of *lac* permease (encoded by fusion 36), showed low activity yet was relatively stable, with a half-life of 43 min. The hybrid protein encoded by fusion 13, a cytoplasmic domain fusion expressing intermediate alkaline phosphatase activity, differed from most other cytoplasmic segment hybrids in that it was degraded to alkaline phosphatase-sized fragments (data not shown). These fragments may result from proteolysis after partial export of the alkaline phosphatase moiety of the hybrid protein.

Toxicity of *lacY–phoA* Fusion Expression. The high level production of hybrid protein was highly toxic to cells carrying certain of the *lacY–phoA* fusion plasmids. The toxicity was observed as a reduction in the size of colonies grown on 5-bromo-4-chloro-3-indolyl phosphate indicator agar under conditions inducing hybrid protein expression (Table 1). Colonies expressing toxic LacY–PhoA fusions generally showed large white secondary colonies, which presumably correspond to mutant cells failing to produce hybrid protein. As a rule, the expression of fusions with junctions corresponding to cytoplasmic sites in *lac* permease was less toxic than those with nearby periplasmic segment junctions.

***lac* Permease Activities of Gene Fusions.** The *lac* permease activities of the various gene fusions were assessed by their abilities to complement a *lacY* mutation (in strain CC181) for lactose fermentation on MacConkey indicator agar. The two longest fusions (fusions 35 and 36) showed strong complementation. Fusions 31, 33, and 34 showed a barely perceptible complementation, while fusions 2 and 29 showed no complementation. The high *lac* permease activity of the fusion 35 hybrid protein was confirmed by direct assay of *lac* permease uptake activity of whole cells (R. Kaback, personal communication). The $LacY^+$ phenotypes of cells carrying the long fusion plasmids are unlikely to be due to intragenic complementation between plasmid and chromosomal *lacY* alleles, since *lac* permease functions as a monomer (1).

DISCUSSION

This report describes an analysis of the membrane topology of *lac* permease using a set of gene fusions encoding *lac* permease–alkaline phosphatase hybrid proteins. The alkaline phosphatase activities of such hybrids appear to correlate with the normal topologies of bacterial cytoplasmic membrane proteins (10). Two models for the topology of *lac* permease based primarily on its amino acid sequence have been proposed, one with 12 membrane-spanning segments (1, 4) and one with 14 membrane-spanning segments (6). The two models differ primarily in a central region of the protein, which is generally hydrophobic but in which transmembrane helices are difficult to assign unambiguously (see Fig. 1). Our results provide strong support for the 12-helix model of *lac* permease: fusions with junctions corresponding to all six proposed periplasmic segments showed high cellular alkaline phosphatase activities and fusions with junctions corresponding to six cytoplasmic segments showed low activities. Four fusions for which the 12-helix and 14-helix models make opposite activity predictions all show properties that fit with the 12-helix alternative.

Earlier studies of *lac* permease topology used proteolysis, antibody binding, and membrane-impermeable reagent modification to position sites of the protein to one side or other of the membrane. By using these methods, the N and C termini and sites near amino acid residues 135 and 190 were found to face the cytoplasm (1, 6, 7). These sites of known subcellular disposition of the *lac* permease polypeptide chain are reference points for checking the validity of using alkaline phosphatase fusions to analyze the topologies of membrane proteins with many membrane-spanning segments. Earlier studies of such proteins using alkaline phosphatase fusions analyzed proteins for which little or no independent topological information was available (9, 20, 21). We found full agreement between the results of our gene fusion analysis and the biochemical studies, an outcome that provides strong empirical support for the use of gene fusions to help analyze the topologies of such proteins. The apparent success of this method in the analysis of *lac* permease implies that even transmembrane segments containing charged residues (i.e., segments 7–11) can function efficiently in generating topology without C-terminal sequences (which are lost in these gene fusions).

Although the majority (32 of 36 fusions) of the *lacY–phoA* fusions could be placed in high or low alkaline phosphatase activity categories, four expressed intermediate alkaline phosphatase activities. Two junction sites of the four (fusions 12 and 13) fell at the C-terminal end of one of the transmembrane segments oriented with its N terminus facing the periplasm, so that C-terminal cytoplasm residues were lost in the corresponding hybrid proteins. Earlier studies indicated that hybrids such as these sometimes show high alkaline phosphatase activities because the cytoplasmic residues can be needed for normal positioning of the transmembrane segment (9). Positively charged residues may be particularly important in this positioning (22). Two periplasmic segment fusions also expressed intermediate alkaline phosphatase activities. The fusion junction of one of these (fusion 29) follows a membrane-spanning segment (tm9) that is one of the two most hydrophilic of *lac* permease. This transmembrane segment may require interaction with C-terminal parts of *lac* permease absent from the hybrid protein for fully efficient insertion.

Most of the LacY–PhoA hybrid proteins examined were found to be proteolytically unstable. In general, the stability of periplasmic segment hybrids decreased as the size of the hybrid increased. The unstable periplasmic segment hybrids generally gave alkaline phosphatase-sized degradation products, as has frequently been observed for exported alkaline phosphatase hybrid proteins (8, 18). LacY–PhoA hybrids with cytoplasmic fusion junctions were more uniformly unstable and generally did not yield alkaline phosphatase-sized degradation products. The striking exception to this rule was

provided by the full-length LacY–PhoA hybrid (encoded by fusion 36), which was relatively stable. These results are compatible with those of Roepe and Kaback (23), who found that deletions from the C-terminal end of *lac* permease that impinged on the final putative membrane-spanning segment greatly destabilized the corresponding mutant proteins. Thus these results suggest that degradation of LacY–PhoA hybrid proteins may be initiated in improperly folded regions of the membrane protein and move processively into the alkaline phosphatase domains. If the alkaline phosphatase moiety is situated in the periplasm, it is folded and relatively resistant to the degradation, so that alkaline phosphatase-sized fragments are released. If the alkaline phosphatase moiety is cytoplasmic and, therefore, not fully folded, it is degraded. For the full-length LacY–PhoA hybrid, the *lac* permease (which is functional) is apparently normally folded so that proteolysis is not efficiently initiated.

Many of the LacY–PhoA fusions had junction points corresponding to putative membrane-spanning segments rather than periplasmic or cytoplasmic segments. The rules that govern the alkaline phosphatase activities of such hybrids differ depending on whether the membrane-spanning segment fused to is oriented with its N terminus in the periplasm ("incoming") or in the cytoplasm ("outgoing"). For incoming membrane-spanning segments, hybrid proteins containing as many as 10 or 11 residues showed high alkaline phosphatase activity (fusions 11 and 18). An incoming segment fusion containing 22 residues (fusion 12) showed reduced activity compared to the preceding periplasmic segment fusions, although this activity was still considerably higher than most cytoplasmic segment fusions. Studies of fusions to the MalF protein of *E. coli* have shown that efficient anchoring of alkaline phosphatase in the cytoplasm can in some cases require cytoplasmic sequences following a transmembrane segment (9).

To determine the pattern of alkaline phosphatase activity for hybrid proteins containing different lengths of outgoing transmembrane sequence, we isolated a series of fusions spanning transmembrane segments 3 and 5. In these studies, nearly periplasmic fusion levels of alkaline phosphatase activity were obtained with 11 but not 8 amino acids of the 25- and 20-residue transmembrane segments. Thus, it appears that the export functions of outgoing transmembrane segments can require as few as half of the amino acid residues of such segments. The two minimum sequences of 9–11 membrane-spanning segment amino acids needed to promote alkaline phosphatase export correspond closely in their lengths and overall polarities to the shortest hydrophobic core regions found in naturally occurring cleavable signal sequences (24). It is worth noting that the three outgoing transmembrane segments of *lac* permease that contain charged residues (segments 7, 9, and 11) all have the charged residues situated at least 12 amino acids from the N-terminal ends of the segments. This placement may reflect the need for at least 9–11 contiguous apolar residues to initiate insertion of membrane-spanning segments.

Why are naturally occurring outgoing membrane-spanning segments normally at least 18–20 residues long if half this number can suffice to promote translocation from the cytoplasm? It seems most likely that the extra nonpolar residues are needed for stable anchoring of the segment in the bilayer once translocation has occurred. Indeed, studies of deletion mutants of a eukaryotic membrane protein have shown that shortening a membrane-spanning segment even from 19 to 17 residues can decrease the strength of its association with the membrane (25). By this interpretation, alkaline phosphatase fusions to outgoing membrane-spanning sequences can provide a genetic separation of the export and membrane anchoring functions of such sequences.

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