Vol. 97, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1980 Pages 802-810

AMINO TERMINAL SEQUENCE OF PORCINE PRE-β-LACTOGLOBULIN. COMPARISON WITH ITS OVINE COUNTERPART

Mercier, J-C., Hazé, G., and F. Addeo*
Laboratoire de Biochimie et Technologie Laitières
Gaye, P., Hue, D., and M-N. Raymond
Laboratoire de Physiologie de la Lactation
Institut National de la Recherche Agronomique
C.N.R.Z., 78350, Jouy-en-Josas, France

Received October 7,1980

SUMMARY

Porcine mammary gland mRNAs were translated in a wheat germ cell-free system in the presence of radioactive amino acids. Automated Edman degradation performed on β-lactoglobulin isolated by immunoprecipitation from the mixture of radiolabeled lactoproteins showed the occurrence of a hydrophobic amino terminal extension made up of 18 amino acid residues. Comparison of the amino terminal sequences of porcine and ovine pre-β-lactoglobulins revealed a high degree of homology in the signal peptide region. This suggests that the efficient transfer of that protein across the endoplasmic reticulum membrane requires the structural integrity of the transient amino terminal extension.

INTRODUCTION

β-lactoglobulin, a major lactoprotein whose biological role is still unknown, was initially thought to be exclusively secreted by the lactating mammary glands of ruminants. However, proteins similar to β-lactoglobulin in terms of molecular weight and amino acid composition have been isolated from milks of at least two non-ruminant species, pig (1) and donkey (F. Addeo, unpublished data), and more recently, a β-lactoglobulin-like protein was reported to occur in human milk (2). We have previously demonstrated by amino acid sequence analysis that the six major ovine lactoproteins, including β-lactoglobulin (3), are synthesized as precursors in a cell-free system (3-5) and co-translationally segregated and processed into their authentic counterparts in the presence of mammary microsomal membranes (6). Furthermore, we have shown that the signal peptides of bovine, ovine, porcine and rabbit pre-β-caseins are almost identical (7). This led us to suggest that the functional properties of the transient amino terminal extension of a given protein depend not only on its overall hydrophobicity and the clustering of hydrophobic amino acid residues, two characteristics shared by all signal peptides hitherto investigated, but also on the primary structure that is itself well-

*Permanent address: F. Addeo, Istituto di Industrie Agrarie dell'Università di Napoli, 80055, Portici, Italy.

0006-291X/80/220802-09$01.00/0

adapted to the efficient transfer of the remaining part of the polypeptide chain across the endoplasmic reticulum membrane. Additional support for this proposal is provided in the present communication in which we report that the amino terminal sequence of porcine pre-β-lactoglobulin has a high degree of homology with its ovine counterpart in the transient signal region.

MATERIALS AND METHODS

Isolation and purification of porcine whey proteins, including β-lactoglobulin

Milk was collected from a lactating *Large White* sow and defatted by centrifugation at 2000g for 30min and subsequent removal of the upper layer of cream hardened by cooling. Caseins were discarded by acid precipitation (8) and subsequent centrifugation at 3000g for 30min from the skim milk diluted with one volume of deionized water. The whey was dialysed against distilled water and then lyophilized. β-lactoglobulin and α-lactalbumin were isolated from the whey protein bulk by chromatography on a column of Ultrogel AcA54 (50mM-Tris/HCl, pH 8.5; 200mM KCl). If necessary, both proteins were respectively repurified by chromatography on CM-cellulose 32 (20mM-sodium acetate/acetic acid buffer, pH 4.9; elution with a linear gradient of NaCl from 0 to 300 mM). Purity of the proteins was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Gel electrophoresis

SDS-PAGE was performed on gel slab gradients (10 to 20%) using the buffer system described by Laemmli (9). Radiolabeled proteins were detected either by autoradiography or fluorography according to Bonner and Laskey (10).

Preparation of antibodies

Antibodies against porcine β-lactoglobulin were prepared as previously described (11).

Preparation of wheat germ extracts and reticulocyte lysates

The wheat germ extract was prepared according to Davies and Kaesberg (12) using the procedure described by Benicourt and Haenni (13) that was slightly modified (4). Reticulocyte lysates treated with staphylococcal nuclease (14) to reduce endogenous globin mRNA activity were prepared according to Lingrel (15).

Protein synthesis in the cell-free system and immunoprecipitation of β-lactoglobulin. The translation of poly(A) RNAs isolated from bound polysomes of a lactating sow mammary gland, as well as the immunoprecipitation of β-lactoglobulin, were carried out according to procedures detailed elsewhere (11, 4). A reticulocyte lysate supplemented with 6 to 10 A_{260} units of mammary microsomal membranes /ml was used as a translational system in the assay devoted to the study of the co-translational proteolytic processing of pre-β-lactoglobulin (6).

Isolation of rough microsomes from rabbit mammary gland and preparation of degranulated microsomal membranes

Degranulated microsomal membranes were prepared according to the procedure of Blobel and Dobberstein (16) from rough microsomes isolated from a rabbit lactating mammary gland (6).

Amino terminal sequence analyses of β-lactoglobulin isolated from milk and from the cell-free system

β-lactoglobulin isolated from milk was reduced and alkylated with iodoacetic acid by the procedure of Crestfield *et al* (17) except that 6M guanidine and dithioerythritol were used as dissociating and reducing reagents, respectively, before sequence analysis. Automated Edman degradation was performed with a Beckman updated model 890B sequencer by using the Beckman dimethylbenzylamine program no 102473. The material released at each cycle was converted with 1M-HCl at 80°C during 10min, and the resulting phenylthiohydantoin (PTH) derivatives of the amino acids were extracted with ethyl acetate and analysed by both thin-layer

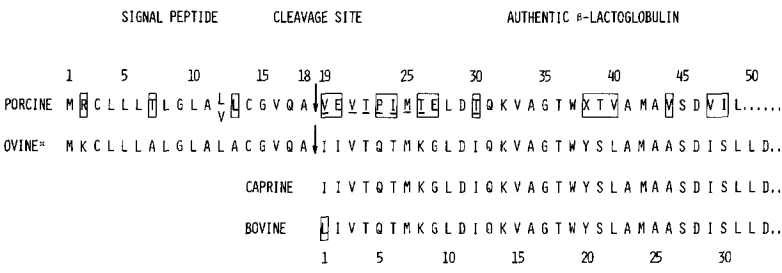

Fig. 1. *Comparative study of the amino terminal sequences of ovine* (3) (Mercier, unpublished results) *and porcine pre-β-lactoglobulins and bovine* (21) *and caprine* (22) *β-lactoglobulins.*

*The ovine amino acid sequence was arbitrarily chosen as reference. Boxes indicate substituted amino acid residues. The arrows indicate the site of cleavage of the pre-protein.

Underlined amino acid residues of authentic β-lactoglobulin were also identified by radiosequence analysis of pre-β-lactoglobulin.

(18) and gas-liquid (19) chromatography, in the latter case after trimethylsilylation. In some instances, isochratic high performance liquid chromatography was performed on a Waters chromatograph (model ALC/GPC 204U) according to the procedure described by Frank and Strubert (20). The aqueous phase was analysed for PTH-Arg and PTH-His. The immunoprecipitates containing *in vitro* synthesized β-lactoglobulin (radiolabeled with one amino acid at a time) were sequenced either individually or as a pool, each sample being supplemented with 3mg of bovine β-lactoglobulin carrier prior to analysis. An aliquot (1/25 v/v) of the converted material was gas-chromatographed to monitor the efficiency of the carrier degradation and calculate the repetitive yield. The remaining portion was used for identification of the radiolabeled PTH amino acids that were co-chromatographed with appropriate PTH amino acid carriers, either on silica gel (Merck F254) or on HPLC columns. The spots scraped off the plates or the fractions recovered from the liquid chromatograph were collected in vials containing toluene-based scintillation fluid and counted for radioactivity in a Packard model 3375 liquid scintillator. The aqueous phase obtained at each conversion cycle was analysed whenever a sample contained radiolabeled Arg or His. [^{35}S]cysteine-labeled β-lactoglobulin was reduced and alkylated with iodoacetic acid, as indicated above, prior to sequencing.

Sources of materials

[^{35}S]Met(610) and sixteen [^{3}H]-labeled amino acids were obtained from CEA France: Asp(10), Asn(22), Ser(38), Glu(21), Pro(20), Gly(10), Ala(50), Val(30), Ile(10), Leu(30), Tyr(45), Phe(30), Lys(30), His(40), Trp(4), Arg(10). [^{3}H]Thr (20), [^{3}H]Gln(21) and [^{35}S]cysteine(640) were obtained from New England Nuclear. Numbers in parentheses refer to specific activity expressed in Ci/mmol.

RESULTS

AMINO TERMINAL SEQUENCE OF β-LACTOGLOBULIN ISOLATED FROM PORCINE MILK

Automated Edman degradation of S-carboxymethylated β-lactoglobulin through 31 cycles yielded the sequence of 30 amino acid residues (Fig.1). Two successive analyses were performed, giving the same results, and subsequent analyses of radiolabeled β-lactoglobulin synthesized *in vitro* confirmed in all cases the proposed amino terminal sequence.

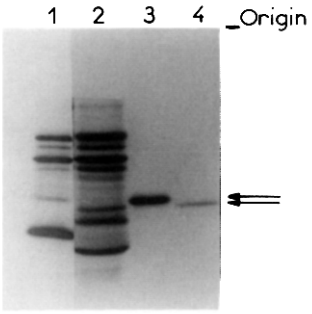

Fig. 2. *Analysis, by SDS polyacrylamide gel electrophoresis and subsequent autoradiography, of polypeptides synthesized by translation of porcine mammary poly(A) RNAs in a nuclease-treated reticulocyte cell-free system containing* [^{35}S]*methionine.*
The electrophoresis was performed on gel slab gradients (10 to 20%)
Lane 1. Total porcine mammary mRNAs translational products
Lane 2. As lane 1, except that the translation was carried out in the presence of mammary microsomal membranes.
Lane 3. Immunoprecipitate of β-lactoglobulin synthesized in the absence of mammary microsomal membranes.
Lane 4. Immunoprecipitate of β-lactoglobulin synthesized in the presence of mammary microsomal membranes.
The upper and lower arrows indicate the positions of pre-β-lactoglobulin and authentic β-lactoglobulin, respectively.

ELECTROPHORETIC AND AMINO TERMINAL SEQUENCE ANALYSES OF IMMUNOPRECIPITATED PORCINE β-LACTOGLOBULIN SYNTHESIZED IN A CELL-FREE SYSTEM

The occurrence of pre-β-lactoglobulin, as suggested by the slower mobility of *in vitro* synthesized β-lactoglobulin co-electrophoresed with its authentic counterpart on SDS-PAGE (Fig.2), was demonstrated by sequencing separate or pooled immunoprecipitates of *in vitro* translated β-lactoglobulin radiolabeled with one amino acid each time. Fig.3 shows the amount of radioactivity recovered at each cycle of the automated Edman degradation carried out on different samples. The amino terminal sequence deduced from these data (Fig.1) is clearly identical with that of authentic β-lactoglobulin after cycle 18, thus demonstrating the occurrence of a precursor protein with an amino terminal extension consisting of 18 amino acid residues. The occurrence at cycle 12 of both a minor peak of [^{3}H] PTH-Val and a smaller peak of [^{3}H]PTH-Leu as compared with that of cycle 13, is best interpretated by assuming that pre-β-lactoglobulin might be actually the translational product of a mixed population of unequally distributed allelic mRNAs, hence, the dual assignment of leucine and valine residues at position 12. The occurrence of a protein contaminant as an alternative explanation is not consistent with the clear Edman degradation patterns obtained with the other samples radiolabeled with the remaining set of amino acids.

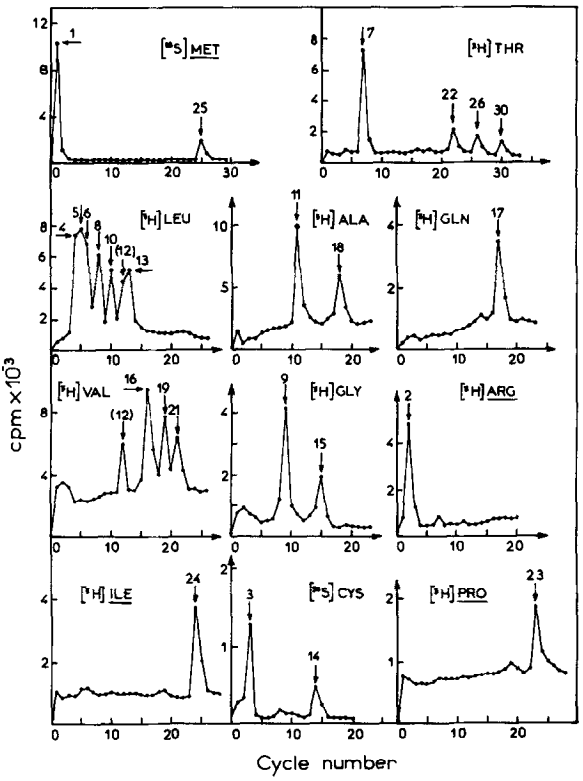

Fig. 3. *Sequence analyses of immunoprecipitates of* in vitro *translated β-lactoglobulin radiolabeled with one amino acid each time*
The data have been corrected neither for background nor any 'out of cycle' radioactivity. Cycle zero represents the blank cycle (4). The automated Edman degradation was performed either on separate ([^{3}H]Thr; [^{3}H]Gln; [^{3}H]Gly; [^{3}H]Val; [^{3}H]Ala; [^{35}S]Cys) or pooled immunoprecipitates ([^{3}H]Arg + [^{3}H]Ile + [^{3}H]Pro + [^{35}S]Met). Amino acids have been underlined whenever a diagram refers to radioactivity recovered from silica gel plates or from HPLC columns. No peak of radioactivity was detected in the first 18 cycles of Edman degradations carried out on samples radiolabeled with other amino acids (diagrams not shown).
Sequence positions assigned to various amino acids are indicated by arrows.

CO-TRANSLATIONAL CLEAVAGE OF PRE-β-LACTOGLOBULIN BY MAMMARY MICROSOMAL MEMBRANES

Sequence analysis of the [^{35}S]methionine-radiolabeled component that was immunoprecipitated with the β-lactoglobulin antiserum from the cell-free system in which translation was carried out in the presence of rabbit mammary microsomal membranes gave methionine at position 7 (Fig.4 and 1). This result, together with the identical electrophoretic migrations of authentic β-lactoglobulin and its counterpart synthesized *in vitro* in the presence of microsomes (Fig.2), demonstrate that the precursor was accurately cleaved to generate authentic β-lactoglobulin.

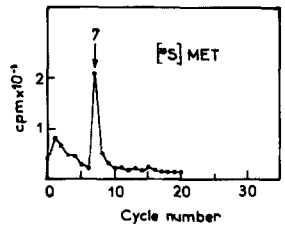

Fig. 4. *Radiosequence analysis of the protein immunoprecipitated with a β-lactoglobulin antiserum, from a reticulocyte cell-free system containing porcine mammary mRNAs, rabbit mammary microsomal membranes and* [^{35}S] *methionine.*
The occurrence of [^{35}S]PTH-Met at cycle 7, i.e at a position occupied by a methionyl residue in the polypeptide chain of authentic β-lactoglobulin, demonstrates that the signal peptide was accurately removed by the microsomal preparation.

DISCUSSION

Amino terminal sequencing of both the major component of porcine milk whey and of its *in vitro* synthesized counterpart has provided confirmatory evidence that this lactoprotein is actually a β-lactoglobulin. Comparison of the sequence data reported here with the known amino terminal sequence of ovine pre-β-lactoglobulin (3) and with the primary structures of bovine (21) and caprine (22) β-lactoglobulins reveals a clear homology, as illustrated in Fig.1.

The most interesting feature is that the amino terminal sequences of porcine and ovine pre-β-lactoglobulins contain an area of minimal variability corresponding to the transient signal peptide. There is 84% homology among the porcine and ovine signal peptides and 2 of the 3 amino acid substitutions (such as the Arg/Lys exchange at position 2) are conservative in nature, thus avoiding any major conformational perturbation. In contrast, the adjacent stretch of 31 amino-acid residues is more variable with a lower degree of homology (less than 60%) and a high ratio of non-conservative amino acid exchanges. Thus, the amino terminal extension of pre-β-lactoglobulin appears to be highly conserved as was also observed with other presecretory proteins: pre-β-casein (7), preproinsulin (23-25) and pre-α-lactalbumin (Raymond *et al*, manuscript in preparation). In the latter case for example, the porcine and ovine (5) signal peptides were found to differ by the amino acid replacements Val/Leu, X/Pro and Ile/Thr at positions 9, 15 and 17, respectively (85% homology), whereas the adjacent stretch of 33 amino acid residues showed only 58% homology.

The striking conservation of the amino acid sequences of the signals suggests that the evolutionary process has carefully selected structures with optimal functional properties. It is therefore suggested that in the case of β-lactoglobulin the amphipatic structure, consisting of a rather long hydrophobic core in presumed

α-helical conformation (26, 27) flanked with a positively charged polar amino end, might be well-adapted for the efficient transfer of the growing remaining part of the polypeptide chain across the ER membrane.

Although the involvement of particular hydrophobic sequences in the translocation of proteins into or across membranes is now well substantiated in both eukarotes and prokaryotes (see recent reviews by Blobel (28, 29), Davies & Tai (30), Wickner (31)), the mechanism(s) by which those generally short-lived amino terminal sequences initiate the transport of polypeptide chains across a given membrane remains a complex and vexed question. According to the 'signal hypothesis' (16, 28), the signal peptide might recognize and interact with putative receptors, thus triggering the formation of a proteinaceous pore through which the growing polypeptide chain could be vectorially transferred. But other mechanisms such as the 'direct transfer model' (32-34) have been proposed: the signal peptide of the nascent polypeptide chain is thought to interact non specifically with the ER membrane by virtue of its high hydrophobicity, thus allowing the spontaneous insertion of the presumably loop-shaped growing polypeptide chain in the lipid bilayer (32-35).

Whichever mechanism is involved, there is some reason to believe that the functional properties of a given signal peptide require, in addition to a minimal overall hydrophobicity (36), the occurrence of clustered hydrophobic amino acid residues in an appropriate conformation as suggested by the minimal variability of the amino acid sequence which presumably reflects a selection pressure. Such a conclusion has been recently supported by biochemical studies of mutant bacteria defective in protein transport (37-39). In most strains, the failure in the secretion of a given protein was found to be related to an alteration of the hydrophobic core of the signal peptide , either a deletion leading to the shortening of the core or an amino acid substitution involving the replacement of a neutral amino acid residue by a charged residue. It should be noted, however, that the latter type of alteration might affect in a few cases (37) the post-translational modifications of the protein without preventing its transport across the membrane as found in the case of a mutant lipoprotein (37).

Since export-defective mutations were not found in the amino terminal basic segments of signal peptides, it has been suggested that this region might be not critical for protein export (38). However, evolutionary data argue somewhat against that interpretation. The occurrence of basic residues near the amino termini of most signal peptides examined so far together with the conservation of these residues through the course of evolution, as observed in the case of pre-β-casein (7) and pre-α_{s1}-casein (40), and also the striking Lys/Arg replacement found at position 2 in the ovine and porcine pre-β-lactoglobulins (this study), suggest an important role of the basic amino terminal segment. It might prevent

for example the degradation of the nascent chain by aminopeptidases or contribute through ionic interaction to the association of the signal peptide with the charged surface of the membrane (26, 32, 33, 35).

Acknowledgment: We are grateful to M. Bonneau and his team for the sample of sow's milk.

REFERENCES

1. Kessler, E. & Brew, K. (1970) *Biochem. Biophys. Acta* 200, 449-458.
2. Conti, A., Liberatori, J. & Napolitano, L. (1980) *Milchwissenschaft* 35, 65-68
3. Mercier, J-C., Hazé, G., Gaye, P. & Hue, D. (1978) *Biochem. Biophys. Res. Comm.* 82, 1236-1245.
4. Gaye, P., Gautron, J-P., Mercier, J-C. & Hazé, G. (1977) *Biochem. Biophys. Res. Comm.* 79, 903-911.
5. Mercier, J-C., Hazé, G., Gaye, P., Pétrissant, G., Hue, D. & Boisnard, M. (1978) *Biochem. Biophys. Res. Comm.* 85, 662-670.
6. Gaye, P., Hue, D., Mercier, J-C. & Hazé, G. (1979) *FEBS Lett.* 101, 137-142.
7. Mercier, J-C. & Gaye, P. (1980) *Annals of the New York Academy of Sciences* 343, 232-251.
8. Mercier, J-C., Maubois, J-L., Poznanski, S. & Ribadeau-Dumas, B. (1968) *Bull. Soc. Chim. Biol.* 50, 521-530.
9. Laemmli, U.K. (1970) *Nature* 227, 680-685.
10. Bonner, W.M. & Laskey, R.A. (1974) *Eur. J. Bidchem.* 46, 83-88.
11. Gaye, P. & Houdebine, L-M. (1975) *Nucleic Acids Res.* 2, 707-722.
12. Davies, J.N. & Kaesberg, P. (1973) *J. Virol.* 12, 1434-1441.
13. Benicourt, C. & Haenni, A.L. (1975) in *'In vitro transcription of viral genomes' INSERM* 47, 189-196.
14. Pehlam, H.R. & Jackson, R.J. (1976) *Eur. J. Biochem.* 67, 247-256.
15. Lingrel, J.R. (1972) in *Methods in Molecular Biology* (Last, J.A. & Laskin, A.I., eds.) vol. 2, pp 231-261, Marcel Dekker, New York.
16. Blobel, G. & Dobberstein, B. (1975) *J. Cell. Biol.* 67, 835-851; 852-862.
17. Crestfield, A.M., Moore, S. & Stein, W.H. (1963) *J. Biol. Chem.* 238, 622-627.
18. Cohen-Solal, M.C. & Bernard, J-L. (1973) *J. Chromatogr.* 80, 140-143.
19. Pisano, J.J., Bronzert, T.J. & Brewer, H. Jr. (1972) *Anal. Biochem.*45, 43-59.
20. Frank, G. & Strubert, W. (1973) *Chromatographia* 6, 522-524.
21. Braunitzer, G., Chen, R., Schrank, B. & Stangl, A. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* 354, 867-878.
22. Préaux, G., Braunitzer, G., Schrank, B. & Stangl, A. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* 360, 1595-1604.
23. Lomedico, P.T., Chan, S.J., Steiner, D.F. & Saunders, G.F. (1977) *J. Biol. Chem.* 252, 7971-7978.
24. Bell, G.I., Swain, W.F., Pictet, R., Cordell, B., Goodman, H.M. & Rutter, W.J. (1979) *Nature* 282, 525-527.
25. Lomedico, P., Rosenthal, N., Efstratiadis, A., Gilbert, W., Kolodner, R. & Tizard, R. (1979) *Cell* 18, 545-558.
26. Garnier, J., Gaye, P., Mercier, J-C. & Robson, B. (1980) *Biochimie* 62, 231-239.
27. Austen, B.M. (1979) *FEBS Lett.* 103, 308-313.
28. Blobel, G., Walter, P., Chang, C.N., Goldman, B., Erickson, A.H. & Lingappa, V.R. (1979) in *Symposium of the Society of Experimental Biology (Great Britain)* (Hopkins, C.R. & Duncan, C. eds.) Cambridge Univ. Press, London, 33, 9-36.
29. Blobel, G. (1980) *Proc. Natl. Acad. Sci. USA* 77, 1496-1500.
30. Davies, B.D. & Tai, P.C. (1980) *Nature* 283, 433-438.
31. Wickner, W. (1979) *Ann. Rev. Biochem.* 48, 23-45.
32. Von Heijne, G.& Blomberg, C. (1979) *Eur. J. Biochem.* 97, 175-181.
33. Von Heijne, G. (1980) *Eur. J. Biochem.* 103, 431-438.
34. Von Heijne, G. (1980) *Biochem. Biophys. Res. Comm.* 93, 82-86.

35. DiRienzo, J.M., Nakamura, K. & Inouye, M.A. (1978) *Ann. Rev. Biochem.* 47, 481-532.
36. Hortin, G. & Boime, I. (1980) *Proc. Natl. Acad. Sci. USA.* 77, 1356-1360.
37. Lin, J.J.C., Kanazawa, H. & Wu, H.C. (1980) *J. Biol. Chem.* 255, 1160-1163.
38. Emr, S.D., Hedgpeth, J., Clément, J-M., Silhavy, T.J. & Hofnung, M. (1980) *Nature* 285, 82-85.
39. Bedouelle, H., Bassford, P.J.Jr., Fowler, A.V., Zabin, I., Beckwith, J. & Hofnung, M. (1980) *Nature* 285, 78-81.
40. Mercier, J-C., Hazé, G., Hue, D., Gaye, P. & Addeo, F. (1980) *Biochimie* submitted for publication.