52. D. A. Allen and R. H. Sanders, *Nature (London)* **319**, 191 (1986).
53. R. L. Brown and K. Y. Lo, *Astrophys. J.* **253**, 108 (1982); J. van Gorkom, K. Y. Lo, M. J. Claussen, unpublished result.
54. D. C. Backer, in *Extragalactic Radio Sources*, D. S. Heeschen and C. Wade, Eds. (International Astronomical Union Symposium 97, Reidel, Dordrecht, Holland, 1982), p. 389.
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Research Article

A Genetic Approach to Analyzing Membrane Protein Topology

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Fusions of the secreted protein alkaline phosphatase to an integral cytoplasmic membrane protein of *Escherichia coli* showed different activities depending on where in the membrane protein the alkaline phosphatase was fused. Fusions to positions in or near the periplasmic domain led to high alkaline phosphatase activity, whereas those to positions in the cytoplasmic domain gave low activity. Analysis of alkaline phosphatase fusions to membrane proteins of unknown structure may thus be generally useful in determining their membrane topologies.

THE AMINO ACID SEQUENCE OF A PROTEIN IS FREQUENTLY known with little or no additional information available about how the sequence is folded in its normal three-dimensional structure. The situation can be less grim if the sequence is that of an integral membrane protein rather than that of a cytoplasmic protein, because of the common occurrence in such membrane proteins of easily identified long contiguous stretches of hydrophobic amino acids. High resolution structural analyses of bacteriorhodopsin and the *Rhodopseudomonas viridis* photosynthetic reaction center polypeptides have shown that such long hydrophobic sequences generally correspond to transmembrane alpha-helical stretches of a membrane protein (*1, 2*). Armed with this fact, and with a plot of the average hydrophobicity along the sequence of a membrane protein (*3*), possible two-dimensional membrane topologies for the polypeptide can be drawn, with each long hydrophobic sequence corresponding to a transmembrane alpha helix. Such a protocol appears to have correctly predicted the transmembrane segments of reaction center polypeptides before the high resolution structures were determined (*2, 4*).

How can models for the membrane topology of a protein be tested in the absence of diffraction analysis? There are a number of ways to determine elements of membrane protein structure. Identifying sites of a membrane protein that interact naturally with other proteins of known cellular location, such as modifying enzymes (*5, 6*) or binding proteins (*7*), position the sites relative to the membrane. Sites can also be positioned by their reaction with membrane-impermeant small molecules, proteases, or antibodies added from one side or other of the membrane (*8*). Reagents that react from within the lipid bilayer can directly identify membrane-spanning sequences (*9*). Spectroscopic analysis of purified membrane proteins can provide an overall measure of their secondary structures, which includes that of their membrane-spanning sequences (*10*).

In this article, we describe a genetic method to help determine membrane protein topology. This approach offers the advantage that it does not depend directly on the exposure and reactivity of amino acid side chains of the polypeptide, and should be a useful complement to the approaches which do.

Rationale of the method. A protein spanning the cytoplasmic membrane of *Escherichia coli* will have different domains exposed to the cytoplasm and periplasm. Using fusions of such a membrane protein to alkaline phosphatase we have sought to distinguish these domains. Alkaline phosphatase appears to require export to the periplasm to show enzymatic activity (*11*). The idea underlying this approach is illustrated in Fig. 1 for a hypothetical membrane protein whose polypeptide chain crosses the membrane six times. If alkaline phosphatase were fused to a site normally facing the periplasm (fusion 1), it is possible that the alkaline phosphatase moiety would be exported to the periplasm and show enzymatic activity. Alternatively, if it were fused to a part of the protein facing the cytoplasm (fusion 2), the alkaline phosphatase moiety would remain cytoplasmic and inactive. Thus, the activities of fusions at different positions would reflect the normal membrane topology of the protein.

Tsr protein fusions. To test the scheme illustrated in Fig. 1, we have analyzed fusions to the *E. coli* Tsr protein, one of a set of four related proteins involved in chemotaxis as chemoreceptors. These proteins appear to exist as tetramers of a simple transmembrane structure in which each polypeptide chain crosses the membrane twice (Fig. 2a). Each polypeptide is divided into a periplasmic domain between the two transmembrane sequences, and a large cytoplasmic domain at the carboxyl terminus of the protein (Fig. 2a). This structure is derived from the amino acid sequences of the proteins (*12, 13*), the properties of proteolytic fragments of one of them (*14*), the sites of covalent modification of one of the proteins

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by a cytoplasmic methyltransferase (*15*), and the properties of chimeric chemoreceptors (*16*). Tsr protein is thus a model membrane protein of simple, well-characterized transmembrane disposition to use in testing the validity of the scheme shown in Fig. 1.

Enzymatically active fusions of alkaline phosphatase to Tsr protein were isolated by insertion of transposon Tn*phoA* into a plasmid carrying the *tsr* gene (*17*). Such fusions eliminate carboxyl-terminal sequences of Tsr protein, replacing them with alkaline phosphatase (Fig. 1). Tn*phoA* shows a low DNA sequence specificity of insertion, and thus has the potential to fuse alkaline phosphatase at many sites in Tsr protein (*17*). Fusion plasmids yielding high and low alkaline phosphatase activities were found, and were easily distinguished on media containing an alkaline phosphatase indicator, 5-bromo-4-chloro-3-indolyl phosphate. The sites of Tn*phoA* insertion in plasmids giving different levels of activity were determined by restriction enzyme and DNA sequence analysis. Strikingly, four independently generated insertions leading to high enzymatic activity all had fusion junctions positioned in or near sequences encoding the periplasmic domain of Tsr protein (Table 1, lines 1 to 3, and Fig. 2b). The hybrid proteins encoded by three of these plasmids tested (identified after precipitation with antibody to alkaline phosphatase) were made in high amounts and were of the sizes expected from the sites of Tn*phoA* insertion (Table 1, lines 1 to 3). Five additional highly active fusions to two of the other chemoreceptors, the Tar and Tap proteins, also had insertions that mapped at sites corresponding to the periplasmic domains of these proteins (Fig. 2b). These results show that the positions of Tn*phoA* insertions leading to fusions with high alkaline phosphatase activities are correlated with the normal membrane topologies of the chemoreceptor proteins.

Table 1. Properties of *tsr-phoA* fusion plasmids. Fusion plasmids are derived from pJFG5, itself a derivative of pBR322 (*27*) with the *tsr* gene replacing *tet* sequences between the Hind III and Bam HI sites (*28*). All plasmids correspond to Tn*phoA* insertions into pJFG5 except pCM234 and pCM235, which are deleted for sequences between the *tsr* Mlu I and Bss HII sites, and pCM251 and pCM252, which are deleted for sequences distal to *phoA*, between the Sal I sites of Tn*phoA* and pJFG5. ND, not determined.

Fusion plasmid	Gene	AP activity (units/ OD_{600})*	Position of fusion junction†	Protein size (kD)		Relative amount protein‖
				Predicted‡	Found§	
pCM204	*tsr*	306	19	50.5	49.5	0.52
pCM201	*tsr*	241	89	58	56	(1.0)
pCM203	*tsr*	343	164	66	68	1.05
pCM210	*tsr*	0.7	170 (OF)	67	46	0.14
pCM206	*tsr*	8	247	75	72	1.93
pCM211	*tsr*	7	433	95	102	1.05
pCM234	*tsr*Δ1	177	402	63	ND	ND
pCM251	*tsr*	10	402	92	95	0.60
pCM235	*tsr*Δ1	172	520	76	81	0.85
pCM252	*tsr*	9	520	104	108	0.62

*Alkaline phosphatase (AP) activity of strains carrying different plasmids was measured in permeabilized cells (*11*). †The most carboxyl terminal amino acid residue of the *tsr* coding sequence (*12*) prior to Tn*phoA* sequence is presented for each fusion. The *tsr* and *phoA* sequences are in the same translational reading frame in all plasmids except pCM210. The nucleotide sequences of different junction fragments were determined by the dideoxynucleotide chain termination method after each was subcloned into a phage M13 derivative (*29*). OF indicates *tsr* and *phoA* sequences in different translational reading frames. ‡Calculated from the position of Tn*phoA* insertion with a value of 107 daltons per Tsr protein amino acid residue and 48,500 daltons for residues contributed by Tn*phoA*. §Determined for hybrid proteins precipitated by antibody to alkaline phosphatase and separated by SDS–polyacrylamide gel electrophoresis by their rates of migration relative to proteins of known molecular size. ‖Expressed relative to the amount of hybrid protein recovered from cells carrying plasmid pCM201, and corrected for the size of each protein. Strain CC118 cells carrying different plasmids were treated with radioactive methionine for 1 or 2 minutes at 37°C, and their hybrid proteins were precipitated with an excess of antibody to alkaline phosphatase (*17*). Proteins were separated by electrophoresis, and the amount of radioactivity present in each hybrid protein band was determined after elution from the polyacrylamide gel by liquid scintillation analysis (*17*).

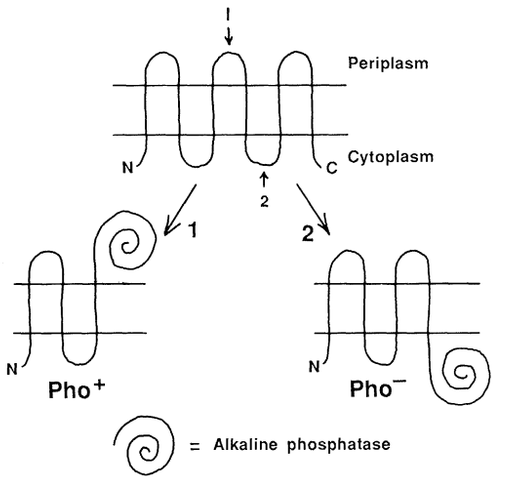

Fig. 1. Scheme for using alkaline phosphatase fusions to identify membrane protein topology. Fusions to positions in periplasmic domains of a hypothetical membrane protein (position 1) may give a periplasmic alkaline phosphatase moiety with high enzymatic activity (Pho^+), whereas fusions to positions in cytoplasmic domains (position 2) may give a cytoplasmic alkaline phosphatase moiety with low enzymatic activity (Pho^-); N, amino terminal; C, carboxyl terminal.

Restriction analysis of *tsr-phoA* fusion plasmids leading to low alkaline phosphatase activity showed the Tn*phoA* to be positioned at sites of *tsr* corresponding to both its periplasmic and cytoplasmic domains (Table 1, lines 4 to 6). The sites of these insertions thus did not correlate with the membrane topology of Tsr protein. However, DNA sequence analysis of three of the plasmids revealed that two classes of low activity fusions could be distinguished, depending on whether the *tsr* and *phoA* sequences were in the same translational reading frame. Two low activity fusions with Tn*phoA* inserted at positions corresponding to cytoplasmic sites of Tsr protein had the *tsr* and *phoA* genes in frame (Table 1, lines 5 and 6). Each of these fusion plasmids directed the synthesis of an approximately equal amount of a hybrid protein of the size expected from the site of Tn*phoA* insertion (Table 1, lines 5 and 6).

In contrast, a low activity fusion with Tn*phoA* positioned at a site corresponding to the periplasmic domain of Tsr protein had the *tsr* and *phoA* sequences out-of-frame relative to each other (Table 1, line 4). Cells carrying this plasmid synthesized a protein that was precipitated by antibody to alkaline phosphatase, but the protein was made in reduced amounts relative to other *tsr-phoA* hybrid proteins and was much smaller than that predicted if the *tsr* and *phoA* genes were in frame (Table 1, line 4). This protein is presumed to result from an internal translation initiation site that is used inefficiently.

In-frame fusions of *phoA* to *tsr* at sites corresponding to its cytoplasmic domain show 20 to 40 times less activity than fusions at periplasmic domain sites, even though the two types of fusions direct the synthesis of comparable amounts of hybrid protein (Table 1, lines 1 to 3 and 5 and 6) (*18*). These findings indicate that in-frame fusions of alkaline phosphatase to the cytoplasmic domain of a membrane protein give low alkaline phosphatase activity, as required by the scheme shown in Fig. 1.

Fusions to a Tsr protein deletion mutant. To further test whether alkaline phosphatase fusions can help reveal membrane protein structure, we analyzed fusions to a mutant Tsr protein expected to show an altered membrane topology. This mutant (*tsr*Δ1) carries a deletion of Tsr protein sequences which includes its second transmembrane sequence (corresponding to the sequence between the *tsr* Mlu I and Bss HII sites; Fig. 3, top). We thought that this transmembrane sequence might help anchor the Tsr protein

chain in the membrane (*19*) and that, in its absence, sequences carboxyl terminal to it which were normally cytoplasmic might pass into the periplasm (Fig. 3, center). Fusions of alkaline phosphatase at such (normally cytoplasmic) positions would be predicted to lead to high enzymatic activity.

Analysis of five Tn*phoA* insertions into the deleted *tsr* gene leading to high activity indicated that all were positioned at sites corresponding to the normally cytoplasmic domain of the protein (Fig. 3, bottom; Table 1, lines 7 and 9). One of these insertions (in pCM235) fused alkaline phosphatase at a site 16 amino acid residues from the end of Tsr protein (Table 1, line 9), indicating that nearly all of the Tsr protein cytoplasmic domain present in the deletion derivative can pass efficiently into the periplasm.

The region of *tsr* missing from the *tsr*Δ1 deletion mutant was placed back into each of the active fusion plasmids, generating *tsr-phoA* fusions with structures the same as if they had been generated by Tn*phoA* insertion into wild-type *tsr*. Cells carrying the resulting fusion plasmids directed the synthesis of normal amounts of hybrid proteins but showed 10 to 20 times less alkaline phosphatase activity than those carrying the parental *tsr*Δ1 fusion plasmids (Table 1, lines 7 to 10) (*18*). Since replacing the second transmembrane sequence is expected to change the location of the alkaline phosphatase moieties of the hybrid proteins from the periplasm to the cytoplasm, the corresponding loss of activity is further evidence for a correlation of activity with topology. This result argues strongly against the possibility that the low alkaline phosphatase activity of fusions at cytoplasmic positions is due to the exact amino acid sequence at the fusion joint rather than being due to the membrane configuration of such hybrid proteins. The positions of in-frame low activity *tsr-phoA* fusions isolated directly (Table 1, lines 5 and 6), or in two steps with the use of *tsr*Δ1 (Table 1, lines 8 and 10) (*18*) are shown in the model for Tsr protein membrane topology in Fig. 2c.

Activation of a low activity fusion. The results described suggested that any manipulation leading to the loss of the second transmembrane sequence from a low activity *tsr-phoA* cytoplasmic domain fusion might increase its activity. As a test of this possibility, a plasmid (pCM211) which encodes a fusion of alkaline phosphatase to the middle of the Tsr protein cytoplasmic domain was deleted for *tsr* sequences by treatment with the restriction enzyme Eco RV. There are three Eco RV recognition sites in the *tsr* DNA sequence (*12*), and none in the remainder of plasmid pCM211. Two of the sites (RV_1 and RV_2) are in positions corresponding to the periplasmic domain just amino terminal to the second transmembrane sequence, and one (RV_3) is carboxyl terminal to the second transmembrane sequence in the cytoplasmic domain (Fig. 4, upper panel). The DNA sequence of *tsr* shows all three sites to be oriented such that deletions between them should retain the original translational reading frame. Thus, we expected that a derivative of plasmid pCM211 lacking either the RV_1–RV_3 fragment or the RV_2–RV_3 fragment would encode a hybrid protein with a periplasmic alkaline phosphatase moiety showing increased activity (Fig. 4, lower panel). Plasmid pCM211 was extensively digested with Eco RV, treated with ligase and transformed into *phoA*$^-$ cells with selection for transformants on nutrient agar containing an alkaline phosphatase indicator. Surprisingly, most (approximately 95 percent) of transformant colonies were Pho$^-$. Plasmids present in three of these Pho$^-$ colonies examined were found by restriction analysis to correspond to precise RV_1–RV_3 deletion derivatives of plasmid pCM211 (one of which is called pw1). Plasmids from four Pho$^+$ colonies were also analyzed. Three of these carried precise RV_2–RV_3 deletions (one of which is called pb1), whereas one of the four (called pb4) apparently contained an RV_1–RV_3 deletion with additional small loss of DNA such that an Eco RV site was not regenerated when the two Eco RV ends were joined.

A simple explanation for these unexpected findings was that the sequence of *tsr* in our plasmid differed from the published (*12*) sequence, such that the RV_1 site was not oriented the same way relative to the *tsr* coding sequence as the RV_2 and RV_3 sites. Thus, a precise RV_1–RV_3 deletion derivative of plasmid pCM211 (for example, pw1) would show little alkaline phosphatase activity because the *phoA* sequence would be out of frame with the amino terminal *tsr* sequence. Deletion of the RV_1–RV_3 fragment and additional DNA (as in pb4) could place the *phoA* sequence in frame and lead to high enzymatic activity. In support of this explanation, we detected hybrid *tsr-phoA* proteins of the appropriate size precipitated by antibody to alkaline phosphatase in cells carrying plasmids pb1 and pb4, but not in cells carrying plasmid pw1 (*18*). Direct DNA sequence analysis confirmed the existence of two differences between the sequence of the *tsr* gene in our plasmid and that previously reported (*12*), leading to a difference in the translational reading frame in the region containing the Eco RV_1 site (*20*). In summary, our results indicated that when the original reading frame was maintained, two different deletions covering the second transmembrane sequence of Tsr protein activated the low activity hybrid protein encoded by a cytoplasmic domain *tsr-phoA* fusion.

Stability and cellular location of fusion proteins. We examined the stabilities of representative *tsr-phoA* fusion proteins in pulse-chase experiments. Of three fusions of alkaline phosphatase to the Tsr periplasmic domain examined, the hybrid protein encoded by one (pCM204) was stable (with a half-life of more than 90

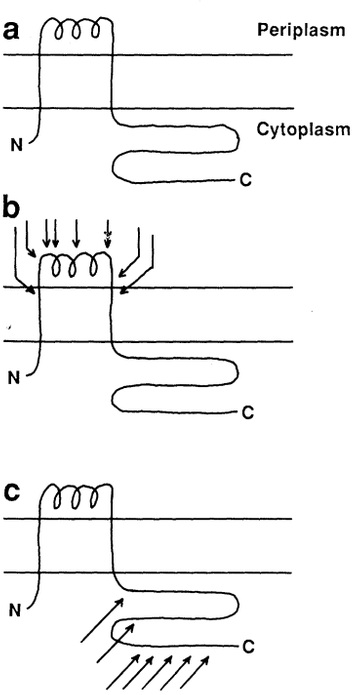

Fig. 2. Fusions of alkaline phosphatase to chemoreceptor proteins. (a) The proposed membrane topology of chemoreceptor proteins. The periplasmic domains of these proteins contain approximately 170 amino acid residues, and the cytoplasmic domains contain approximately 330 amino acid residues. (b) Sites of alkaline phosphatase fusion to chemoreceptor proteins leading to high alkaline phosphatase activity. (c) Sites of in-frame alkaline phosphatase fusions to Tsr protein leading to low alkaline phosphatase activity. The positions of fusions to Tsr protein (four fusions), Tar protein (four fusions), and Tap protein (one fusion) are presented together in (b). Two independently isolated high activity *tsr-phoA* fusions had their fusion junctions at identical positions, and are indicated by the double arrowhead. Of the seven in-frame low activity fusions, two were isolated after Tn*phoA* insertion into a plasmid carrying *tsr*, and five were isolated in two steps after insertion of Tn*phoA* into a plasmid carrying a deletion derivative of *tsr* (Fig. 3). Plasmids carrying Tn*phoA* insertions were isolated either as described before (*17*) using F42 *lacI3* zzf-2::Tn*phoA*, or using an integration-deficient, replication-deficient phage lambda derivative carrying Tn*phoA* (*31*). With either technique, plasmids carrying Tn*phoA* were isolated after transformation of Pho$^-$ cells by selection for the presence of a drug-resistance marker (kanamycin resistance) carried by Tn*phoA*. The selection plates also contained the alkaline phosphatase indicator 5-bromo-4-chloro-3-indolyl phosphate, making it possible to distinguish transformant colonies exhibiting different levels of alkaline phosphatase activity. Fusion plasmids leading to increased alkaline phosphatase activity were isolated and analyzed by restriction mapping to determine the sites of Tn*phoA* insertion. The insertion positions of nine *tsr-phoA* plasmids were further analyzed by DNA sequence analysis (Table 1). Plasmids carrying the chemoreceptor genes (pJFG5 for *tsr*, and pMK1 for *tar* and part of *tap*) were provided by M. Manson.

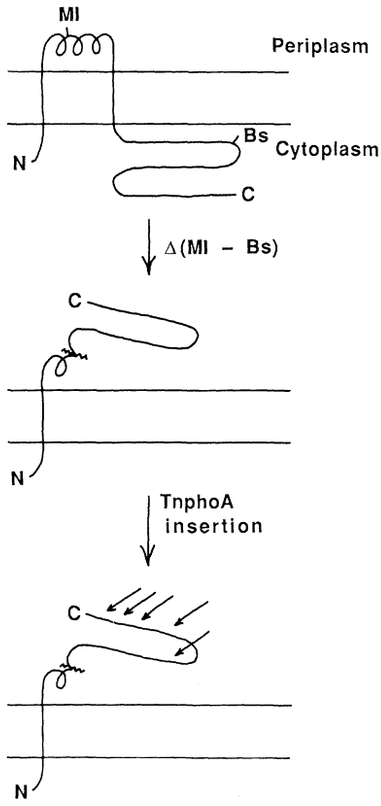

Fig. 3. Fusions of alkaline phosphatase to a deletion derivative of Tsr protein. Sequences encoding a part of Tsr protein that includes its second transmembrane segment were removed by deleting the *tsr* gene of plasmid pJFG5 of DNA between its only Mlu I (MI) site and Bss HII (Bs) site (upper panel). This manipulation results in an in-frame deletion in which the normally cytoplasmic carboxyl terminal domain of Tsr protein may be translocated into the periplasm (middle panel). The positions of five different highly active fusions of alkaline phosphatase to this deletion derivative are shown in the lower panel. Five of the fusions shown in Fig. 2c were generated by replacing the deleted *tsr* sequence of these fusions with the normal sequence by means of recombinant DNA techniques.

minutes), whereas those of two others (pCM201 and pCM203) were degraded (with half-lives of approximately 20 minutes) to give polypeptides about the size of wild-type alkaline phosphatase (48 kD). The protein encoded by plasmid pCM235 was also degraded to give a fragment the size of alkaline phosphatase (with a half-life of about 16 minutes). Earlier studies showed that β-lactamase–alkaline phosphatase hybrids secreted to the periplasm generally were degraded to give a fragment the size of alkaline phosphatase (*17, 21*). Thus, it appears that many (but not all) fusion proteins expected to have their alkaline phosphatase moieties in the periplasm are degraded to yield alkaline phosphatase-sized fragments. Perhaps this degradation results from the action of a periplasmic protease on amino acid sequences near the fusion junctions of such proteins.

Two fusions of alkaline phosphatase to the cytoplasmic domain of Tsr protein (encoded by plasmids pCM211 and pCM252) were both somewhat stable (with half-lives of 30 to 60 minutes). Cytoplasmic alkaline phosphatase is generally quite unstable (with a half-life of less than 10 minutes), and these two hybrid proteins present the first apparent exceptions to this general behavior.

We examined the cellular locations of three *tsr-phoA* fusion proteins and their breakdown products by cell fractionation. The full-length hybrid proteins of both periplasmic and cytoplasmic domain fusions fractionated predominantly with the membrane (Table 2). Two of the hybrid proteins examined were ones which were degraded to fragments the size of alkaline phosphatase, and these fragments were recovered in both the periplasmic and membrane fractions (Table 2). These findings are consistent with a periplasmic location of the alkaline phosphatase moieties of these hybrid proteins, with release of alkaline phosphatase fragments into the periplasm by proteolysis near the fusion junctions.

Identifying membrane protein topology with protein fusions. In this article we describe an analysis of Tsr protein, a protein involved in chemotaxis whose topology in the *E. coli* cytoplasmic membrane is relatively well established. We have found that a strict correlation exists between the cellular location of the domain of Tsr protein to which alkaline phosphatase is fused and the level of alkaline phosphatase activity observed. Hybrid proteins with alkaline phosphatase fused at sites in or near the Tsr protein periplasmic domain gave at least 20 times more activity than those with alkaline phosphatase fused at sites in the cytoplasmic domain. An analysis of alkaline phosphatase fusions may thus be generally useful in identifying the transmembrane topology of cytoplasmic membrane proteins. Unlike the most commonly used biochemical techniques for determining membrane protein topology, this genetic approach does not rely on the exposure or chemical reactivity of amino acid sequences positioned at one side or the other of the membrane. The approach thus provides an independent method for acquiring topological information.

Although the correlation we have observed for Tsr protein is striking, there are a number of potential limitations in using alkaline phosphatase fusions to identify membrane protein topology. The method relies on the cytoplasmic or periplasmic location of a membrane protein domain not being altered by fusion of the domain to alkaline phosphatase. Such a behavior requires both that sequences of the membrane protein carboxyl terminal to the fusion junction of a hybrid protein be nonessential to the normal localization of the domain, and that the alkaline phosphatase does not itself dominate the localization behavior of the hybrid. We can imagine instances in which these conditions would not be met. For example, if a domain were stably held in a cytoplasmic position by interac-

Table 2. Fractionation of *tsr-phoA* hybrid proteins. Cells [strain CC118 (*17*) carrying F42 *lacI3* and the plasmid listed] were fractionated as described (*30*) except for slight modifications. Exponentially growing cells (1.5 ml) in M63 medium supplemented with 19 amino acids were exposed to [^{35}S]methionine (40 to 80 μCi) for 5 to 15 minutes at 37°C. Cultures were then cooled and centrifuged (3 minutes, 15,600*g*, 4°C). The cell pellets were resuspended in 150 μl of cold spheroplast buffer [100 m*M* tris-HCl, *p*H 8.0, 0.5 m*M* EDTA, 0.5 m*M* sucrose, and phenylmethylsulfonyl fluoride (PMSF) (20 μg/ml)] and incubated for 5 minutes on ice. A 50-μl sample was frozen and considered as the whole cell fraction. The remaining 100 μl was centrifuged (3 minutes, 15,600*g*, 4°C); the pellet was warmed to room temperature, and then resuspended with shaking in 100 μl of ice-cold water. After 45 seconds on ice, 5 μl of 20 m*M* $MgCl_2$ was added. The osmotically shocked cells were centrifuged (3 minutes, 15,600*g*, 4°C), and the supernatant was saved as the periplasmic fraction. The pellet was resuspended in 150 μl of cold spheroplast buffer, to which was added 15 μl of lysozyme at 2 mg/ml and 150 μl of cold water. Cells were incubated for 5 minutes on ice, and then centrifuged (3 minutes, 15,600*g*, 4°C). The pellet was resuspended in 600 μl of 10 m*M* tris-HCl, *p*H 8.0, and PMSF at 20 μg/ml and subjected to three cycles of freezing and thawing; 20 μl of 1*M* $MgCl_2$ and 6 μl of deoxyribonuclease I at 1 mg/ml were added. The lysed spheroplasts were then centrifuged (25 minutes, 15,600*g*, 4°C). The pellet corresponded to the membrane fraction, and the supernatant (which was normally concentrated sixfold by precipitation with trichloroacetic acid before immunoprecipitation) corresponded to the cytoplasmic fraction. Radioactively labeled protein in each fraction was quantitated after precipitation by antibody to alkaline phosphatase and electrophoresis (*17*). The behavior of proteins of known cellular location in this protocol has been described (*17*). In all cases analyzed, the total protein profile of each fraction subjected to immunoprecipitation was analyzed by SDS–polyacrylamide gel electrophoresis to check qualitatively that it corresponded to that characteristic of the fraction and did not show excessive contamination with proteins of other fractions.

Plasmid	Protein size* (kD)	Protein distribution (percent)		
		Cytoplasm	Membrane	Periplasm
pCM203	66	<1	97	3
pCM203	48	7	23	70
pCM235	76	1	98	1
pCM235	48	7	26	67
pCM252	104	<1	97	2

*Calculated from the Tn*phoA* insertion position or determined using SDS–polyacrylamide electrophoresis.

tions between the transmembrane sequences on each side of it, the loss of the carboxyl terminal transmembrane sequence in a fusion protein would lead to the domain (and its attached alkaline phosphatase) being abnormally localized to the periplasm. For a protein with transmembrane segments in a beta conformation, a structure requiring hydrogen bonds between the transmembrane sequences to be stable (*22*), misleading results from a fusion analysis would seem likely. Fusions of alkaline phosphatase within transmembrane sequences have not been fully enough analyzed to predict their behaviors. Although cytoplasmic location of alkaline phosphatase in a fusion protein is never expected to give high enzymatic activity, low activity does not guarantee a cytoplasmic position of the junction site. For example, it appears that alkaline phosphatase must dimerize to show enzymatic activity (*23*), and it is possible that a hybrid protein with a periplasmic alkaline phosphatase moiety could lack activity because dimerization was sterically hindered. In addition, it may not even be possible to isolate fusions at some positions because production of the corresponding hybrid protein would be detrimental to the cell. Because of these potential limitations, we believe that it is essential that this gene fusion approach be used in conjunction with established biochemical techniques to determine membrane protein topology.

It is not yet known whether alkaline phosphatase fusions can be successfully used to analyze proteins with membrane topologies more complex than that of Tsr protein. However, the initial results of an analysis of one such protein involved in maltose uptake (the MalF protein) appear to be compatible with the topology of the protein predicted from its amino acid sequence (*24*).

Implications for membrane protein insertion. The shortest active *tsr-phoA* fusion has its junction at the end of the first transmembrane sequence of Tsr protein. A similar result has been obtained for MalF protein (*18*). These findings suggest that a single transmembrane sequence can function like a signal sequence to promote alkaline phosphatase export to the periplasm. Presumably, such sequences function analogously in promoting the translocation of periplasmic domains of the normal (unfused) membrane proteins. Perhaps such transmembrane sequences differ fundamentally from the signal sequences of proteins secreted completely through the membrane only in their not being cleaved after export from the cytoplasm has occurred. Because they are not removed, such sequences help anchor the proteins in the membrane after insertion and contribute to the proteins' cellular functions (for example, in chemotaxis or transport).

Seven different hybrid proteins with alkaline phosphatase fused to the cytoplasmic domain of Tsr protein showed low enzyme activity, evidently because the alkaline phosphatase moiety is sequestered on the cytoplasmic side of the cytoplasmic membrane (Fig. 1). Cytoplasmic alkaline phosphatase does not appear to exhibit enzymatic activity (*11, 17, 21*). These results thus indicate that localizing the alkaline phosphatase moiety close to the membrane does not suffice to promote its efficient export. This finding argues against a model in which the sole function of the normal alkaline phosphatase signal sequence in export is to bring the protein close to the cytoplasmic membrane where it can spontaneously translocate into the periplasm.

Fusions of alkaline phosphatase to the Tsr protein cytoplasmic domain do show a low but significant level of alkaline phosphatase activity (Table 1). The source of this activity is not known, but it may arise from an inefficient export of the alkaline phosphatase moiety of such hybrids.

Hybrid proteins with alkaline phosphatase fused to the cytoplasmic domain of Tsr protein showed high activity in derivatives lacking the second transmembrane sequence of Tsr protein. Three different deletions gave such increased activity, and what at first

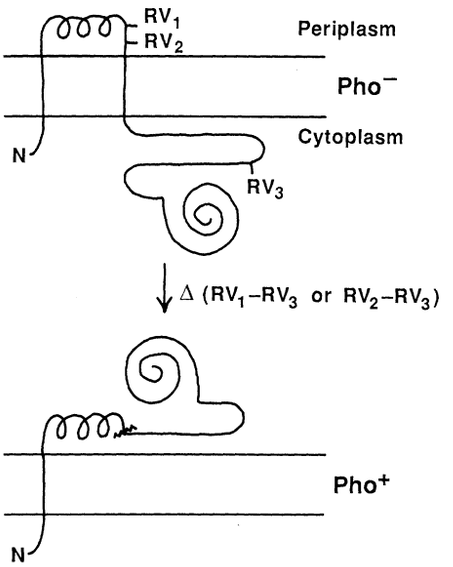

Fig. 4. Activation of the alkaline phosphatase in a cytoplasmic domain hybrid protein. The expected structure of the low activity *tsr-phoA* hybrid protein encoded by plasmid pCM211 is shown in the upper panel along with the positions in the hybrid protein corresponding to the three Eco RV recognition sites. Alkaline phosphatase is represented as a spiral. Deletion of sequences between RV_1–RV_3 or RV_2–RV_3 removes the second transmembrane sequence of Tsr protein and may allow translocation of the alkaline phosphatase moiety to the periplasm, where it is expected to show increased enzymatic activity (lower panel).

appeared to be an exception to this general behavior led to the discovery of a difference between the published *tsr* DNA sequence and that in the plasmid we have analyzed. These findings suggest that the second transmembrane sequence (and surrounding sequences) is responsible for holding the alkaline phosphatase moiety of such hybrids in the cytoplasm. Such a model is consistent with other evidence indicating that hydrophobic sequences can anchor proteins in membranes (*19*). Our results further indicate that much of the Tsr protein cytoplasmic domain sequence can pass through the cytoplasmic membrane into the periplasm. This behavior contrasts markedly with that of the cytoplasmic protein β-galactosidase, which appears to "jam" in the membrane if the cell attempts to export it (*25*).

Our studies indicate that the two transmembrane sequences rather than the entire structure of the Tsr protein are the primary determinants of how the protein inserts into the membrane. It is simple to picture the insertion process as being due to a sequential functioning of the transmembrane sequences as export signals. The first transmembrane sequence could initiate translocation of the Tsr polypeptide chain and the second transmembrane sequence could terminate its export. This sequential functioning of export signals need not correspond to actual cotranslational translocation across the lipid bilayer. It could also correspond, for example, to a sequential interaction with components of a secretory apparatus, with actual translocation occurring later relative to translation (*26*).

REFERENCES AND NOTES

1. D. Leifer and R. Henderson, *J. Mol. Biol.* **163**, 451 (1983).
2. J. Deisenhofer, O. Epp, K. Miki, R. Huber, H. Michel, *Nature (London)* **318**, 618 (1985).
3. J. Kyte and R. F. Doolittle, *J. Mol. Biol.* **157**, 105 (1982); D. M. Engelman, T. A. Steiz, A. Goldman, *Annu. Rev. Biophys. Chem.* **15**, 321 (1986).
4. J. C. Williams, L. A. Steiner, G. Fehler, M. I. Simon, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7303 (1984); D. C. Youvan, E. J. Bylina, M. Alberti, H. Begusch, J. E. Hearst, *Cell* **37**, 949 (1984).
5. W. Lennarz, *Methods Enzymol.* **98**, 91 (1983).
6. W. Springer and D. E. Koshland, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 533 (1977).
7. J. A. Walder *et al.*, *J. Biol. Chem.* **259**, 10238 (1984); S. O. Nelson, J. K. Wright, P. W. Postma, *EMBO J.* **2**, 715 (1983).

8. H.-D. Lemke, J. Bergmeyer, J. Straub, D. Oesterhelt, *J. Biol. Chem.* **257**, 9384 (1982); M. Dumont, J. Trewhella, D. M. Engelman, F. M. Richards, *J. Membr. Biol.* **88**, 233 (1985); N. Carrasco, D. Herzlinger, W. Danho, H. R. Kaback, *Methods Enzymol.* **125**, 453 (1986).
9. J. Brunner, *Trends Biochem. Sci.* **6**, 44 (1981).
10. J. P. Rosenbusch, *J. Biol. Chem.* **249**, 8019 (1974); J. A. Reynolds and W. Stoeckenius, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2803 (1977); H. Vogel, J. K. Wright, F. Jaehnig, *EMBO J.* **4**, 3625 (1985).
11. S. Michaelis, H. Inouye, D. Oliver, J. Beckwith, *J. Bacteriol.* **154**, 366 (1983).
12. A. Boyd, K. Kendall, M. I. Simon, *Nature (London)* **301**, 623 (1983).
13. A. Boyd, A. Krikos, M. I . Simon, *Cell* **26**, 333 (1981); A. Russo and D. E. Koshland, *Science* **220**, 1016 (1983); J. Bollinger, C. Park, S. Harayama, G. L. Hazelbauer, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3287 (1984).
14. Mowbray, D. L. Foster, D. E. Koshland, *J. Biol. Chem.* **260**, 11711 (1985).
15. M. R. Kehry, M. W. Bond, M. W. Hunkapiller, F. W. Dahlquist, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3599 (1983).
16. A. Krikos, M. Patricia-Conley, A. Boyd, H. Berg, M. I. Simon, *ibid.* **82**, 1326 (1985).
17. C. Manoil and J. Beckwith, *ibid.*, p. 8129.
18. C. Manoil, unpublished results.
19. M. J. Gething and J. Sambrook, *Nature (London)* **300**, 598 (1982); G. Adams and J. Rose, *Cell* **41**, 1007 (1985); N. Davis, J. Boeke, P. Model, *J. Mol. Biol.* **181**, 111 (1985).
20. The sequence of *tsr* in the plasmid that we have used (pJFG5) differs from the published sequence (*12*) in containing an additional adenine residue at the codon corresponding to lysine-147, and one, rather than two, adenine residues at the codon corresponding to asparagine-161 (C. Manoil, M. I. Simon, J. Beckwith, in preparation).
21. C. Hoffman and A. Wright, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5107 (1985).
22. C. Tanford, *The Hydrophobic Effect* (Wiley, New York, 1980), p. 208.
23. S. McCracken and E. Meighen, *J. Biol. Chem.* **254**, 2396 (1979).
24. S. Froshauer and J. Beckwith, *ibid.* **259**, 10896 (1984); D. Boyd and C. Manoil, unpublished results.
25. P. Bassford, T. J. Silhavy, J. Beckwith, *J. Bacteriol.* **139**, 19 (1979).
26. C. Lee and J. Beckwith, *Annu. Rev. Cell Biol.*, in press.
27. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1982).
28. J. Gebert, M. Manson, W. Boos, unpublished data.
29. J. Messing, *Methods Enzymol.* **101**, 20 (1983).
30. D. Koshland and D. Botstein, *Cell* **30**, 893 (1982); B. R. Copeland, R. J. Richter, C. E. Furlong, *J. Biol. Chem.* **257**, 15065 (1982).
31. J. Barondess and C. Manoil, unpublished results.
32. We dedicate this paper to Hiroshi Inouye, who died on 24 July 1986. Hiroshi's pioneering work on alkaline phosphatase was essential to the evolution of the present study. We thank D. Boyd for useful discussions, C. Lee for artistic contributions, and M. Manson, J. Gebert, and M. Kossmann for the gift of plasmids. Supported by a research grant from the American Cancer Society (J.B.) and a fellowship (C.M.) from the Arthritis Foundation.

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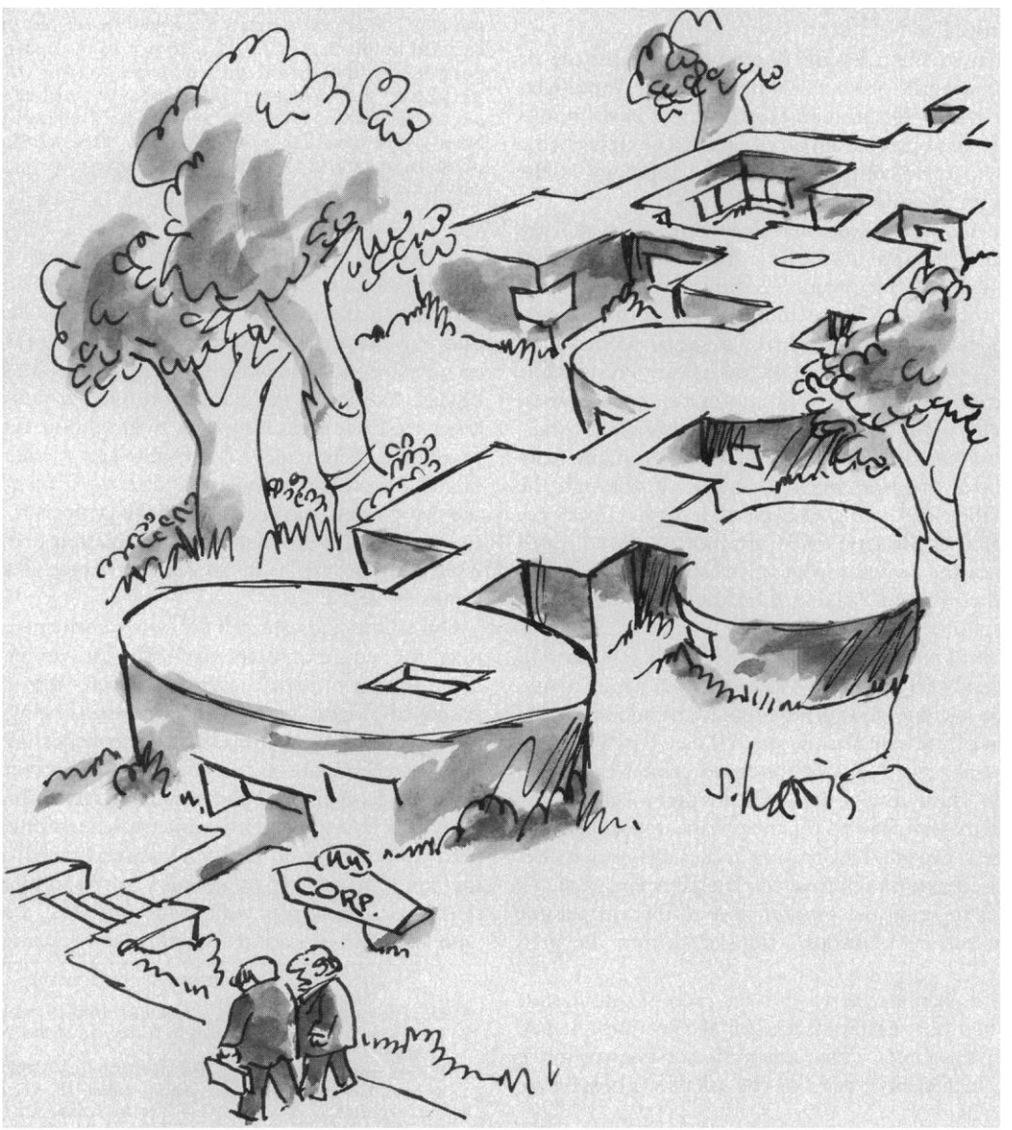

"It's bound to be efficient. It's a flow chart."