Resolution of whether the GS and FPC syndromes are due to lesions in the same gene should now be possible with linked genetic markers. Although our studies have not yet permitted a rigorous conclusion, very little of our information was derived from the two families segregating Gardner's syndrome, K109 and K1498. Moreover, clinical studies in extended pedigrees (*5*), have suggested that FPC is a single genetic disease with a wide spectrum of phenotypic expression. An accurate gene marker for FPC-GS would be a useful diagnostic tool for directing clinical screening efforts, such as colonoscopy, to the individuals who are at risk.

Note added in proof: Since submission of this paper, a similar finding has been reported by Bodmer *et al.* (*18*).

REFERENCES AND NOTES

1. E. J. Gardner, *Am. J. Hum. Genet.* **3**, 167 (1951).
2. ———, *ibid.* **14**, 376 (1962).
3. R. A. Lewis, W. E. Crowder, L. A. Eierman, R. L. Nussbaum, R. E. Ferrell, *Ophthalmology* **91**, 916 (1984).
4. A. L. Watne, *Curr. Probl. Cancer* 7, 3 (1982).
5. H. J. R. Bussey, *Familial Polyposis Coli* (Johns Hopkins Univ. Press, Baltimore, 1975), p. 50.
6. C. E. Dukes, *Ann. Eugen. London* **17**, 1 (1952).
7. A. M. O. Veale, *Intestinal Polyposis* (Monograph, 101 pp.) Eugenics Laboratory Memoirs XL, London (Cambridge Univ. Press, New York, 1965).
8. L. Herrera, S. Kakati, L. Gibas, E. Pietrzak, A. A. Sandberg, *Am. J. Med. Genet.* **25**, 473 (1986).
9. G. M. Lathrop, P. Cartwright, J. M. Lalouel, *Cytogenet. Cell Genet.*, in press. [abstract from Human Gene Mapping 9 Workshop, Paris (September 1987)].
10. G. M. Lathrop, J. M. Lalouel, C. Julier, J. Ott, *Am. J. Hum. Genet.* **37**, 482 (1985).
11. ———, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3443 (1984).
12. J. Wasmuth, unpublished data.
13. J. L. Bos *et al.*, *Nature (London)* **327**, 293 (1987).
14. H. T. Lynch, *Cancer* **56**, 934 (1985).
15. J. Turcot, J. P. Despres, F. Pierre, *Dis. Colon Rectum* **2**, 465 (1959).
16. S. R. Woodward, E. J. Gardner, R. W. Burt, L. K. Neff, *Encyclia* **58**, 21 (1983).
17. R. Burt *et al.*, *N. Engl. J. Med.* **312**, 1540 (1985).
18. W. F. Bodmer *et al.*, *Nature (London)* **328**, 614 (1987).
19. W. Cavenee, T. Mohandas, P. Pearson, R. White, *Am. J. Hum. Genet.* **36**, 10 (1984).
20. K. C. Reed and D. A. Mann, *Nucleic Acids Res.* **13**, 7207 (1985).
21. J. Dausset, *La Presse Medicale* **15**, 1801 (1986).
22. R. White *et al.*, *Nature (London)* **313**, 101 (1985).
23. L. R. Carlock, D. Skarecky, S. L. Dana, J. J. Wasmuth, *Am. J. Hum. Genet.* **37**, 839 (1985).
24. J. Overhauser, A. Beaudet, J. J. Wasmuth, *ibid.* **39**, 562 (1986).

25. We thank L. Ballard, M. Culver, J. R. Eldridge, J. Gill, S. Gillilan, M. Hadley, L. Jerominski, C. Martin, M. Mitchell, L. Nelson, B. Ogden, L. Rowe, L. Sargeant, T. Sears, K. Smith, and J. Stevens for technical assistance in this project. R. Foltz assisted in the editing and preparation of the manuscript. Supported in part by NIH grant CA40641 (R.B.). R.W. and J.-M.L. are investigators of the Howard Hughes Medical Institute.

23 July 1987, accepted 14 October 1987

Bacteriophage M13 Procoat Protein Inserts into the Plasma Membrane as a Loop Structure

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The major coat protein of bacteriophage M13 is synthesized as a precursor, the procoat, with a typical leader (signal) sequence of 23 residues at its NH_2-terminus. A fusion protein that contains the NH_2-terminal 141 residues of cytoplasmic ribulokinase and all but the first ten residues of M13 procoat was made. The fusion protein inserts into the plasma membrane of *Escherichia coli* and is processed by leader peptidase to give rise to a leader peptide of 155 residues and the mature coat protein of 50 residues. The NH_2-terminus of the leader peptide remains in the cytoplasm and is protected from protease added to the medium outside of the cell. This indicates that M13 procoat inserts into the membrane as a loop structure and that the NH_2-terminus of a leader peptide remains within the cytoplasm during membrane insertion.

INSERTION OF PROTEINS INTO THE membrane is mediated either by a cleavable leader (signal) sequence or by an uncleaved signal or insertion sequence (*1*). Cleavable leader sequences of bacterial proteins and proteins inserted across the endoplasmic reticulum are located at the NH_2-termini of pre-proteins, are structurally similar, and share a similar length (13 to 26 residues). Each one consists of a polar, basic region at the NH_2-terminus followed by a stretch of hydrophobic residues and a region coding for the recognition site for the processing enzyme called leader peptidase (*2*). Since the enzymatically active site of the leader peptidase is located on the outer surface of the inner membrane (*3*), only those precursor proteins that are translocated across the membrane are processed to their mature form (*4, 5*). Mutations that alter the hydrophobicity of the leader region inhibit membrane insertion and consequently inhibit processing (*5, 6*). Although the involvement of leader sequences in initiating membrane insertion is now well established, the molecular mechanism of this process is unknown.

The insertion of the coat protein of bacteriophage M13 into the inner membrane of *Escherichia coli* has been extensively studied as a model system (*7, 8*). The coat protein is synthesized in the cytoplasm as a precursor protein, the procoat, with a typical 23-residue cleavable leader sequence at its NH_2-terminus. Mutations that alter the hydrophobic region of the procoat leader strongly inhibit its membrane insertion (*5*). In addition, it has recently been shown that the procoat leader can substitute for the leader sequence of the outer membrane protein A (OmpA) without affecting its membrane insertion (*9*). We have proposed that the hydrophobic regions in the leader and in the mature (membrane anchor) regions of M13 procoat directly partition into the hydrophobic core of the membrane bilayer (*4, 5*). Transmembrane electrochemical potential is then required to translocate the central, acidic region of the procoat across the membrane, leaving the NH_2- and COOH-termini at the inner face of the membrane (Fig. 1A). If this model is correct, fusion of other polar peptides to the NH_2- or COOH-terminus of procoat should not interfere with its insertion into the membrane. In contrast, if this protein inserts linearly across the plasma membrane with its leader peptide first, fusion of extra, polar residues on the NH_2-terminus would block membrane assembly.

To test if the NH_2-terminus of procoat remains in the cytoplasm, I have constructed a fusion protein of 205 amino acids (M_r 21,955) that encompasses the first 141 amino acids of ribulokinase (*ara*B), 63 amino acids of procoat, and a glycyl residue at the fusion point (Fig. 1B). In this construction, the first 10 amino acids of the procoat leader were deleted.

Expression of this plasmid-encoded fusion protein was induced by addition of L-arabinose into the growth medium, and the cells were pulse labeled with [^{35}S]methionine. The fusion protein was immunoprecipitated by antibodies to ribulokinase (Fig. 2, lane 1) and was of higher molecular weight than the nonfused ribulokinase fragment of 162 amino acids (Fig. 2, lane 4). In addition, a small amount of pulse-labeled ribulokinase was seen at a lower molecular weight; its electrophoretic mobility suggests that it is the result of cleavage of the fusion protein. When the same sample was immunoprecipitated with antibody to M13 coat protein (Fig. 2, lane 3), both the full-length fusion protein and a protein which comigrated with M13 coat protein (see markers in Fig. 2, lane 2) were observed. As expected, the ribulokinase fragment itself was not immu-

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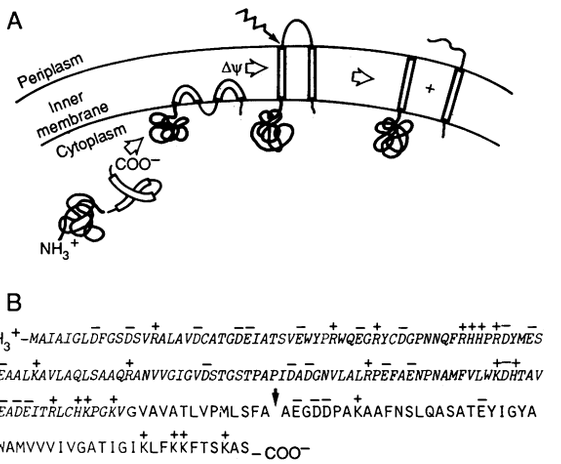

Fig. 1. (**A**) Loop model for insertion of ribulokinase-procoat fusion protein into the membrane. The ribulokinase part is drawn as a coil and the hydrophobic parts of procoat are drawn as rectangles. (**B**) Amino acid sequence and distribution of charged residues of the ribulokinase-procoat fusion protein. A fragment of the *ara*B gene coding for 141 amino acids of ribulokinase was fused in frame with M13 gene VIII coding for procoat. Plasmid pQN8 encoding wild-type procoat (*9*, *11*) and its derivative pQN8 OL14 (*5*) have been described. Plasmid pQN180 was constructed by cleaving pQN8 OL14 with Sma I and Sal I. After inactivation of the enzymes, the plasmid was incubated with the Klenow fragment of DNA polymerase I in the presence of deoxycytidine 5′triphosphate (dCTP), deoxyguanosine 5′ triphosphate (dGTP), and deoxythymidine 5′ triphosphate (dTTP) at 30°C for 1 hour. The plasmid was then circularized with T4 ligase and transformed into MC 1061. The deletion was verified by analysis of fragments produced after digestion with Dde I and by sequencing of the fusion region. All enzyme reactions and DNA preparations were as described (*18*). The *ara*B-derived amino acid sequence is shown in italics to distinguish it from the procoat-derived sequence, and the site of cleavage by leader peptidase is indicated by an arrow.

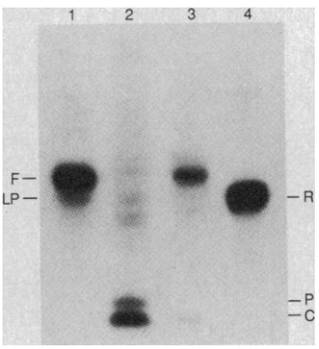

Fig. 2. Expression of the ribulokinase-procoat fusion protein. *Escherichia coli* MC 1061 [Δ*lacX74*, *araD139*, Δ(*ara leu*)*7697*, *galU*, *galK*, *hsr*, *hsm*, strA] containing plasmid pQN180 encoding the fusion protein (lanes 1 and 3), or cells containing plasmid pQN8 encoding the nonfused ribulokinase fragment and procoat (lanes 2 and 4), were labeled with 5 μCi [^{35}S]methionine for 1 minute (lanes 1 to 4) and subsequently incubated for 1 minute with an excess of unlabeled L-methionine (lanes 1 and 3). The cells were mixed with equal volumes of 40% trichloroacetic acid (TCA). The precipitates were collected by centrifugation and immunoprecipitated (*11*) with antibodies to ribulokinase (lanes 1 and 4) and to M13 coat (lanes 2 and 3). The precipitates were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography (*19*). F designates *ara*B-procoat fusion, LP *ara*B-procoat leader peptide, R ribulokinase, P procoat, and C coat.

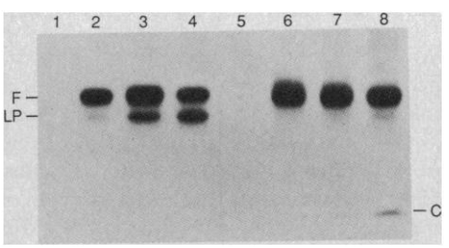

Fig. 3. Kinetics of in vivo processing of the ribulokinase-procoat fusion protein. Exponentially growing cultures of pQN180/MC 1061 were pulse-labeled with [^{35}S]methionine for 30 seconds in the absence of L-arabinose (lanes 1 and 5) or 10 minutes after induction with 0.4% L-arabinose. The induced cultures were incubated with an excess of unlabeled L-methionine (lanes 2 to 4 and 6 to 8) for the indicated times. Samples were chilled, precipitated with 20% trichloroacetic acid, and immunoprecipitated with antibodies to ribulokinase (lanes 1 to 4) or M13 coat (lanes 5 to 8).

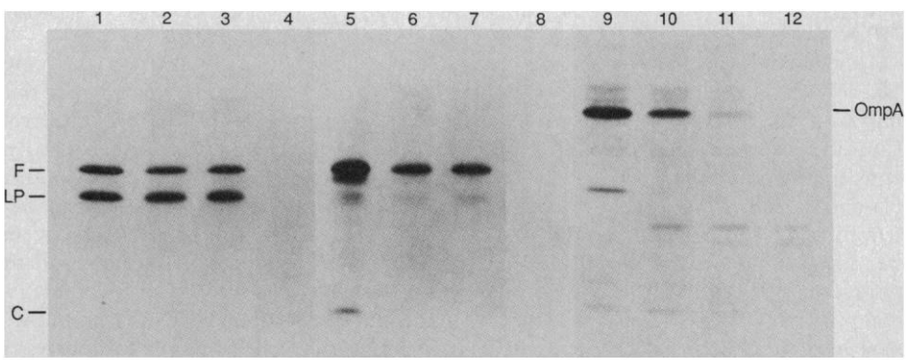

Fig. 4. The NH_2-terminus of the leader remains in the cytoplasm during membrane assembly of M13 procoat. Exponentially growing cultures of pQN180/MC 1061 were labeled with [^{35}S]methionine for 2 minutes and incubated with an excess of unlabeled L-methionine for 5 minutes. The cells were osmotically shocked by mixing with 2 volumes of ice-cold tris-HCl (*p*H 8), 50% sucrose, and 20 m*M* EDTA. Proteinase K (Boehringer) was added to a final concentration of 1 mg/ml, and the samples were incubated (lanes 1, 5, and 9, 0 minutes; lanes 2, 6, and 10, 30 minutes; lanes 3, 4, 7, 8, 11, and 12, 60 minutes). A portion of the cells was lysed by adding 2% Triton X-100 before proteolysis (lanes 4, 8, and 12). After digestion, the proteinase was inactivated by adding phenylmethanesulfonyl fluoride (Boehringer) and the samples were precipitated by 20% TCA and immunoprecipitated with antibodies to ribulokinase (lanes 1 to 4), to coat (lanes 5 to 8), and to OmpA (lanes 9 to 12).

noprecipitated by antibody to M13 coat protein.

After incubating with an excess of unlabeled L-methionine (Fig. 3), the ribulokinase-procoat fusion was cleaved to a fusion protein of ribulokinase plus the leader peptide that was recognized by antibodies to ribulokinase (Fig. 3, lanes 3 and 4), but not by antibodies to M13 coat (Fig. 3, lanes 7 and 8). As a further cleavage product, a lower molecular weight protein appeared after further incubation which was detected by M13-coat antibodies (Fig. 3, lanes 7 and 8) but not by *ara*B antibodies (Fig. 3, lanes 3 and 4). This protein comigrated with the mature M13 coat protein (Fig. 2, lane 3). These results suggest that the fusion protein inserted into the cytoplasmic membrane in vivo and was normally processed by leader peptidase, although at a slower rate. After a 5-minute chase period 54% of the fusion protein was cleaved, as estimated by the method of Suissa (*10*); the half-life of wild-type procoat was 15 seconds (*11*). The cleavage of the coat protein was not taken as a measure of the efficiency of cleavage since it is known that the membrane-inserted coat protein is degraded with a half-life of about 2 minutes (*11*).

As another means of showing that the fusion protein can indeed be processed by the leader peptidase enzyme, the protein was synthesized in an in vitro transcription-translation system and subsequently incubated either with or without purified leader peptidase (*12*). In the presence of leader peptidase, the precursor protein was cleaved to a large peptide with ribulokinase antigenicity and a small peptide comigrating with M13 coat protein. The in vivo and in vitro experiments indicate that an internal leader sequence can be cleaved by leader peptidase.

To investigate the location of the precursor and the cleavage products, cells were pulse-labeled with [^{35}S]methionine for 2 minutes and incubated in unlabeled medium for 5 minutes. Accessibility of the proteins

was analyzed by adding proteinase K to the medium (outside of the cells). The precursor form was mainly protected from proteinase K by the plasma membrane (Fig. 4, lanes 1 to 3) and only digested after disruption of the membrane by detergent (Fig. 4, lane 4). Most of the precursor was therefore located in the cytoplasm indicating that translocation across the membrane, and not processing, is the rate-limiting step for coat production. The coat protein, however, was digested by proteinase K (Fig. 4, lanes 5 to 8) and had therefore been normally inserted into the inner membrane of *E. coli*. The proteolysis of OmpA was taken as an internal control (Fig. 4, lanes 9 to 12). The other cleavage product, consisting of the ribulokinase fragment fused to the leader peptide of procoat, was not accessible to the protease (Fig. 4, lanes 1 to 3). This result indicates that the NH_2-terminal part of the leader peptide remains in the cytoplasm during the membrane-insertion process in vivo. An alternative interpretation, assuming a portion of the ribulokinase-derived sequence enters the membrane, is unlikely since a cluster of 11 charged amino acids precedes the leader sequence.

A cleavable leader sequence therefore does not need to be placed at the NH_2-terminus of a protein to retain its function of initiating membrane insertion and subsequent cleavage. The reason why leader (signal) sequences are located at one end of the protein might simply be that this allows an easy removal of this hydrophobic region. Such a removal is probably often required since a leader region might interfere with the function or with the location of a protein (*13*). An NH_2-terminal position of the leader sequence might be preferred since it allows the protein to interact with the membrane rapidly, before its synthesis is completed or before its folding has become too compact (*8*). A crucial requirement for membrane insertion of a pre-protein is probably the conformational arrangement of the leader sequence so that it is exposed rather than buried in the folded pre-protein. Conformational protrusion is probably also required for uncleaved internal leader sequences. Both cleavable and uncleavable internal signal sequences share the basic mechanism of membrane insertion. The internal signal sequence of the asialoglycoprotein receptor can initiate membrane insertion of rat α-tubulin when placed at the NH_2-terminus (*14*). Dalbey *et al.* have recently shown that the internal signal sequence of leader peptidase can functionally replace the NH_2-terminal leader sequence of OmpA and M13 procoat (*15*). These results taken together show that internal signal sequences and cleavable leader sequences are functionally exchangeable and, in principle, not restricted to a definite location in the protein.

Earlier studies with pro-lipoprotein (*16*) have shown that a fusion of two signal peptides allows cleavage at both cleavage sites, suggesting that internalized signal peptides are still functional, although less efficient than the NH_2-terminal signal peptide. Although pro-lipoprotein differs from other pre-proteins in its insertion pathway and is cleaved by a different signal peptidase, the effect of an internal leader (signal) peptide on the translocation rate is similar to that of procoat. The internal location of a leader may slow membrane insertion if it is buried in the already folded NH_2-terminal part of the protein. In a similar study with a fusion protein of α-globin and pre-prolactin it was observed that both signal-sequence–flanking protein regions were secreted into dog pancreas microsomes (*17*). However, these investigators observed that only a portion of the signal peptide fusion had been sequestered into the microsomes, suggesting the possibility that proteins insert into the endoplasmic reticulum by two different mechanisms.

In support of a loop-like insertion mechanism Kuhn *et al.* have recently shown that the COOH-terminus of procoat is necessary for membrane insertion but remains in the cytoplasm (*4*). Similar to NH_2-terminal fusion, COOH-terminal fusion does not prevent membrane insertion or processing by leader peptidase. Protease mapping experiments led to the conclusion that the COOH-terminal fused peptide was located in the cytoplasm while the acidic coat region was translocated across the membrane. Taken together, these results are in agreement with the idea that M13 procoat, in vivo, initially inserts into the membrane as a loop structure, leaving both termini (NH_2 and COOH) in the cytoplasm.

REFERENCES AND NOTES

1. G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1486 (1980); W. T. Wickner and H. F. Lodish, *Science* **230**, 400 (1985).
2. G. von Heijne, *Eur. J. Biochem.* **133**, 17 (1983); A. Kuhn and W. Wickner, *J. Biol. Chem.* **260**, 15914 (1985); R. Dierstein and W. Wickner, *EMBO J.* **5**, 427 (1986).
3. P. B. Wolfe and W. Wickner, *Cell* **36**, 1067 (1984); R. E. Dalbey and W. Wickner, *J. Biol. Chem.* **260**, 15925 (1985).
4. A. Kuhn, W. Wickner, G. Kreil, *Nature (London)* **322**, 335 (1986).
5. A. Kuhn *et al.*, *EMBO J.* **5**, 3681 (1986).
6. S. D. Emr, M. N. Hall, T. Silhavy, *J. Cell Biol.* **86**, 701 (1980); V. A. Bankaitis, B. A. Rasmussen, P. I. Bassford, *Cell* **37**, 243 (1984).
7. W. T. Wickner, *Trends Biochem. Sci.* **8**, 90 (1983).
8. R. Zimmermann and D. I. Meyer, *ibid.* **11**, 512 (1986).
9. A. Kuhn *et al.*, *EMBO J.* **6**, 501 (1987).
10. M. Suissa, *Anal. Biochem.* **133**, 511 (1983).
11. A. Kuhn and W. Wickner, *J. Biol. Chem.* **260**, 15907 (1985).
12. A. Kuhn *et al.*, unpublished results.
13. R. E. Dalbey *et al.*, in *Microbiology*, L. Leive *et al.*, Eds. (American Society of Microbiology, Washington, DC, 1986), pp. 234–237; M. Inukai and M. Inouye, *Eur. J. Biochem.* **130**, 27 (1983).
14. M. Spiess and H. F. Lodish, *Cell* **44**, 177 (1986).
15. R. E. Dalbey *et al.*, *J. Biol. Chem.*, in press.
16. J. Coleman *et al.*, *Cell* **43**, 351 (1985).
17. E. Perara and V. R. Lingappa, *J. Cell. Biol.* **101**, 2292 (1985).
18. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
19. K. Ito, T. Date, W. Wickner, *J. Biol. Chem.* **255**, 2123 (1980).

20. I thank R. Imber for the antibodies to ribulokinase and R. Dalbey, W. Wickner, and M. Yld-Rice for their continuous support. Technical assistance of H. Jütte and typing by E. Amstutz are acknowledged. Supported by the Swiss National Science Foundation (grant 3.533.-0.86).

27 May 1987; accepted 25 September 1987

A Rapid Cold-Hardening Process in Insects

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Traditionally studies of cold tolerance in insects have focused on seasonal adaptations related to overwintering that are observed after weeks or months of exposure to low temperature. In contrast, an extremely rapid cold-hardening response was observed in nonoverwintering stages that confers protection against injury due to cold shock at temperatures above the supercooling point. This response was observed in nondiapausing larvae and pharate adults of the flesh fly, *Sarcophaga crassipalpis*, nondiapausing adults of the elm leaf beetle, *Xanthogaleruca luteola*, and the milkweed bug, *Oncopeltus fasciatus*. The rapid hardening response is correlated with the accumulation of glycerol.

MANY INSECTS RESPOND TO THE approach of winter by entering a period of dormancy (diapause) and by making physiological adjustments that increase their ability to tolerate low temperatures. For the many species that cannot tolerate tissue freezing, preparation for winter usually involves a gradual accumula-

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