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Genetics

Genetic analysis of membrane protein topology by a sandwich gene fusion approach

(alkaline phosphatase/MalF protein/*phoA* cassette)

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ABSTRACT **We describe a cloning vector that allows the construction of *phoA* sandwich fusions in which mature alkaline phosphatase is inserted into target proteins. In contrast to previous fusions obtained using the Tn*phoA* transposon, the entire amino acid sequence of the target protein is present in the fusion product. We have constructed a series of sandwich fusions of alkaline phosphatase to the multispanning cytoplasmic membrane protein MalF. Despite the fact that the alkaline phosphatase was tethered to MalF at both its N and its C terminus, the enzyme exhibited high activity when it was fused to a periplasmic domain of the membrane protein. Cells harboring an alkaline phosphatase sandwich fusion to the end of the first membrane-spanning segment of MalF exhibited both MalF and alkaline phosphatase activity. When alkaline phosphatase was inserted into a cytoplasmic domain of MalF, its specific activity was very low. Our results suggest that the alkaline phosphatase activity of *phoA* sandwich fusions provides a more sensitive monitor than previous methods of the cellular localization of the domain of the target protein to which the enzyme is fused. Thus, the sandwich fusion approach can give a more accurate picture of membrane protein topology.**

A previous paper (1) described a genetic strategy for analyzing the topology of integral membrane proteins. The approach involves the use of fusions to the *phoA* gene, which codes for alkaline phosphatase (AP) of *Escherichia coli*. The method depends on four features of AP. (*i*) The enzyme is only active when it is translocated through the cytoplasmic membrane into the periplasm. It is inactive when localized to the cytoplasm. (*ii*) The signal sequence of AP can be replaced by export signals derived from other proteins, including those found in integral membrane proteins. (*iii*) The mature part of AP is neutral with respect to export. It contains no export information itself. (*iv*) Extensive amino acid sequences from other proteins can be fused to the N terminus of AP without interfering with its activity. Thus, with a cytoplasmic membrane protein, only AP fusions to periplasmic domains of the protein, which are preceded by an export signal, show high AP enzymatic activity. Fusions to cytoplasmic domains exhibit low activity. As a result, by determining the AP activity of fusions to different hydrophilic domains of such proteins, it is possible to distinguish cytoplasmic from periplasmic domains and, in this way, determine the membrane topology of the protein.

Both *in vivo* and *in vitro* systems for generating *phoA* fusions have been developed. Tn*phoA*, a derivative of Tn*5*, allows the *in vivo* isolation of *phoA* fusions to plasmid-encoded as well as chromosomal genes (2, 3). *In vitro*-constructed fusions can be obtained by the use of plasmids containing the *phoA* gene with conveniently placed restriction sites (4, 5).

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The AP fusion approach has been successfully used to study the topology of a number of cytoplasmic membrane proteins (6). However, we have noted a potential limitation to the approach. The fusions are generated in such a way that AP replaces a C-terminal portion of the membrane protein. If assembly into the correct topological structure depends upon interactions between N-terminal and C-terminal portions of the membrane protein, the fusion approach could give an incorrect picture. While this potential limitation does not appear to have been a problem in most of the analyses done so far, we have found situations in which C-terminal information is required for proper assembly (7, 8). In the case of two cytoplasmic membrane proteins, MalF and leader peptidase, we have found that *phoA* fusions to the beginning (N-terminal) of cytoplasmic domains of the proteins exhibit 7- to 20-fold higher AP activity than fusions to the end (C-terminal) of the same domains. In an extreme case, a fusion to a cytoplasmic domain of the MalF protein exhibited almost as much AP activity as a fusion to a periplasmic domain. In these cases, the AP enzymatic activity corresponds to AP exported to the periplasm (C. A. Lee, D.B., and J.B., unpublished results).

We have shown (9) that the C-terminal information, which is missing in certain fusion proteins and which is required for proper topological assembly of the MalF fusion protein, involves the positively charged amino acids usually found (10) in cytoplasmic domains of such proteins. Positively charged amino acids appear to be required to stably anchor the fused AP as well as the hydrophilic segment of MalF in the cytoplasm.

In this study, in order to overcome the limitation to the Tn*phoA* fusion approach described above, we have constructed a new class of AP fusions. In these fusions, rather than replacing the C-terminus of the membrane protein MalF with AP, we constructed fusions in which AP is inserted into an otherwise intact MalF protein. This was done either by a series of steps using the AP fusions obtained before or by the use of a newly constructed cassette that allows the direct generation of what we call sandwich fusions. In a periplasmic sandwich fusion, AP exhibits normal enzymatic activity even though it is tethered at both its N and C termini. In contrast, cytoplasmic Tn*phoA* fusions that resulted in high levels of AP export, when converted into sandwich fusions, show very low levels of AP activity. The sandwich fusion approach thus provides a more accurate way of examining membrane protein topology as well as providing a tool for the analysis of a number of other biological problems.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. *E. coli* strains used were ME 1201, which is a *recA*::*cat* (taken from strain BW 10724, which was a gift of Barry Wanner, Purdue University)

Abbreviation: AP, alkaline phosphatase.

derivative of DHB4 [*araD139* Δ(*ara–leu*)7697 *lacX74 phoA* Δ[*Pvu* II]*phoR malF3 galE galK thi rpsL* F'*lacI*^{Q} *pro*; ref. 7] and DHB24, which is DHB4 *pcnB zad*::Tn*10* (11). Phage f1R408 (12) was used to prepare single-stranded DNA template from plasmids, which were all derivatives of pDHB32 (7). Phage m13tg130 (13) was used to construct the polylinker at the 3' end of *phoA* in pSWFII. pDHB32 contains the *E. coli malF* gene and part of the *malG* gene expressed under the *tac* promoter of pKK223-3 (14). It also contains the M13 intergenic region (15).

Media, Cell Growth, and Enzymes. Media are according to Miller (16). Proteins were labeled in cultures growing exponentially at 37°C in M63/0.2% glycerol, supplemented with each common amino acid except methionine, by exposing them to [^{35}S]methionine at 50 μCi/ml (1 μC = 37 kBq) for 2 min. T4 gene *32* protein was obtained from Boehringer Mannheim. All the other enzymes were obtained from New England Biolabs.

AP Assay and Maltose Transport Assay. AP activity in strains was assayed by measuring the rate of *p*-nitrophenyl phosphate hydrolysis in permeabilized cells (17). Data for multicopy plasmids were obtained from uninduced logarithmic-phase cultures. Single-copy data were obtained as described (7), after 160 min of induction of log-phase cultures with 5 mM isopropyl β-D-thiogalactoside. The initial rate of maltose uptake was determined as described by Brass *et al.* (18).

Antibody Precipitation and Gel Electrophoresis. These were done as described by Ito *et al.* (19) and Laemmli (20), respectively.

Oligonucleotide-Directed Mutagenesis. This was done as described (7) except that DNA remaining single-stranded was not digested with mung bean nuclease. Where mentioned in the text, transformants were screened for the altered genotype by colony hybridization using the ^{32}P-end-labeled oligonucleotide as a probe (21).

Construction of Sandwich Fusions *in Vitro*. To construct the *malF–phoA* sandwich fusion at the *Pvu* II site, the *Sal* I–*Bst*EII fragment containing *phoA* of pSWFII was filled in with Klenow DNA polymerase and cloned into the *Pvu* II site of *malF*. To construct the malF–phoA sandwich fusion at the *Bss*HII site, the *Xma* I–*Bst*EII *phoA* fragment of pSWFII was filled in with Klenow DNA polymerase and cloned into the *Bss*HII site of *malF* that had been cut back with mung bean nuclease. Both fusions do have two additional amino acids at the C terminus of AP: glycine and tyrosine. At the N terminus of AP the *Pvu* II sandwich fusion has Ser-Thr-Leu-Glu-Asp-Pro-Arg-Val-Pro-Asp added to the 5' Tn*phoA* linker region (2). In the *Bss*HII sandwich fusion, one alanine of MalF was deleted due to cutting back the *Bss*HII site. At the N terminus of AP, Arg-Val-Pro-Asp was added to the 5' Tn*phoA* linker region.

Conversion of *malF*–Tn*phoA* Fusions to *malF'–phoA–'malF* Sandwich Fusions. This set of sandwich fusions was constructed in three steps (Fig. 1). A 56-mer oligonucleotide was introduced into each fusion strain (Fig. 1*A*) in order to remove the stop codon in *phoA* and to extend the reading frame into a polylinker containing the three restriction sites *Bst*EII, *Bss*HII, and *Sac* I which are present in *malF* as unique sites (Fig. 1*B*). In each case this alteration had no effect on phenotype. The desired candidates were identified by colony hybridization using the oligonucleotide as a probe and subsequently tested for the presence of the additional restriction sites. From these constructs a *Bst*EII fragment was isolated that contained *malF* material 5' to *phoA* and *phoA* itself lacking its stop codon (Fig. 1*B*). In the second step, this *Bst*EII fragment was cloned in frame into the *Bst*EII site of wild-type *malF* (Fig. 1*C*). At this stage, a rather complex construct had been generated: *malF'–phoA–'malF'–'malF* (Fig. 1*D*). The size of the 'malF' material that had been duplicated during the cloning step was dependent on the fusion joint. It was only 12 base pairs (bp) in the case of the B fusion, but was 824 and 835 bp for the K and L fusions, respectively. The M fusion was converted to a sandwich fusion somewhat differently. Here, instead of using the *Bst*EII site for subcloning of the altered *malF–phoA* fragment, a *Sac* I fragment was used. Thus the duplicated *'malF'* material was only 354 bp in size. To fuse the 3' end of *phoA* to the fusion joint in *malF* located downstream of *phoA*, the duplicated *'malF'* material was deleted by using specific oligonucleotides for each fusion.

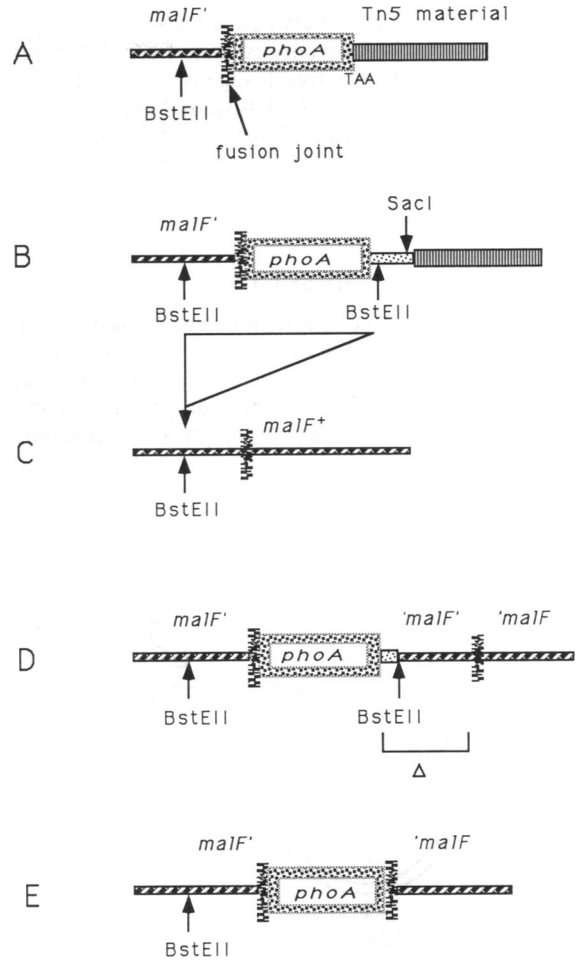


FIG. 1. Conversion of *malF*–Tn*phoA* fusions to *malF'–phoA–'malF* sandwich fusions *in vitro*. (*A*) *malF*–Tn*phoA* fusion. TAA is the termination codon. (*B*) An oligonucleotide is introduced that deletes the stop codon of *phoA* and inserts a linker (stippled box) containing restriction sites. (*C*) The *Bst*EII fragment is excised and cloned into the *Bst*EII site of wild-type *malF*. (*D*) The 3' end of *phoA* is fused to the original downstream fusion joint by deleting the part of *malF* that had been duplicated during the previous cloning step. (*E*) The *malF'–phoA–'malF* sandwich fusion.

Insertion of Sandwich Fusions into the Chromosome in Single Copy. Sandwich fusions cannot be inserted into the chromosome by homologous recombination at the *phoA* locus as *malF*–Tn*phoA* fusions have been (7). Therefore, we have constructed a λ shuttle vector, λDBK261, which permits transfer of fusion genes between plasmids and bacteriophage λ by homologous recombination. This phage contains the Tn*903* kanamycin-resistance gene flanked on one side by the *tac* promoter of DHB32 (7) and on the other side by part of the *bla* gene and part of the M13 intergenic region from the same plasmid. Lysates of DBK261 grown on strains containing pDHB32 derivatives contain recombinant ampicillin-resistant transducing phage in which the missing part of the

bla gene and whatever is between it and the *tac* promoter have replaced the kanamycin-resistance gene. Ampicillin-resistant, kanamycin-sensitive lysogens derived from such phage containing *malF*–Tn*phoA* fusions have AP activities similar to those reported previously (7). In some cases the identity of the λ recombinants was confirmed by recombination back onto the plasmid shuttle vector pDBK6, which is similar to the phage.

RESULTS

Construction of a *phoA* Sandwich Fusion Cassette. We have constructed a plasmid (pSWFII, Fig. 2) that contains a *phoA* cassette that can be inserted by a simple cloning step at restriction sites in structural genes for proteins. With the appropriate choice of restriction enzymes, the resulting fusion will encode a hybrid protein with AP tethered at both its N and C termini to the target protein. All the sequences from the native target protein are present in the hybrid protein. The plasmid contains most of *phoA* but lacks the coding region for its signal sequence and five additional amino acids. The *phoA* gene is flanked by polylinkers containing multiple restriction sites. The open reading frame of *phoA* extends into both linker fragments. The polylinker at the 5′ end of *phoA* was taken from pPHO7 (5), a plasmid used for the construction of Tn*phoA* fusions *in vitro*. The polylinker at the 3′ end of *phoA* has a TAG stop codon in the *Xba* I site. It is therefore possible to construct *phoA* sandwich fusions as well as Tn*phoA* fusions by using the same cassette. If the restriction sites upstream of the stop codon are used, sandwich fusions are generated. If the sites downstream of the stop codon are used, fusions comparable to those obtained with Tn*phoA* are generated. The difference from the original Tn*phoA* fusions is the presence of five additional amino acids at the C terminus of AP. These Tn*phoA*-like fusions could be converted to sandwich fusions in a strain carrying an amber suppressor.

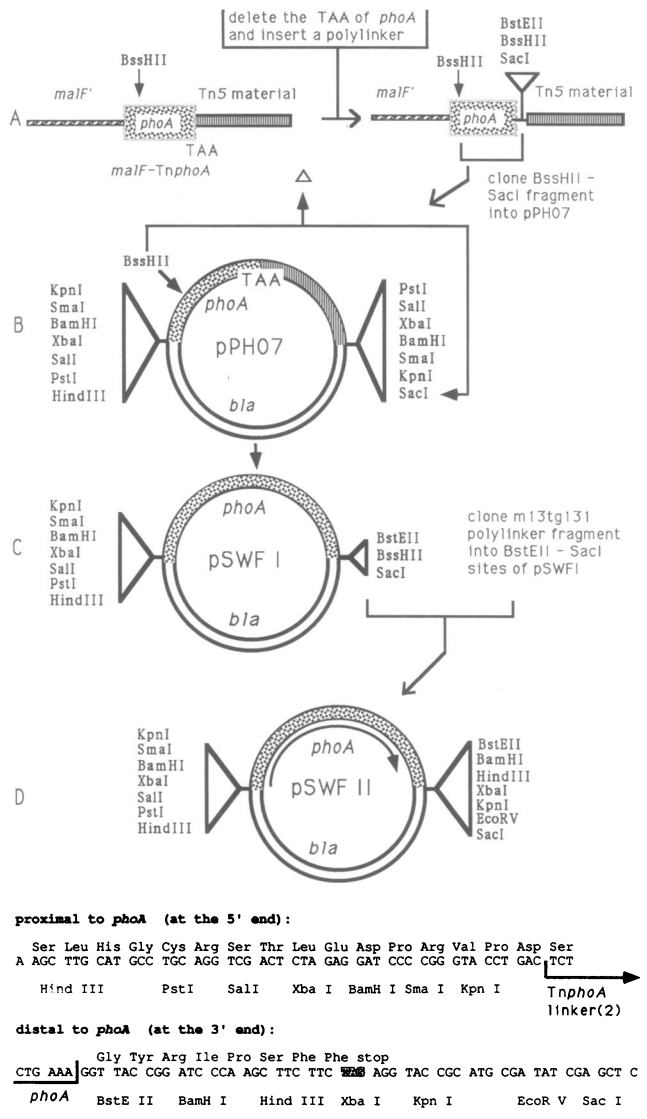
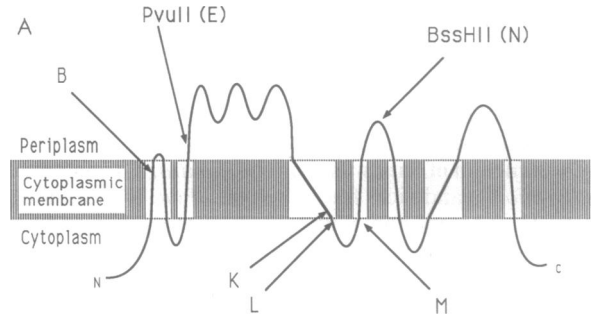


FIG. 2. The construction of the *phoA* sandwich fusion cassette pSWFII. pSWFII was made by inserting a polylinker at the 3′ end of *phoA* between the last codon of *phoA* and its stop codon, using the Tn*phoA* cassette pPHO7. (*A*) Starting with *malF*–Tn*phoA* fusion B (7), the stop codon of *phoA* was removed and a polylinker containing *Bst*EII, *Bss*HII, and *Sac* I sites was introduced using an oligonucleotide. (*B* and *C*) A 1-kbp *Bss*HII–*Sac* I fragment of *malF*–Tn*phoA* was exchanged with a 2.3-kbp *Bss*HII–*Sac* I fragment of Tn*phoA* cassette pPHO7 (5), yielding pSWFI. (*D*) The polylinker of pSWFI was extended by cloning parts of the phage m13tg131 polylinker into the *Bst*EII–*Sac* I sites of the pSWFI linker. The linker regions of pSWFII are shown below the plasmid diagram.

pSWFII was used to construct one *malF*–Tn*phoA* fusion and two *malF*–*phoA* sandwich fusions *in vitro* by using the *Pvu* II (bp 297) and *Bss*HII (bp 1059) sites of *malF*. The fusion joints are in regions that code for the second and third periplasmic domains of MalF, respectively. The fusion joints are very close to the previously isolated *malF*–Tn*phoA* fusions E (bp 296) and N (bp 1051) (7). As with these Tn*phoA* fusions, the two sandwich fusions had high specific AP



B

Fusion-joints	Tn*phoA* fusions		sandwich fusions	
	Multi copy	Single copy	Multi copy	Single copy
B	1345	30.8	970	6.5
*PvuII (E)	1190	(30)	631	7.0
K	627	8.7	6	<0.1
L	723	17.5	24	0.1
M	230	2.4	5	<0.1
*BSSHII (N)	735	(26)	302	2.7

* these fusions were constructed using pSWFII.

FIG. 3. AP activities of *malF′–phoA–′malF* fusion and *malF′*–Tn*phoA* fusion strains. (*A*) The location of the fusion joints of *malF*–Tn*phoA* and *malF′–phoA–′malF* sandwich fusions used in this study. Each arrow points to the site at which the fusion joint is located in the topological model of MalF. The fusions were present on a multicopy plasmid. The exact fusion joints were as follows: B at bp 94, *Pvu* II (E) at bp 297, K at bp 907, L at bp 921, M at bp 958, and *Bss*HII (N) at bp 1059. (*B*) Comparison of the AP activities of Tn*phoA* and sandwich fusions. Fusions were present on a multicopy plasmid or in single copy inserted into the chromosome. Tn*phoA* fusions were inserted at the *phoA* locus as described (7). Values for the E and N fusions in parentheses are from previously published data (7). Single-copy data have been corrected by subtracting a background value (0.1 unit) measured in the *phoA*-deletion strain DHB4.

activities (Fig. 3). Thus, tethering AP at both its N and C termini does not severely interfere with the enzymatic activity of the protein.

Conversion of *malF*–Tn*phoA* Fusions to *malF'–phoA–'malF* Sandwich Fusions. Since sandwich fusions to periplasmic domains of MalF exhibited AP activities similar to those of related Tn*phoA* fusions obtained earlier, we wished to see whether sandwich fusions to cytoplasmic domains would display different properties. As we have described (7), *malF–phoA* fusions to the beginning of the third cytoplasmic domain of MalF (K and L) export almost as much AP to the periplasm as *phoA* fusions to the preceding and following periplasmic domain of MalF. A fusion to the end of the same cytoplasmic domain (M) exported much less AP, showing about 10 times lower AP activity. We suggested that C-terminal information might be necessary to determine the correct topology of the hybrid protein. To test this hypothesis, we have taken the K, L, and M fusions and, in a series of steps, restored the missing C-terminal portions of MalF. The same procedure was used with one of the previously obtained periplasmic fusions, B. In this way, we converted the *malF*–Tn*phoA* fusions B, K, L, and M to *malF'–phoA–'malF* sandwich fusions. Subsequently, the final constructs (Fig. 1*E*) were tested for the amount, the correct size, and the stability of hybrid protein made as well as for their AP activities.

The AP activities of sandwich fusions to the first, second, and third periplasmic domains were almost as high as those of Tn*phoA* fusions to the same domains (Fig. 3). The sandwich fusions derived from the K, L, and M fusions, all having cytoplasmic fusions joints, exhibited very low AP activities. This is in contrast to the activities seen with the original Tn*phoA* fusions. In particular, the K and L sandwich fusions gave activities 30–100 times lower than the corresponding Tn*phoA*-obtained fusions. The same difference in activities was obtained after transfer of the sandwich fusions and Tn*phoA* fusions in single copy onto the chromosome (Fig. 3). The low AP activity of sandwich fusions K, L, and M are not due to the breakdown of the hybrid proteins (unpublished data). Thus, it appears that we were able to stabilize the cytoplasmic location of the AP in the K and L fusions by adding back the C-terminal part of MalF that had been deleted in the Tn*phoA* fusions to the same base pair of *malF*.

Size, Stability, and Amount of Sandwich Fusion Proteins Made. The size, stability, and amount of sandwich fusion proteins made were tested by doing pulse–chase experiments. To be able to detect AP as well as MalF breakdown products, we used polyclonal antibodies raised against a MalF–PhoA fusion protein. The antibodies recognize various MalF–PhoA fusion proteins, wild-type AP, and wild-type MalF (B. Traxler and J.B., unpublished results).

Since all sandwich fusions contained the complete MalF and AP sequences, they all exhibited the same size in 10% polyacrylamide gels (data not shown). They migrated at about 95 kDa, whereas their predicted molecular mass is about 106 kDa. The difference between predicted and observed molecular mass is probably due to the hydrophobic character of MalF. Wild-type MalF itself migrates at about 17 kDa lower than its actual molecular mass in the same type of gel (22). The difference sites of insertion of AP within MalF apparently did not influence the behavior of the hybrid proteins in polyacrylamide gels.

All six hybrid proteins tested showed little breakdown during 30 min of chase. We could not detect any breakdown products (see Fig. 4). The amount of sandwich fusion proteins made was a little lower than that of Tn*phoA* fusions (data not shown), which is reflected in the observed lower AP activities (Fig. 3). It was also observed that after about 15 min of induction of the *tac* promoter with 10 mM isopropyl

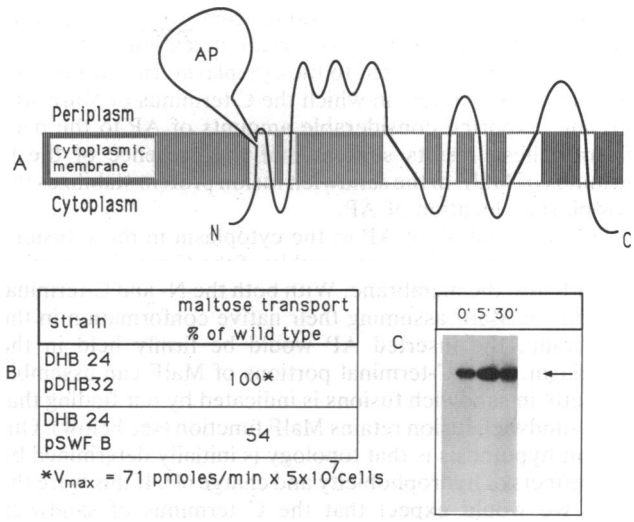


FIG. 4. A bifunctional sandwich fusion. (*A*) Proposed topology of the bifunctional protein encoded by the sandwich fusion *malF'–phoA–'malF* B. (*B*) Maltose transport activity of *malF'–phoA–'malF* sandwich fusion B. For maltose transport assays cells were grown to midlogarithmic phase in M63/0.2% glycerol/0.2% maltose. [^{14}C]-Maltose was applied at a concentration of 6.5 μM. (*C*) The stability of the sandwich fusion protein B examined in a pulse–chase experiment. Cells growing exponentially in M63/0.2% glycerol were labeled with [^{35}S]methionine for 2 min. Zero, 5, and 30 min after the addition of 25 mM L-methionine the cells were lysed and the proteins were precipitated using antibodies that recognize AP as well as MalF.

β-D-thiogalactoside, the rate of *de novo* synthesis of the hybrid proteins began to decrease (data not shown).

A Bifunctional Sandwich Fusion. Cells carrying the *malF'–phoA–'malF* fusion B to bp 94 of *malF* were both AP$^+$ (Fig. 3) and MalF$^+$ (Fig. 4). The cells were able to transport maltose effectively (54% of wild-type rates). The high transport activity of these cells was not due to the overproduction of the sandwich fusion protein. The amount of MalF and AP crossreacting material produced in the strain used in the transport assay was less than is seen from a wild-type chromosomal *malF* gene (data not shown). Since we could not detect any breakdown of the hybrid protein, we believe that both AP and MalF activities are exhibited by the sandwich fusion protein itself. The finding that the sandwich fusion protein B is able to transport maltose indicates that the C terminus of the hybrid protein must have inserted into the membrane with the correct topology.

DISCUSSION

We have shown that when AP of *E. coli* is tethered at both its N and C termini to another protein, it retains its enzymatic activity. We have inserted AP in each of three periplasmic domains of the multispanning cytoplasmic membrane protein MalF. These sandwich fusion proteins exhibit high specific AP activity comparable to that of MalF–PhoA fusion proteins obtained with Tn*phoA*, in which a C terminal portion of MalF is missing. The high levels of activity of the sandwich fusion proteins are somewhat surprising, since AP must dimerize in order to be enzymatically active (23). One might have thought that fusing the protein at both ends would interfere with this process. Our findings provide further testimony to the extent to which proteins can be manipulated without interfering with their activity.

We have also constructed sandwich fusions in which AP is inserted in a cytoplasmic domain of MalF. These were derived from the previously isolated Tn*phoA* fusions in the same domain, which are missing the C terminus of MalF. The sandwich fusions showed 30–100 times lower enzymatic

activity than the original fusions from which they were constructed. The lower activity is an indication that the AP is predominantly localized to the cytoplasm. In contrast, the original fusion strains, in which the C terminus of MalF was missing, exported considerable amounts of AP to the periplasm. These results suggest that the presence of the C terminus of MalF in the sandwich fusion protein stabilizes the cytoplasmic location of AP.

The stabilization of AP to the cytoplasm in these fusions may be due to the proper assembly of the C-terminal portion of MalF into the membrane. With both the N- and C-terminal portions of MalF assuming their native conformation in the membrane, the inserted AP would be firmly held in the cytoplasm. That C-terminal portions of MalF can assemble correctly in sandwich fusions is indicated by our finding that the B sandwich fusion retains MalF function (see below). Our current hypothesis is that topology is initially determined by local effects of hydrophobicity and charge (7). If this were the case, we would expect that the C terminus of sandwich fusions would, in most cases, be correctly inserted in the membrane. The interactions that result in the appropriate localization of AP in fusions to cytoplasmic domains would be the same interactions that stabilize the native protein.

Our results indicate that the use of sandwich fusions for analyzing the arrangement of proteins in the membrane provides results that are better correlated with actual topology than those obtained with Tn*phoA* fusions. It appears that sandwich fusions to cytoplasmic domains may always show AP activities 20 times or more lower than fusions to periplasmic domains. This approach could be particularly useful when studies with the Tn*phoA* fusions yield ambiguous results.

We have shown in this study that it is possible to isolate bifunctional sandwich fusions. Cells carrying the *malF'–phoA–'malF* fusion B exhibited both AP and MalF activity. During purification of this hybrid protein any detectable AP activity was strictly correlated with the presence of the sandwich fusion protein (B. Traxler and J.B., unpublished results). This indicates that the hybrid protein is stable and is most likely itself responsible for both activities. (We can only assume this for the MalF activity, since it is not easy to monitor. The reconstitution of the multicomponent maltose transport system with separately purified components has not as yet been reported.) We also do not know whether all of the fusion protein is bifunctional or whether one subpopulation of the hybrid molecules is responsible for AP activity and another for MalF activity. Our results indicate that the insertion of the large AP molecule does not disrupt the structure of MalF in this fusion protein. We have recently shown that deletions of the first transmembrane segment of MalF also produce a protein that functions in maltose transport. Thus, this N-terminal segment of MalF is not required for its function.

The sandwich fusions make slightly less protein than the corresponding Tn*phoA* fusions. We have observed that high levels of production of the MalF'–PhoA–'MalF hybrid proteins interfere with cell growth, often leading to cell death. Fifteen minutes after induction of the sandwich fusion genes the cells show reduced protein synthesis. These effects could explain the lesser synthesis seen with sandwich fusions.

Sandwich fusions may have uses besides the study of topology: for example, the AP hybrid proteins could be used for the production of antibodies against proteins that are hard to purify. Since the entire target protein is present in the sandwich fusions, such antibody preparations may be directed against epitopes throughout the protein. *phoA* sandwich fusions could be used to study the export of proteins that pass through the outer membrane and are released from the cells, such as proteases or bacteria toxins. If inserted AP can also be exported to the medium, then halos of blue color should be seen with colonies plated on medium containing the 5-bromo-4-chloro-3-indolyl phosphate. This would provide a convenient phenotype for genetic studies. Sandwich fusions may also be used to construct nonpolar insertion mutations.

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