# gene expression and regulation:

# the legacy of LUIGI GORINI

Proceedings of the Symposium, Gene '87, held in Milan, Italy, 12–14 October 1987

Editors:

# MINA BISSELL

Lawrence Berkeley Laboratory Berkeley, California, USA

# GIANNI DEHÒ

Department of Genetics University of Milan Milan, Italy

# GIANPIERO SIRONI

Department of Genetics University of Milan Milan, Italy

# ANNAMARIA TORRIANI

Department of Biology Massachusetts Institute of Technology Cambridge, Massachusetts, USA



© 1988 Elsevier Science Publishers B.V. (Biomedical Division)

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without the prior written permission of the publisher, Elsevier Science Publishers B.V., Biomedical Division, P.O. Box 1527, 1000 BM Amsterdam, The Netherlands.

No responsibility is assumed by the Publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the material herein. Because of rapid advances in the medical sciences, the Publisher recommends that independent verification of diagnoses and drug dosages should be made.

Special regulations for readers in the USA – This publication has been registered with the Copyright Clearance Center Inc. (CCC), 27 Congress Street, Salem, MA 01970, USA. Information can be obtained from the CCC about conditions under which photocopies of parts of this publication may be made in the USA. All other copyright questions, including photocopying outside the USA, should be referred to the copyright owner, Elsevier Science Publishers B.V., unless otherwise specified.

International Congress Series No. 784 ISBN 0 444 80993 7

Published by: Elsevier Science Publishers B.V. (Biomedical Division) P.O. Box 211 1000 AE Amsterdam The Netherlands

Sole distributors for the USA and Canada: Elsevier Science Publishing Company Inc. 52 Vanderbilt Avenue New York, NY 10017 USA

Library of Congress Cataloging in Publication Data:

Gene expression and regulation : the legacy of Luigi Gorini ; proceedings of the symposium held in Milan, Italy, 12-14 October 1987 / editors, Mina Bissell ... [et al.]. cm. -- (International congress series ; no. 784) D. Includes bibliographies and index. ISBN 0-444-80993-7 (U.S.) 1. Gene expression--Congresses. 2. Genetic regulation--Congresses. 3. Gorini, Luigi, 1903-1976. I. Gorini, Luigi, 1903-1976. II. Bissell, Mina. III. Series. [DNLM: 1. Gene Expression Regulation--congresses. W1 EX89 no. 784 / OH 450 G3263 1987] QH450.G4618 1988 574.87'322--dc19 DNLM/DLC 88-16507 for Library of Congress CIP



# LIST OF PARTICIPANTS

Abir-Am, Pnina G. Alano, Pietro Albertini, Alessandra Atlung, Tove Ballario, Paola Ballou. Lisa Maria Barlati, Sergio Barsacchi, Giuseppina Barthelmess, Ilse B. Bazzicalupo, Marco Bellincampi, Daniela Benfante, Roberta Bickle, Thomas A. Birago, Cecilia Bissell, Montgomery D. Bissell, Mina J. Blobel, Gunter Boera, Giuliana Bogdanov, Alexei Bongcam-Rudloff, Erik Boquet, Paul L. Borgese, Nica Borghetti, Angelo F. Bossi, Lionello Breviario, Diego Brickman, Edith R. Brzoska, Pius Burger, Richard M. Burket, Anne E. Cammarano, Piero Carignani, Giovanna Carlomagno, Maria Stella Carpousis, Agamemnon J. Caruso, Marinella Cassani, Giovanni Chatellard, Cristine Chetverin, Alexander B. Chroboczek, Jadwiga Ciampi, Sofia Citarella, Franca Cohn, Melvin Colombo, Anna Luisa Cundliffe. Eric D'Ari. Richard Darnell, James Davies, Julian De Carli, Luigi De Felice. Maurilio De Ferra, Francesca De Franciscis. Vittorio De Giorgi, Carla Defez, Roberto Dehò, Gianni Denaro, Maurizio

Di Fonzo, Natale Di Girolamo, Mario Di Liegro, Carlo Di Nocera, Paolo Donini, Pierluigi Donini, Silvia Dragani, Tommaso A. Drubin, David, G. Drury, Lucy S. Duncan, Margaret J. Eilers. Martin Elizur, Erela Epstein, Wolfgang Fabiani, Lucia Fantoni, Antonio Figueroa-Bossi, Nara Forti, Giorgio Franchi, Elisabetta Frontali, Laura Frova, Carla Frunzio, Rodolfo Gaetani, Sancia Gallant, Ionathan A. N. Galli, Enrica Galli, Gabriella Gallo, Gabriella Gavazzi, Giuseppe Ghisotti. Daniela Giannattasio, Sergio Gianni, Maurizio Giladi, Hilla Giuliano, Giovanni Glansdorff, Nicolas A. M. Gobbi, Emanuela Goldberg, Edward B. Goldstein, Alan H. Gorini, Giovanni Gottesman, Susan Grassi, Fabio M. Gualerzi, Claudio O. Guerrini-Spagnolo, Anna Maria Guidolin, Angelo Hanahan, Douglas Hardy, Simon J. S. Honigman, Alik Iaccarino, Maurizio Inselburg, Joseph Ishida, Masaaki Isono, Katsumi Jacoby, Lee B. Jacoby, George A. Iorgensen. Eva Cecile Kaariainen, Leevi Kanan, Joao Enrique Correa

Karlström, Olle H. Kikuchi, Akihiko Kim, Jiyoung Kimchi, Adi\* Kitakawa, Madoka Kogut, Margot Kramer, Helmut Krisch, Henry M. Kurland, Charles G. La Farina, Mario L. Lampen, J. Oliver Lancini, Giancarlo Lanzavecchia, Giulio Legault-Demare, Lucienne Levi-Montalcini, Rita Londei, Paola Lucchini, Giovanna Maaloe, Ole Maas, Werner K. Macer, Darryl R. J. Magasanik, Boris Magni, Giovanni Mahadevan, Subramony Makarow, Marja Mangiarotti, Giorgio Margarit, Immacolata Marguerie, Gérard Martegani, Enzo Mastromei, Giorgio McCarter, Linda L. Metzenberg, Robert L. Mignatti, Paolo Minozzi, Gianni Mintz, Beatrice Monaci, Paolo Monier, Roger Morandi, Carlo Mormeneo, Salvador Morpurgo, Giorgio Mortimer, Robert K. Muller, Gunter Murgola, Emanuel J. Nasi, Sergio Neidhardt, Frederick C. Newman, Elaine B.S. Nicosia, Alfredo Nierlich. Donald P. Ninfa, Alexander J. Nomura, Masayasu Olins, Peter Ottaviano, Ercole Ottolenghi, Sergio Pahel, Greg L. Palla, E. Pappenheimer, Alwin M. Pardee, Arthur Pedroni, Paola Picard, Marguerite Piechowska, Miroslawa Pilone-Simonetta, Mirella

Plevani, Paolo Ouadrifoglio, Franco Quagliariello, Ernesto Rao, Narayana Ravazzolo, Roberto Ricca, Ezio Ricciardi-Castagnoli, Paola Rickenberg, Howard V. Risuleo, Gianfranco Riva, Silvano Rosset, Roland Rossi, Nicoletta Rotman, Boris M; Russomanno, Giorgio P. M. Saccone, Cecilia Sager, Ruth Scarino, Maria Laura Schaechter, Moselio W. Schekman, Randy Scheuermann, Richard H. Schrank, Augusto Schrank, Irene Semenza, Giorgio Sgorbati, Barbara Shammah, Suzy Shen, Shi-Hsiang Sironi, Gianpiero Sitia, Roberto M. Spirin, Alexander S. Stanier (Cohen-Bazire), Germaine Stirpe, Fiorenzo Strbàňová, Sona Sussman, Martin V. Szulmajster, Yekisiel Tandeau de Marsac, Nicole Taramelli, Roberto Tenca, Stefano Torriani, Annamaria Torti, Francesca Toussaint, Ariane C. Ullmann, Agnes Ursini. Matilde Valeria Van den Broek, Peter J. A. Vanoni, Marco Venetianer, Pal Viotti, Angelo Vitale, Alessandro Von Wettstein, Diter Von Wilcken-Bergmann, Brigitte Vos, Yvonne J. Walker, Graham C. Wandersman, Cécile Weglenski, Piotr Weisblum, Bernard Yanofsky, Charles Yarmolinsky, Michael B. Yashphe, Jacob Yura, Takashi Zagorski-Ostoja, Wlodzimierz W. Zucchi, Ileana

# CONTENTS

## INTRODUCTION

Luigi Gorini: As I knew and remember him	
R. Levi-Montalcini	3
The legacy of Luigi Gorini	
J. Davies	13

## TRANSCRIPTIONAL CONTROL IN PROKARYOTES

Introduction	
A. Ullmann	19
Regulons and multiregulon systems: Overview of global regulation	
F.C. Neidhardt	21
Global regulation of nitrogen utilization in enteric bacteria	
B. Magasanik	23
Physiological roles and regulation of the heat shock sigma factor ( $\sigma^{32}$ ) in	
Escherichia coli	
T. Yura, C. Wada, R. Yano, N. Kusukawa and H. Nagai	31
A new dimension to SOS regulation	
J.R. Battista, T. Nohmi, C.E. Donnelly and G.C. Walker	41
Protein degradation in Escherichia coli: Recovering from stress	
S. Gottesman, Y. Katayama-Fujimura, J. Trempy and M.R. Maurizi	49
The unitary hypothesis for the repression mechanism of arginine biosynthesis in	
E. coli B and E. coli K12 - revisited after 18 years	
D. Lim, J. Oppenheim, T. Eckhardt and W.K. Maas	55
Regulation of alternative intracellular states in the phage-plasmid P4	
G. Dehò, D. Ghisotti, S. Zangrossi, P. Alano, M.T. Neri, S. Fattore and G. Sironi	65
The past of gene regulation in E. coli and the present of the phosphate global	
regulation	
A. Torriani, N.N. Rao and J. Yashphe	73

# **REGULATION OF TRANSLATIONAL PROCESSES**

Introduction	
C.O. Gualerzi	83
The Screenless Screen	
C.G. Kurland	89
Feedback regulation of ribosome biosynthesis in Escherichia coli	
M. Nomura	97
Active sites within ribosomal RNA	
E. Cundliffe	109
Erythromycin-inducible resistance in Streptomyces by translational attenuation	
S. Kamimiya and B. Weisblum	119
Ribosome-inactivating proteins from plants	
F. Stirpe	129
Genetic and mechanistic aspects of translational initiation in bacteria	
C.L. Pon and C.O. Gualerzi	137
Phosphorylation and ADP-ribosylation of eukaryotic elongation factors	
A.S. Spirin, L.P. Ovchinnikov, E.K. Davydova, A.S. Sitikov and A.G. Ryazanov	151

## POST-TRANSLATIONAL EVENTS

Intracellular protein topogenesis <i>G. Blobel</i>	163
Export of proteins from bacteria	
L.L. Randall and S.J.S. Hardy	173
Genetics of protease secretion in E. chrysanthemi	
C. Wandersman, S. Létoffé and P. Delepelaire	177
Molecular requirements for protein translocation into the endoplasmic reticulum	
R. Deshaies, J. Rothblatt, S. Sanders, M. Werner-Washburne, E. Craig and	
R. Schekman	187
Import of honeybee prepromelittin into the endoplasmic reticulum	
G. Müller and R. Zimmermann	199
Translational and post-translational control of Ig gene expression	
R. Sitia, C. Alberini and C. Valetti	209

# GENE EXPRESSION IN YEAST AND FUNGI

Introduction	
G. Magni	221
Molecular genetic analysis of the yeast actin cytoskeleton	
D. Drubin and D. Botstein	223
Gene expression in Neurospora crassa	
M. Orbach, J. Paluh, A. Roberts, M. Springer, M. Sachs, K. Hager, D. Burns,	
T. Schmidhauser and C. Yanofsky	231
A molecular analysis of mating type in the genus Neurospora	
N.L. Glass, J. Grotelueschen, R.L. Metzenberg, C. Staben, C. Yanofsky and	
S.J. Vollmer	233
Regulation of gene expression in higher plants and industrial yeast	
D. von Wettstein	235
Characterization of a temperature-sensitive mutant and DNA sequence of the	
DNA polymerase I gene of Saccharomyces cerevisiae	
G. Lucchini, C. Mazza, A. Pizzagalli, P. Valsasnini, M. Foiani, S. Francesconi,	
C. Santocanale and P. Plevani	237
Trans-acting genes and cis-acting upstream regulatory regions affecting the	
expression of maltase in budding yeast	
M. Vanoni, S.H. Hong, M. Goldenthal and J. Marmur	247
GSTI: A homolog of polypeptide chain elongation factor is responsible for the	
stability of mini-chromosomes in Saccharomyces cerevisiae	
Y. Kikuchi and A. Kikuchi	257

## DEVELOPMENTAL GENE EXPRESSION IN HIGHER EUKARYOTES

Introduction <i>J.E. Darnell, Jr.</i> Hereditary persistence of fetal hemoglobin and regulation of human globin gene	267
expression	
S. Ottolenghi, R. Mantováni, N. Malgaretti, B. Giglioni, S. Nicolis, P. Comi, T. De Feo, C. Camaschella and G. Saglio	269
Regulation of gene expression by extracellular matrix in higher eukaryotes	070
M.J. Bissell, T.G. Ram and LH. Chen	279
Features of insulin gene regulatory elements revealed in transgenic mice	
S. Alpert, S. Efrat, G. Teitelman and D. Hanahan	289
Nuclear DNA binding proteins in hemopoietic differentiating cells	
R. Ravazzolo, C. Garre, G. Bianchi-Scarra, R. Barresi, P. Fiorentini, V. Capra	
and F. Ajmar	291
Structure and function of the developmentally regulated rat growth factor II	
L. Chiariotti, A.L. Brown, M.M. Rechler, R. Frunzio and C.B. Bruni	299

Study of pyrimethamine resistance in the malaria parasite, Plasmodium falciparum J. Inselburg, D.J. Bzik, T. Horii and Wo-Bo Li	309
POSITIVE AND NEGATIVE CONTROL IN EUKARYOTES	
Introduction <i>R. Monier</i>	321
Protein and RNA synthesis and degradation in growth regulation G.B. Knight, J.M. Gudas and A.B. Pardee	325
Regulation of S6 phosphorylation during the mitogenic response L.M. Ballou, P. Jenö, B. Friis and G. Thomas	333
Tumor suppression and the gro gene <i>R. Sager</i> Expression of alkaline phosphatase in human cell lines is regulated at both	343
transcriptional and post-transcriptional level <i>P. Mignatti, C. Fognani, F. Tredici, M. Negri, G. Della Valle and L. De Carli</i> Suppression of c-myc oncogene by growth inhibitors and loss of responses in tumor cells	353
A. Kimchi	363
CLOSING REMARKS	

AUTHOR INDEX	385
Luigi Gorini: Courage and genius <i>H. Kalckar</i>	383
The legacy of Luigi Gorini <i>M. Cohn</i>	375

# IMPORT OF HONEYBEE PREPROMELITTIN INTO THE ENDOPLASMIC RETICULUM

## GÜNTER MÜLLER and RICHARD ZIMMERMANN

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestr. 33, D-8000 München 2, Federal Republic of Germany.

# INTRODUCTION

Every polypeptide has a specific function as well as a unique functional location, i.e. an intra- or extracellular location where it fulfills its function (1,2). There are two facts which turned the latter into a central problem in our understanding of gene expression in the eukaryotic cell: i) Neglecting mitochondria and chloroplasts, there is only one site of protein synthesis, the cytoplasm, but there are many different potential functional locations, including the cell organelles and the extracellular space. ii) The site of synthesis is separated from these locations by phospholipid bilayers. Therefore, there must exist mechanisms which guarantee the specific transport of proteins across membranes and the assembly of proteins into membranes, respectively.

We are employing honeybee prepromelittin (70 amino acid residues), the precursor of the secretory protein promelittin (49 amino acid residues), as a tool for elucidating the mechanism of import of proteins into the endoplasmic reticulum (3-8). The logic behind using this small precursor protein is that it has a typical signal sequence (21 amino acid residues), is processed by signal peptidase and thus presumably shares some steps of the import pathway with larger precursor proteins, and that it may be able to bypass certain steps which are obligatory for larger precursors, possibly because of a lower tendency for folding into structures that are incompatible with membrane insertion or transport. An additional reason for choosing this small precursor protein is that it can be expected to be imported posttranslationally because of its size and, therefore, to be a technical advantage. Since approximately 40 amino acid residues are shielded within the ribosome, there is not much time between the point where a 21 residue long signal peptide emerges from the ribosome, and becomes free to interact with a receptor, and the point of termination of protein synthesis.

MKFLVNVALVFMVVYISYIYAAPEPEPAPEPEAEADAEADPEAGIGAVLKVLTTGLPALISWIKRKRQ pre I pro I melittin	06 C
MKFLYNVALVFMVVYISYIYAAPEPEPAPEPEAEADAEADPEAGIGAVLKYLTTGLPALGIMVDFCWL	. 1
GIMVLRRRF	Ϋ́ C
OIMVLSSSS	1
MKFLYNVALVFMVVYISYIYAAPEPEPAPEPEAEADAEADPEALVIMORKTWFSIPEKNRPLDFCWI	LI
++ -+ ++++ I VIMGRKTWFSIPEKNLRRRR	C
LVIMORKTWFSIPEKNLSSSS	1
MKFLVNVALVFMVVVISYIYAAPEPGIMVRPLNCIVAVSQNMGIGKNGDLPWPPLRNEFKFL	c
GIMYRPLNCIVAVSQNMGIGKNGDLPWPPLRNEFKFLRRRR + + - + - + - +	1
OIMYRPLNCIVAVSQNMOIOKNOOLPWPPLRNEFKFLSSSS	C
GIMVRPLNCIVAVSQNMGIGKNGDLPWPPLRNEFTTTTISYL	1
MKFLVNVALVFMVVVISVILSØIMVRPLNCIVAVSQNMGIGKNODLPWPPLRNEFTTTTISVL	C
LSGIMVRPLNCIVAVSQNMGIGKNGDLPWPPLRNEFKFLRRRR	I
LSOIMVRPLNCIVAVSONMOIOKNODLPWPPLRNEFKFLSSSS	I

Figure 1. Amino acid sequence of prepromelittin and related precursor proteins, derived from hybrid proteins between prepromelittin and dihydrofolate reductase. Four classes of small precursor proteins related to prepromelittin are shown (for terminology refer to: 4). The different classes of precursors are identical with prepromelittin to varying degrees (indicated for one protein within a class). The different precursors within a class are different from each other within the region which is not related to prepromelittin (indicated for all proteins within a class). The precursors are termed competent (C) or incompetent (I) with respect to import into microsomes (4). Amino acid sequences are given in single letter code. Charged amino acid side chains and the boundaries between the pre, pro, and mature regions are indicated. Three precursor proteins were subjected to oligonucleotide directed mutagenesis which gave rise to three derivatives, containing a single substitution (leucine to cysteine) (for terminology see: 5). This is indicated by superscribed C.

## MATERIALS AND METHODS

Our experimental system involves plasmids, containing the cDNAs of interest and suitable for transcription <u>in vitro</u>, rabbit reticulocyte lysates and dog pancreas microsomes (3-12). We assay association of precursor proteins with microsomes in binding experiments where precursors are incubated with microsomes at low temperature and microsomes are reisolated by gradient centrifugation (5). Membrane insertion is assayed as removal of the signal peptide by signal peptidase on the luminal side of the membrane (3). The assays for complete translocation involve testing sequestration, i.e. protection against externally-added protease in the absence of detergent but sensitivity in the presence of detergent (3,4), and localization, i.e. subfractionation of microsomes at neutral and alkaline pH-values (4).

### RESULTS

### Competence of Prepromelittin for Import into Microsomes

Prepromelittin is correctly processed and imported by dog pancreas microsomes (3). Import of prepromelittin is not dependent on signal recognition particle (SRP) and docking protein (3,7) and, as concluded by analogy with frog prepropeptide GLa and M13 procoat protein (9-11), ribosomes and a ribosome receptor (see below). The primary structure of prepromelittin, however, is crucial for import competence (4): Hybrid carboxy-terminally proteins between prepromelittin, or truncated derivatives, and the cytoplasmic protein dihydrofolate reductase from mouse were constructed. These hybrid proteins were analyzed for membrane insertion and sequestration into microsomes. The results suggest the following: i) The signal sequence of prepromelittin is capable of interacting with the SRP/docking protein-system, but this interaction is not mandatory for import of small precursor proteins (<80 amino acid residues) (Figure 1). This view is supported by the facts that the interaction between a signal peptide and SRP requires the nascent chain to be bound to a functional ribosome (13) and that the small precursor proteins should be completed and released from the ribosome by the time an interaction with SRP could occur (see INTRODUCTION). ii) In prepromelittin and related small precursor proteins a single (or a cluster of) negatively charged amino acid(s) near the amino terminus of the mature part must be balanced by a single (or a cluster of) positively charged amino acid(s) near the carboxy terminus (or charged amino acid residues must be absent from both these positions altogether) in order to create a precursor protein competent for import (Figure 1). Furthermore, in most cases the presence of ATP and, whenever the

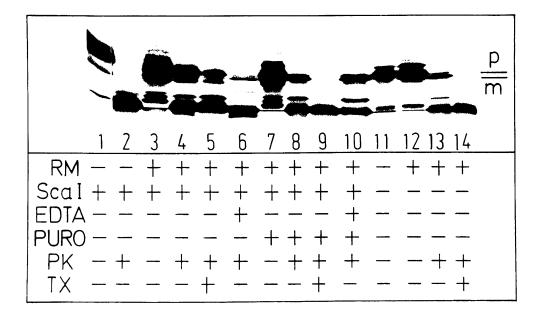


Figure 2. Posttranslational import of precursor proteins containing more than 80 amino acid residues into microsomes.

The plasmid coding for hybrid protein ppm<sub>▲</sub>-DHFR/1 (4) was linearized within the coding region by the restriction enzyme Sca I and was transcribed in vitro, resulting in an mRNA without stop codon. When this transcript was used to program a rabbit reticulocyte lysate in the presence of radioactive methionine (30 min at 37°C), the major translation product was a peptidyltRNA (lane 1) which contained the same 94 amino acid residues as precursor ppmA-DHFR/2 (4) (lane 11). When the peptidyl-tRNA was synthesized in the presence of microsomes, little mature protein was produced and was apparently due to spontaneous release of the polypeptide from the tRNA (lane 3). This mature protein behaved like the mature protein produced during the synthesis of precursor ppmA-DHFR/2 (lanes 12-14) as it was protected against externally-added protease in the absence of detergent (lane 4) and in the presence of EDTA (5 mM) (lane 6), but was sensitive in the presence of detergent (lane 5). In addition to the mature protein, part of the precursor was protease-resistant in the absence and presence of detergent (lanes 4 and 5), but sensitive in the presence of EDTA (lane 6). Because no such precursor was observed after synthesis in the absence of microsomes (lane 2), we conclude that the protease-resistant precursor corresponds to the peptidyl-tRNA which was still hidden in the ribosome. This view is supported by the fact that the protected precursor disappeared during a subsequent incubation in the presence of puromycin (2.5 mM) and resulted in an increase of mature protein (7), i.e. gave rise to a peptidylpuromycin which was protease-resistant in the absence of detergent (lane 8) and in the presence of EDTA (lane 10), but sensitive in the presence of detergent (lane 9).

respective precursor contains two cysteine residues, reducing conditions are a prerequisite for import (5).

# Posttranslational Import of Prepromelittin into Microsomes

Import of prepromelittin is not coupled to translation (3). Furthermore, import of unrelated (9-11) and related (Figure 1) (5) precursor proteins of less than 80 amino acid residues occurs in the absence of protein synthesis and ribosomes (see above). So far, however, we have not succeeded in finding conditions for the posttranslational import of related precursor proteins (5) having more than 80 amino acids other than by artificially keeping the precursor proteins in the form of peptidyl-tRNAs associated with ribosomes (also see: 14). Such precursor proteins can associate with microsomes, through the action of SRP and docking protein, and can subsequently be chased to the mature and imported form by treatment with puromycin (Figure 2). The latter result suggests that the ribosomes, probably in collaboration with the ribosome receptor, have a function in the import of large precursor proteins can be exported in E.coli, without any "tricks", in the absence of protein synthesis (8).

# <u>Competence of Prepromelittin for Association with, Insertion into and</u> <u>Translocation across the Microsomal Membrane</u>

The import pathway of prepromelittin-related precursor proteins was resolved into three sequential steps (3-5): i) Binding of precursors to microsomes; ii) insertion of precursors into the membrane; and iii) complete transfer of the mature proteins across the membrane:

Association and Insertion. Binding of precursor proteins involves the counter-balancing of charged amino acid residues within the mature part, i.e. competence for import is identical with competence for binding (5). Formation of an intramolecular disulfide bridge within the mature part of competent precursor proteins containing two cysteine residues allowed association of the oxidized precursors with the microsomal membrane but reversibly inhibited their membrane insertion (5). Furthermore, formation of an intramolecular disulfide bridge within an incompetent precursor did not result in binding (Table 1), which means that the compensation of charges is the crucial feature of the competent precursor. There was no effect of ATP depletion on binding. Membrane insertion, however, was reversibly inhibited by ATP depletion (5). The effect of ATP was linked to a component of the rabbit reticulocyte lysate. Different prepromelittin derivatives were found to depend on ATP and the reticulocyte component to varying degrees (5). We conclude that binding of prepromelittin-derived precursor proteins to microsomal membranes involves a competent conformation which

# TABLE I ASSOCIATION OF PRECURSOR PROTEINS WITH MICROSOMES AT LOW TEMPERATURE

Precursor proteins were synthesized in rabbit reticulocyte lysates for 10 min at 37°C. After incubation in the presence of cycloheximide (100 µglml) and RNase A (80 uglml) for 5 min at 37°C, the samples were divided into four aliquots. Two aliquots were supplemented with water (reducing conditions), the other two aliquots with  $K_3Fe(CN)_6$  (10 mM) (oxidizing conditions). One aliquot of each set of two was incubated with water (-RM), the other aliquot with microsomes (+RM) for 5 min at 4°C. The samples were divided; one part was diluted with an equal volume of double strength sample buffer, the other part was layered onto a two step sucrose gradient, subjected to centrifugation in a Beckman Airfuge (5 min at 25 psi, rotor A-100/30) and subsequently fractionated as described (5). The supernatants and the interfaces were diluted with an equal volume of double strength sample buffer and the pellets were resuspended in sample buffer. All samples were analyzed by gel electrophoresis and fluorography. Densitometric analysis of the resulting X-ray films was carried out on a LKB densitometer and the amount of precursor protein, recovered in the various fractions, was calculated as a percentage of the total precursor protein used in the assay. The existence of intramolecular disulfide bridges in the competent precursor  $p_{\Delta}$  -DHFR/3Thr<sup>C</sup> and the incompetent precursor  $p_{\Delta}$ -DHFR/3Ser<sup>C</sup> under oxidizing conditions was proved by gel electrophoresis in the absence of reducing agents (refer to 5).

conditions	ass	ociated precursor	(% of total preci	ursor)
	p <b>∆</b> -DHF	R/3Thr <sup>C</sup>	₽ <b>&amp;</b> -DHFI	R/3Ser <sup>C</sup>
	reducing	oxidizing	reducing	oxidizing
RM	- +	- +	- +	- +
supernatant	85.1 62.7	72.3 49.1	79.4 72.5	68.8 59.1
interface	5.4 19.8	3.3 12.9	4.2 5.7	7.2 4.9
pellet	1.7 2.9	5.1 4.2	3.5 2.6	7.9 6.7
sum	92.2 85.4	80.7 66.2	87.1 80.7	83.9 70.7

guarantees compensation of charged residues within the mature part of the precursor proteins and that this conformation has to be altered in order to allow membrane insertion to occur. In general, this conformational change is accomplished with the help of both a cytoplasmic component and ATP.

<u>Translocation</u>. A previously uncharacterized proteinaceous component of the microsomal membrane is required for completion of membrane transfer of promelittin and promelittin-related proteins which contain the hydrophobic part of the melittin domain (3,4).

# Competence of Prepromelittin for Insertion into Detergent Micelles

On the basis of the fact that insertion of M13 procoat protein into detergent micelles is very similar to or possibly identical with membrane insertion, the effects of ATP depletion and the presence of intramolecular disulfide bridges in small precursor proteins related to prepromelittin was assayed with respect to processing by purified E.coli leader peptidase in detergent (6). Prepromelittin is correctly processed by purified leader peptidase in detergent (8). Processing in the presence of detergent showed the same prerequsites (compensation of charged amino acid residues, ATP dependence, reducing conditions in the case of precursors with two cysteine residues) as processing by microsomes. We conclude that precursors of eukaryotic secretory proteins behave similarly in this respect as compared to M13 procoat protein .

# DISCUSSION

We propose the following mechanism for the import of prepromelittin into the endoplasmic reticulum (Figure 3):

A) Prepromelittin is released from the ribosome before an interaction between either the signal peptide and SRP or the ribosome and the ribosome receptor can occur. Its primary structure, however, has evolved in a way which allows it to stay competent for membrane association and insertion without the aid of these components. Possibly, a loop is formed within the mature part, thereby bringing the amino- and carboxy-terminal parts in close proximity of each other. Ionic interactions may stabilize this structure, whereas the presence of unbalanced charges in these positions may destabilize it.

B) This structure can associate with the microsomal membrane only if there is a compensation of charged amino acid residues (or absence of charged amino acid residues) at the termini of the mature part and if the signal peptide is exposed. Even disulfide bridges are allowed at this stage, but they probably never occur <u>in vivo</u>. It is presently unclear whether a signal

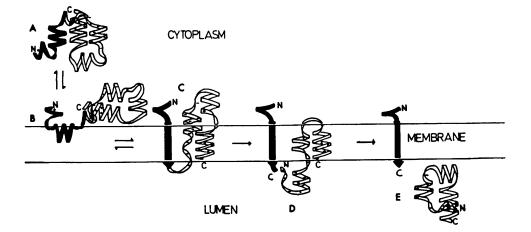


Figure 3. Working model for the import of prepromelittin and related precursor proteins into microsomes. The import of prepromelittin into microsomes occurs as a sequence of the following steps: i) Specific association of prepromelittin with the membrane (B), ii) membrane insertion of prepromelittin (C), removal of the signal peptide by signal peptidase on the luminal side of the membrane (D), and iii) complete translocation of promelittin across the membrane (E). Association involves a competent conformation and a signal peptide; a possible involvement of a signal sequence receptor (SSR) is currently under investigation. Insertion involves the signal peptide and a conformational change which typically is mediated by a soluble cytoplasmic component and the hydrolysis of ATP. Processing involves signal peptidase on the luminal side of the membrane. Translocation involves proteinaceous membrane components, at least in certain cases. Form B can be trapped in binding experiments at low temperatures, in the absence of ATP or in the presence of oxidizing agents in the case of precursors with two cysteine residues (5). Form D was observed in pretrypsinized microsomes after incubation with prepromelittin or related precursors which contain the hydrophobic melittin domain (3,4). The membrane association and insertion steps are likely to be reversible, the steps following processing are presumably irreversible. The proposed conformations are based on standard prediction methods but are, nevertheless, purely hypothetical. The signal peptide is shown in its two most probable conformations (indicated in black).

sequence receptor (SSR) (15) is involved at this step or whether it is a pure protein/lipid interaction. Strikingly, prepromelittin export in E.coli does not involve the secY protein (8).

C and D) We assume that an unfolding, i.e. destabilization of this structure, has to precede membrane insertion. Typically, this is catalzed by a cytoplasmic protein which depends on the hydrolysis of ATP for its action (also see: 11,12). Strikingly, the protein without any charged amino acid residues at the mature termini does not depend on this activity. Disulfide bridges cannot be dissociated by the action of the ATP-dependent protein. Insertion may occur in the form of a hairpin structure between the signal peptide and the mature amino terminus. This event leads to exposure of the cleavage site to the luminal side of the membrane and results in processing by signal peptidase. We assume that this takes place in a lipid environment because we observed the same characteristics for insertion into detergent micelles (also see: 11).

E) The subsequent completion of translocation of the mature protein may occur in one or more steps and may or may not involve membrane components. So far we have defined one protease-sensitive component which seems to be necessary in order to expel the hydrophobic melittin domain within promelittin out of the membrane. Accordingly, proteins which do not contain this domain do not depend on this component (also see: 9). The alternatives for the action of this component are still the ones proposed by either the signal hypothesis or the membrane triggered folding hypothesis or something in between. There may be transient proteinaceous pores involved or just the phospholipids, possibly in the form of a transient bilayer distortion, or there may be membrane proteins which do not form a pore but trigger and/or control the bilayer distortion.

While the import characteristics of small precursors appear to be exceptional in the cells of higher eukaryotic organisms, they seem to be shared by a number of proteins in lower eukaryotic cells (15-20): Import of prepro-x-factor (165 amino acid residues) into microsomes in a cell free Sacccharomyces cerivisiae from also occurs system derived (15-20)and does not involve ribosomes posttranslationally (16).Furthermore, it also depends on the primary structure of the precursor protein (18) and a cytoplasmic component which involves the hydolysis of ATP in its action (15-20).

Therefore, it seems that different strategies have evolved in order to keep precursor proteins competent for import into microsomes which depend upon both the nature of the respective precursor protein and the particular cell type.

#### ACKNOWLEDGEMENTS

We are thankful to Dr Günter Kreil for mRNA and cDNA coding for prepromelittin, to Drs Hermann Bujard and Dietrich Stueber for cDNA coding for dihydrofolate reductase, and Dr William Wickner for a gift of E.coli leader peptidase. Furthermore, we are grateful to Maria Sagstetter and Birgitta Kaßeckert for excellent technical assistance, to Dr Donald W. Nicholson for critical comments on the manuscript, and to Hans Wiech for artfully drawing our working model. This work was supported by grants Zi234/2-1 and Zi234/2-2 from the 'Deutsche Forschungsgemeinschaft', by grant B10 from the 'Sonderforschungsbereich 184: Molekulare Grundlagen der Biogenese von Zellorganellen', and by the 'Fonds der Chemischen Industrie'.

#### REFERENCES

- 1. Zimmermann R, Meyer DI (1986) Trends Biochem Sci 11: 512-515
- Zimmermann R (1986) In: Strauss AW, Boime I, Kreil G (eds) Protein Compartmentalization. Springer, Berlin Heidelberg New York, pp 119 136
- 3. Zimmermann R, Mollay C (1986) J Biol Chem 261: 12889-12895
- 4. Müller G, Zimmermann R (1987) EMBO J 6: 2099-2107
- 5. Müller G, Zimmermann R (1988) EMBO J, in press
- 6. Müller G, Zimmermann R, in preparation
- 7. Zimmermann R, Sagstetter M, Schlenstedt G, Wiech H, Kaßeckert B, Müller G (1988) In: Op den Kamp JAF (ed) New Perspectives in the Dynamics of Assembly of Biomembranes. Springer, Berlin Heidelberg New York, in press
- 8. Cobet W, Mollay C, Müller G, Zimmermann R, in preparation
- 9. Schlenstedt G, Zimmermann R (1987) EMBO J 6: 699-703
- Watts C, Wickner W, Zimmermann R (1983) Proc Natl Acad Sci USA 80: 2809-2813
- 11. Wiech H, Sagstetter M, Müller G, Zimmermann R (1987) EMBO J 6:1011 1016
- 12. Sagstetter M, Zimmermann R, submitted for publication
- 13. Wiedmann M, Kurzchalia TV, Bielka H, Rapoport T (1987) J Cell Biol 104: 201-208
- 14. Perara E, Rothman RE, Lingappa VR (1986) Science 232: 348-352
- 15. Wiedmann M, Kurzchalia TV, Hartmann E, Rapoport T (1987) Nature 328: 830-833
- 16. Hansen W, Garcia PD, Walter P (1986) Cell 45: 397-406
- 17. Rothblatt JA, Meyer DI (1986) EMBO J 5: 1031-1036
- Rothblatt JA, Webb JR, Ammerer G, Meyer DI (1987) EMBO J 6: 3455 3464
- 19. Waters GM, Blobel G (1986) J Cell Biol 102:1543-1550
- 20. Waters GM, Chirico WJ, Blobel G (1986) J Cell Biol 103: 2629-2636