COMPARTMENTATION OF NEWLY SYNTHESIZED PROTEINS

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INTRODUCTION

Proteins synthesized in the cytoplasm have a myriad of functional destinations. Some are transported to the nucleus, others reach their final destination in the extracellular space and still others will traverse pathways leading to specific organelles and the membranes thereof.

Taken together, these observations raised the question: what is the molecular mechanism that specifies the segregation of subsets of proteins to specific organelles? Most of the early experimental work on this question dealt with compartmentalization of secretory proteins. The early morphological experiments of Palade and his colleagues and subsequent cell fractionation studies on pancreatic tissue showed that during the early stages of protein biosynthesis, secretory proteins were concentrated in the lumen of the endoplasmic reticulum. It was also observed that secretory proteins were primarily synthesized on membrane-bound polysomes and proteins remaining in the cell cytoplasm were synthesized on free polysomes.

One of the major hypotheses which attempts to explain this discrimination states that information directing polysomes bearing secretory peptide chains to the endoplasmic reticulum is contained in the nascent chains and that such information is lacking in nonsecreted proteins. This point forms the central theme of this review. We will present an historical account of the experimental findings that led to this postulation; what aspects of protein compartmentation that are not explained by it; as well as current speculations concerning the role of the nascent chains in protein compartmentalization of other organelle proteins. We will confine our review to eukaryotic proteins. For a discussion pertaining to the secretion and processing of prokaryotic proteins, the reader is referred to recent reviews.[1,2]

I. COMPARTMENTALIZATION OF SECRETORY PROTEINS

A. Historical Aspects

The intial observations which suggested a role for the nascent polypeptide chain in the formation of membrane-bound polysomes were studies using puromycin. (Puromycin aborts protein synthesis by releasing nascent chains from peptidyl tRNA.) In 1966, Chefurka and Hayashi[3] showed that ribosomes could be effectively released from RER vesicles following treatment with puromycin. Independently, Redman and Sabatini[4] also made similar observations. In the same year, Aronson[5] studied the binding of polysomes to isolated membranes derived from *Bacillus megaterium*. They showed a strong

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correlation between the amount of polypeptides removed from polysomes by pronase and the loss of binding capacity of these polysomes to membranes. These three groups independently suggested that polysomes engaged in synthesizing secretory proteins were bound to ER membranes via nascent peptide chains. Redman et al.[6] demonstrated that pancreatic amylase synthesized on membrane-bound polysomes was transferred to the luminal side of the ER. Also Redman[7] presented evidence that nascent chains released from microsomal bound ribosomes were transferred to the contents of the microsomal vesicles.

Based on the above observations, Blobel and Sabatini in a 1971 note[8] postulated that the amino terminal region of the nascent chain is responsible for binding. Further support for the role of nascent chains in the binding of polysomes to ER membranes was provided by two major studies in 1972. Swan et al.[9] demonstrated that messenger RNA derived from a myeloma tumor MOPC 41 (an immunoglobulin light chain producer) directed the synthesis in ascites cell-free lysates of not only authentic light chain (M_r 23,000), but also of a protein migrating slightly slower on SDS gels. The latter contained identical tryptic peptides as observed in digests of authentic light chain. Of interest, this protein comigrated with a protein synthesized in a run-off cell-free system containing polysomes isolated from MOPC tissue and ribosome-free supernate from ascites tumor cells. These authors considered that the slower migrating protein represented some precursor form of light chain and that it might be extended at its NH_2-terminus.

Later that year, Milstein et al.[10] showed that translation of myeloma mRNA in a reticulocyte lysate also resulted in synthesis of a heavier form of authentic light chain. They examined synthesis of light chain related proteins in a run-off cell-free system containing either total myeloma microsomes or detergent-released polysomes from microsomal membranes. In lysates containing microsomes, only mature light chain was synthesized, whereas in the system supplemented with microsome-derived polysomes, both light chain and some of the slower migrating protein were observed. Milstein et al. suggested that the latter was a light chain precursor which was modified at the NH_2-terminus, and that its conversion to light chain was the result of a proteolytic activity in the microsomes. They attempted to convert completed forms of the precursor synthesized in reticulocyte lysate by adding microsomes to the cell-free system in a second incubation: no conversion was obtained. They thus suggested that the activity is required during, rather than after, synthesis. However, the addition of membranes at the beginning of translation was not reported. These authors concluded that segregation of proteins destined for export were determined by the presence of a small, cleavable, NH_2-terminal signal. They went on to suggest that "binding to a membrane would then occur after translation of the first few codons of mRNA." They argued that such larger proteins would not appear in the extracellular compartments since they would be cleaved during synthesis.

Schechter showed that mRNA derived from another MOPC line also directed the synthesis of a heavier form of the light chain and that it was modified at the NH_2-terminus.[11] Importantly, Schechter et al.[11] showed that the sequence of the NH_2-terminal region of this larger form contained several leucine residues. It was concluded that this hydrophobic region in the nascent chains facilitated their interaction with the ER membrane.

The next important observation in the field came from studies on biosynthesis of parathyroid hormone.[12] Translation of mRNA isolated from the parathyroid gland in wheat germ lysates resulted in synthesis of a heavier molecular weight form containing authentic sequences found in mature parathyroid hormone. This protein was called "pre-pro-para-thyroid hormone." (The "pro" designation defines a hexapeptide sequence at the amino terminus of parathyroid hormone and serum albumin and this

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sequence is not contained within the "pre" segment [see below]. These "pro" forms are stable intracellular intermediates.) Cyanogen bromide cleavage studies revealed that pre-proparathyroid was modified only at the NH_2-terminal region of the protein; no modification at the COOH-terminus was seen.[12]

At this time, Suchanek et al.[13] observed that translation of bee venom gland mRNA in reticulocyte or wheat germ lysates resulted in synthesis of the pre-form of the secretory protein melittin. As observed above, the extra protein portion was located at the NH_2-terminus. This result demonstrated that synthesis of pre-secretory proteins was not limited to mammalian mRNAs.

Thus, from at least three different tissues, mRNAs encoding specific secretory proteins directed the synthesis of heavier forms, modified at the NH_2-terminus, of the corresponding mature products. It was not clear, however, if these proteins were generated as artifacts of the cell-free systems or reflected authentic physiologic intermediates. Thus, the central issue was whether or not a precursor-product relationship existed between a pre-protein and its secreted product.

The direct conversion of pre-protein forms to the corresponding mature protein was demonstrated independently by two laboratories.[14,15] Szczesna and Boime showed that although hPL (M_r = 22,000) was synthesized in ascites S-30, when this lysate was fractionated into ribosomes and ribosome-free supernate devoid of membranes (S-100), only pre-hPL was synthesized. Conversion of pre-hPL to hPL could be affected by adding microsomal membranes to the reconstituted lysate.[14] However, this proteolytic processing was only observed when the membranes were added within the first few minutes of incubation. If the membranes were added to translation mixtures containing completed chains (i.e., pre-hPL), no cleavage was observed.

In a different translation system containing reticulocyte ribosomes, S-100 from ascites tumor cells, and MOPC 41 mRNA, Blobel and Dobberstein[15] showed that pre-light chain was converted to authentic light chain by the addition of microsomal membranes isolated from dog pancreas. These membranes were stripped of ribosomes by EDTA treatment. Processing also occurred only when membranes were added at the start of incubation. In a set of crucial experiments they showed that the processed form could be segregated into the microsomal vesicles in a form resistant to the action of exogenously added trypsin. Processed light chain was protected from the protease, whereas the pre-form was completely degraded. They also showed that the products of globin mRNA translation were degraded in the same lysates, despite the presence of membranes. These crucial data formed the basis of the signal hypothesis, which was unified and stated in detail by Blobel and his colleagues.[15]

B. The Signal Hypothesis for Secretory Proteins

The hypothesis states that translation of messenger RNAs for pre-proteins containing signal sequences begins on free polyribosomes, i.e., all polyribosomes are initially equivalent. Elongation will proceed on free polysomes for all classes of proteins, until 10 to 40 amino acid residues of nascent chains have emerged from the ribosome. The polysomes will then bind to ER membranes if the nascent chains contain the signal sequence. If they do not contain this sequence, they will not attach to the membranes. It was also suggested that the nascent chain bearing the signal sequence creates a pore or tunnel in the membrane through which the growing polypeptide chain will cross. During this translocation step, the pre-sequence is removed and the protein is then sequestered within the lumen of the vesicle. It should be clear that the signal hypothesis does not require the direct attachment of the messenger RNA to the membranes.

Since these observations, there have been several studies showing that secretory proteins are synthesized via nascent polypeptide chains containing NH_2-terminal

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cleavable pre-peptides. While there is one exception to this rule, which we will discuss below, it appears that the nascent chains of all other secretory proteins contain this signal sequence.

C. Structure of the Pre-Peptide

The amino acid sequence of pre-peptides has provided minimal information regarding its function. It is clear that while there is a central hydrophobic domain in these peptides, there is wide variation in the amino acid composition of this region.[16] For example, many pre-peptides are rich in leucine, but others contain less leucine and more isoleucine or valine. Some pre-peptides contain cysteine and proline, whereas others are devoid of these amino acids. There is also great variability in the length of the signal sequence, ranging from 15 to 30 amino acids. One characteristic feature for most pre-peptides is the presence of one or more charged amino acids in the first 10 residues. The amino acid at the COOH-terminus of the signal sequence is usually a neutral residue of either alanine, glycine, or serine. Thus it seems that the protein cleaves only on the COOH-terminal side of uncharged, relatively small amino acids. It also appears that signal sequences possess α helical and β sheet conformation,[16] and thus secondary structure of the signal peptide may be involved in one or more recognition steps of the pre-protein cleavage reaction.

D. Translocation Apparatus

The major component of the signal hypothesis is that structural information in the signal sequence is required for translocating the nascent chain across the ER membrane. The translocation step can be considered as a summation of the following steps: (a) binding of the nascent chain to the membrane; (b) insertion of those chains through the membrane; and, (c) scission of the signal peptide.

1. Informational Content in the Nascent Chains

An approach to investigating regions of the pre-peptides is through the use of analogs of amino acids that are prevalent in the pre-peptide. The pre-peptides of bovine prolactin and placental lactogen are rich in leucine, and are thus excellent substrates for examining the effects of leucine analogs on the cleavage reaction or on the structure of these pre-proteins and their processing. The rationale for using amino acid analogs to prevent proteolytic processing is not new. Studies of polio viral proteins revealed that similar to pre-protein processing, certain viral protein precursors are cleaved during synthesis and thus, full length translation products of virion RNA were not observed in vivo. Substitution of amino acid analogs for arginine, proline, and phenylalanine in the polio virus polypeptide modified the protease cleavage sites, which then permitted isolation of the full-length polyprotein.[17]

When beta-hydroxyleucine was added to translation mixtures containing human placental or bovine pituitary mRNA, processing of pre-hPL and pre-prolactin respectively, was inhibited. This co-translational inhibition by the analog was readily reversed by simultaneous addition of leucine with the analog at the beginning of the translation reaction.[18]

These pre-proteins synthesized in the presence of analog were not sequestered into microsomal vesicles. These experiments provide direct demonstration that binding and/or translocation and processing of secretory proteins requires structural features determined by the primary amino acid sequence.[18]

2. Membrane Associated Elements

Recently, it has been demonstrated that pre-protein processing can also be inhibited by the sulfhydryl modifying agent, N-ethyl-maleimide.[19] Thibodeau and Walsh showed that while N-ethyl-maleimide prevented the translocation and the processing of ovomucoid,

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binding of the nascent chains to the membrane was apparently unaffected, since messenger RNA encoding ovomucoid was bound to the membranes. The appearance of membrane-bound mRNA in the presence of NEM was shown directly with a homogeneous ovomucoid cDNA. This mRNA was bound via nascent peptide chains, since no ovomucoid mRNA was associated with membranes in translation mixtures containing pactamycin or puromycin.

Walter et al.[20] reported that mild trypsinization of microsomal membranes released a tryptic fragment which, when recombined with the membrane treated fraction, restored translocation activity. These studies were extended by Jackson et al.,[21] who showed that the factor released by trypsin was sensitive to N-ethyl-maleimide. The addition of the pre-treated factors to trypsin-treated membranes resulted in a preparation that was incompetent for translocating the nascent chains.

Warren and Dobberstein[22] showed that washing microsomal membranes with solutions of high ionic strength released a factor from the membranes which rendered them inactive, but that translocation activity could be restored by addition of this factor. It is not clear if this factor is related to the trypsin releasable protein discussed above.

Thus, at least one component of the translocation apparatus contains an essential sulfhydryl group which is apparently exposed to the cytoplasmic side of the ER membrane.

3. The Cleavage Enzyme

An important question that is also raised by the above data concerns the nature of the protease present in the microsomal membranes. Is the cleavage an exoproteolytic or an endoproteolytic event? It is clear from several studies that in vitro removal of the pre-protein results in the corresponding secretory form with no heterogeneity at the amino terminus.[23,24] This is also observed for pro-proteins, as well; cleavage of the pre-pro-parathyroid hormone and pre-pro-albumin give rise to the pro-form of these proteins.[25]

Initial characterization of the pre-protein cleavage activity was performed by Jackson and Blobel[27] and by Strauss et al.[26] Jackson and Blobel[27] showed that deoxycholate solubilized extract of pancreatic microsomes cleaved the pre-portion from released pre-proteins in post-translational assays. This was important since it removed the constraint of examining the pre-protein cleavage reaction only under co-translational conditions. Strauss et al. studied the protease activities in detergent solubilized extracts of pancreatic microsomes against synthetic fluorogenic amino coumarin peptide substrates, which can differentiate between endopeptidase and exopeptidase activities present in the solubilized mixture.[26] It was observed that the solubilized activity had endopeptidase action, which was inhibited by ortho-phenanthroline. These data were also supported by the effects of streptomyces protease inhibitors on the co-translational processing of pre-hPL. Leupeptin, antipain, or elastinal had no effect on co-translational processing, whereas chymostatin, an inhibitor of chymotrypsin, significantly inhibited cleavage. These data suggested that processing of pre-proteins involved at least an endopeptidase component with chymotrypsin-like activity.

Other attempts at inhibiting the pre-protein cleavage reaction involved the use of calcium ions which, at less than 1 m*M* concentrations, inhibited the processing and the sequestration of hPL nascent chains in ascites tumor lysates.[28] The mechanism of Ca^{++} action is not clear, but it likely involves a Ca^{++} induced perturbation of the binding properties of the microsomal membranes which resulted in their failure to bind either nascent chains or the ribosomes.

E. An Exception

One point that arises is whether or not cleavage of the pre-peptide is obligatory for translocation of the nascent chain. The signal hypothesis does not require a scission event

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for sequestration of the polypeptide. It only states that such a signal peptide exists to aid in the unidirectional discharge of the protein. One example of a secretory protein that does not contain a cleavable signal is ovalbumin. Cell-free translation studies of ovalbumin mRNA and DNA sequence analyses of the ovalbumin gene revealed the absence of a distinct pre-peptide in the structural gene.[29,30] Despite the absence of a cleavable peptide, ovalbumin is sequestered into microsomal vesicles when examined in vitro.[31] However, efficiency of sequestration was much less than observed for pre-proteins.[32] Other oviduct secretory proteins such as ovomucoid, conalbumin, and lysozyme are synthesized with cleavable signal peptides. (Studies defining the subcellular distribution of ovalbumin and whether or not its nascent chains are membrane-bound in the hen oviduct have not been explored in detail. Thus, it is conceivable that secretion of ovalbumin does not traverse the identical pathway as pre-proteins.)

In any case, these data led to a major revision of the signal hypothesis, i.e., that the signal peptide was confined to the NH_2-terminal region. Lingappa et al.[33] presented evidence that ovalbumin is translocated via determinants contained in a central region of the protein. This presumed internal signal, which is present in a tryptic fragment between residues #229 and #276 of mature ovalbumin, contained a region of homology with NH_2-terminal signals of two other oviduct secretory proteins. Addition of this fragment to cell-free lysates resulted in the inhibition of pre-prolactin processing.

Cleavage and sequestration of pre-proteins occur when membranes are present within the first few minutes of incubation, that is, when the nascent chains are no longer than 70 to 90 amino acids.[34b] Thus the time interval between the start of protein synthesis and membrane addition when translocation of nascent chains can occur should be greater for ovalbumin than that for pre-secretory proteins. This was in fact observed; whereas efficient segregation of prolactin required membrane addition within 2 min of incubation, sequestration of nascent ovalbumin chains occurred even if the addition of membranes was delayed 5 min after the start of protein synthesis.

While the possible existence of an internal signal peptide is intriguing, this report is nevertheless preliminary. The concentration of tryptic fragment added to the cell-free lysate is considerably greater (106-fold) than the amount of protein synthesized in the cell-free system. There was insufficient screening of other peptide fragments which would indicate if the effect was specific or some nonphysiological effect of the peptide inhibiting the binding of the nascent chain. It is also unclear if this fragment acted as a detergent which perturbed the co-translational segregation activity of the membranes.

Recent experiments of Meek et al.[34a] on the kinetics of a co-translational binding of ovalbumin and ovomucoid mRNA to microsomal membranes are not consistent with the above model. If the ovalbumin signal is located in the central portion of the system, binding of ovalbumin mRNA to the membrane would temporally follow the attachment of an mRNA encoding a pre-secretory protein. It was observed, however, that ovalbumin and ovomucoid mRNAs bound to the microsomes at about the same time following the start of translation. However, the early binding of these nascent chains may be nonproductive, i.e., the binding of these chains are not responsible for initiating translocation of ovalbumin. Thus, further evidence is necessary to elucidate the role, if any, for internal signal sequences in the translocation of certain secretory proteins, such as ovalbumin and other compartmentalized proteins.

F. Rough vs. Smooth Endoplasmic Reticulum

It has been presumed that processing of nascent polypeptide chains occurs only in the rough endoplasmic reticulum (RER) and not in the smooth ER (SER). Kreibich et al.[35] suggested that the RER differs from the SER in that the former contains specific sites for

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the binding of polysomes. They proposed that the determinants for binding were conferred to the RER by the presence of two proteins termed ribophorins ($M_r = 65,000$ and 63,000). These proteins were apparently absent in the smooth microsomal fractions. Their studies were primarily based on observations with rough and smooth microsomal preparations isolated from livers of fed, phenobarbital-treated rats. They showed that in the absence of protein biosynthesis and under certain salt conditions ribosomes could bind to stripped rough but not to smooth membranes. However, Bielinska et al.[36] showed that smooth liver microsomal membranes isolated from phenobarbital-induced animals removed the signal sequence from the nascent pre-hPL chains and sequestered the processed protein. The procedure used to isolate these smooth membranes was identical to that used by Kreibich et al.[35] Smooth membranes isolated from Kreb's ascites tumor cells also translocated and processed nascent secretion chains very efficiently. Moreover, smooth membranes derived from the bovine adrenal cortex were very active in removing the signal sequence and translocating the cleaved protein into the lumen of the vesicle. This is significant since the adrenal cortex is composed of greater than 90% smooth ER. Thus, these experiments—which involve a functional assay, that is, *de novo* synthesis and processing of a secretory protein—demonstrate that smooth endoplasmic reticulum and the rough endoplasmic reticulum are equivalent with respect to binding polysomes bearing nascent chains. The data also provide indirect evidence that the ribophorins are not involved in binding or the translocation of nascent peptide chains.

Although the translation experiments were performed with ascites tumor lysates, it was also observed that all of the smooth fractions described above process pre-hPL and pre-hCG-α in a recticulocyte lysate. Nevertheless, it is conceivable that a factor in both lysates was bound to the membranes and resulted in their interaction with polysomes.

Not consistent with the data of Bielinska et al. are results from Blobel's laboratory which showed that smooth membrane preparations from the pancreas were not active in pre-protein processing.[33] However, this is readily explained by the fact that the entire endoplasmic reticulum of the pancreas is essentially in a rough configuration and that little, if any, SER is present. The smooth preparations they used were primarily composed of Golgi membranes and secretory vesicles.

Although the SER is a component of the secretory network, its functional relationship to the RER, is not clear. Dallner et al.[37] suggested that membranes of RER and SER are closely related. The data presented by Belinska et al., as well as previous studies showing that the same enzymes are present in both RER and SER,[38] support this idea. Because it is well established that cleavage of the pre-peptide of the protein can occur only during protein synthesis, it seems inescapable that when cleavage takes place on SER, the ribosomes in SER must, at least transiently, be bound together, i.e., the SER has become RER *pro tem*. Perhaps the distinction between RER and SER has been overemphasized. Mechler and Vasalli presented evidence that a significant proportion of membrane bound ribosomes can be released after translation and that they can enter the free ribosome pool.[39] Conceivably then, the RER that is observed morphologically in vivo represents the population of ribosomes bearing nascent chains which are attached to the ER. Moreover, the distribution of RER to SER in tissues probably reflects the functional state of that tissue. For example, the pancreas contains primarily RER and it is a highly secretory tissue, whereas the adrenal cortex, which is abundant in SER, does not secrete much protein. The ER population of liver would lie betwen these two extremes. Therefore, it seems that the functional state of the tissue, i.e., whether it is secretory or nonsecretory, determines the ratio of RER to SER, and that ribosomes bound to the ER in vivo are through functional interactions between the nascent chains and the ER membrane.

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II. BIOSYNTHESIS OF INTEGRAL MEMBRANE PROTEINS OF EUKARYOTIC ENDOPLASMIC RETICULUM AND PLASMA MEMBRANE

The biosynthesis of eukaryotic plasma membrane and endoplasmic reticular integral membrane proteins has been the subject of intense investigation. Recent developments are briefly summarized because they suggest similarities with the biosynthesis of secretory proteins on the one hand, and with other organellar membrane proteins on the other. This area has been reviewed extensively,[40-42] occasionally rather hypothetically.[41,42]

It is conceptually useful to classify proteins associated with membranes as integral or peripheral.[40] Peripheral membrane proteins, from a biosynthetic viewpoint, are best considered as secretory proteins[43] or as cytoplasmic proteins because they are soluble in an aqueous environment and do not have a membrane-anchoring hydrophobic region. Biosynthesis of peripheral membrane proteins is not further discussed here. Integral membrane proteins are amphipathic; that is, they contain both hydrophilic and hydrophobic domains. Further subclassification of these proteins may prove to be useful in understanding biosynthesis of these proteins.[41,42] One group is transmembrane (ectoproteins), bitopic in their orientation with hydrophilic domains present in the aqueous environment on both sides of the membrane and hydrophobic domain(s) within the lipid bilayer. For glycoproteins, the hydrophilic sugar residues are localized to the noncytoplasmic side of the membrane (extracellular space or lumen of the ER). While most transmembrane proteins are oriented such that the NH_2-terminus is extracytoplasmic, a few are oriented with the NH_2-terminus in the cytoplasm. Moreover, some transmembrane proteins traverse the membrane more than once (polytopic). A second group of integral membrane proteins is localized to one side or the other of the membrane (endoprotein, monotopic). This categorization may become blurred when protein complexes are considered, as some sub-units of the complex may be peripheral, some integral transmembrane, and some endoproteins. In the discussion of the signal hypothesis, Dobberstein and Blobel[15,42] raised the possibility that transmembrane or extra cytoplasmic monotopic proteins might be made on membrane-bound polysomes as larger precursors with cleavable signal peptides, since these proteins must partially cross the membrane. Monotopic membrane proteins localized to the cytoplasmic side, however, might be made on free ribosomes.

From the studies of Palade[44] and many others,[45] it is clear that many plasma membrane integral proteins are synthesized in the rough ER and transported post-translationally via vesicles through the secretory pathway to their final destination. During this vesicular transport, they may undergo several post-translational modifications, including O-linked glycosylation, modifications of asparagine-linked oligosaccharides, phosphorylation and methylation.

Recent progress concerning compartmentalization of newly synthesized integral membrane proteins has been possible through use of the techniques of mRNA translation in cell-free lysates and in vitro reconstitution of compartmentalization through addition of partially purified membrane fractions to the translation reactions. At present, information is somewhat fragmentary; but at least for some integral membrane proteins, compartmentalization does follow the signal hypothesis model. However, proven exceptions also occur suggesting that the mechanism of compartmentalization differs among various integral membrane proteins.

A. Most Transmembrane Proteins are Co-translationally Incorporated RER Membranes

1. Envelope Virus Glycoproteins are Synthesized with Cleavable Signal Peptides

The concept that transmembrane integral membrane proteins are synthesized on

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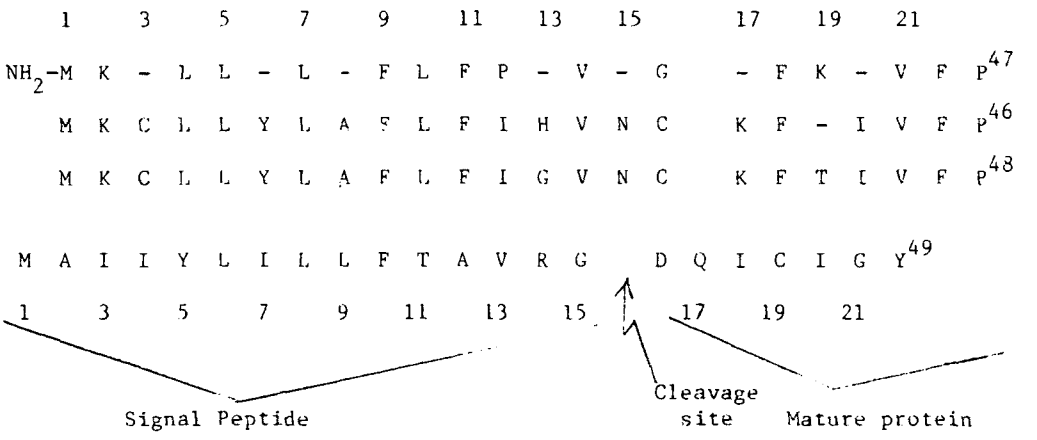

FIGURE 1. Amino acid sequence of the signal peptides of virus glycoproteins. (A) Vesicular stomatitis virus G. (B) Influenza virus hemagglutinin.

membrane-bound polysomes of the RER with cleavable, NH_2-terminal signal sequences was derived from studies with a viral envelope protein — vesicular stomatitis virus glycoprotein (G).[41,46,47] Several groups of investigators independently demonstrated by in vitro translation of mRNA encoding G protein that the complete product of translation contained an NH_2-terminal extension of 16 amino acid residues.[46,47] Nucleotide sequencing of G cDNA[48] provided confirmation of these results (Figure 1). Unfortunately, some discrepancy in sequence (Residues 12, 16, 17) exists in the published reports. This G signal sequence shared structural features in common with secretory protein signal peptides (see above). Several investigators[46,47] also showed that:

1. G signal peptide was removed co-translationally when microsomal membranes were added during translation
2. The in vitro cleavage was correct, resulting in G protein with the appropriate NH_2-terminal sequence
3. Co-translational core glycosylation could be reproduced in vitro
4. Nascent pre-G and nascent pre-prolactin appeared to compete for microsomal membrane translocation "sites"
5. Cleaved, partially glycosylated G was correctly oriented in the microsomal membrane (as assayed after addition of exogenous protease)

These results provided evidence that this integral membrane protein and secretory proteins shared a common translocation apparatus. However, clearly G and secretory proteins differed in that G was not translocated into the RER lumen but became anchored in the membrane near its $-COOH$ terminus. It has been postulated that the "arrested" translocation of G is due to a "stop-transfer signal", in all likelihood the hydrophobic portion of this transmembrane protein.[42]

Analogous results have been reported for the hemagglutinin glycoprotein of influenza virus, as determined by sequencing of the radio-labeled in vitro product of translation and by nucleotide sequencing,[50] and shown in Figure 1.

2. Several Cellular Transmembrane Proteins are also Synthesized with Cleavable Signal Sequences

It could be argued that virus membrane proteins must utilize the cell's secretory machinery, and might not be true models for synthesis of endogenous plasmalemmal

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transmembrane proteins. However, similar conclusions exist for the biosynthesis of the heavy chain (H) of histocompatibility antigens (HLA) A and B.[50] The mature H chains are transmembrane glycoproteins oriented with their NH_2-terminii outside and COOH terminii in the cytoplasm. mRNAs encoding HLA-A and HLA-B isolated from the JY lymphoblastoid cell line were used in these studies. The complete products of in vitro cell-free translation of both H chain mRNAs were larger (by ~2000 daltons) than nonglycosylated mature H chains (derived from tunicamycin-treated cells). Partial NH_2-terminal sequence analyses convincingly demonstrated that the signal peptides were 20 and 24 amino acids long for HLA-A2 and HLA-B7, respectively. Co-translational in vitro proteolytic processing, presumed core glycosylation, and sequestration were also demonstrated. These results strongly suggested that endogenous transmembrane glycoproteins follow the same pathway as viral envelope glycoproteins, possibly sharing a common translocation apparatus with secretory proteins.

Glycophorin A biosynthesis has also been studied in vitro.[51] This transmembrane glycoprotein (M_r 31,000) spans the red cell membrane with its glycosylated NH_2-terminus externally located and its COOH-terminus cytoplasmically localized. These experiments were performed using pulse-chase labeling of an erythroid leukemia cell line. Therefore, the initial product of translation was not characterized. However, the results were consistent with the interpretation that glycophorin A follows the same synthetic pathway as the HLA heavy chains. In vitro studies[52] with membrane-bound (sarcoplasmic reticular, SR) and free polysomes isolated from muscle cells support the interpretation that Ca^{++}-transport ATPase, an integral membrane protein, is also co-translationally incorporated into the SR membrane. These preliminary reports did not demonstrate whether these proteins were synthesized with cleavable signal peptides.

The biosynthesis of microsomal cytochrome P-448 has been studied in more detail.[53] Synthesis of P-448 is induced by 3-methyl-cholanthrene. Translation of P-448 mRNA demonstrated that the in vitro product was larger than mature P-448 (M_r 59,000 vs. 53,000). Co-translational addition of microsomal membranes resulted in cleavage of this pre-448 to its mature size. These results suggested that P-448 is initially made with a cleavable (?NH_2-terminal) signal sequence.*

Thus, for the integral membrane proteins discussed, biosynthesis proceeds analogously to secretory protein synthesis, perhaps sharing elements of the same translocation apparatus.

3. Some Integral Membrane Proteins are Synthesized Without Cleavable Signal Peptides

In 1977, Coon et al.[54] determined the NH_2-terminal sequence of rabbit liver cytochrome P-450. Based on these results, they suggested that the NH_2-terminus of the mature protein might act as a signal sequence directing its nascent chain to the microsomes by binding to the same receptor as pre-secretory proteins. This hypothesis might be compared to the situation of ovalbumin — which appears to contain a noncleavable, internal signal sequence which may utilize the same translocation apparatus as the secretory proteins (see above).

Evidence to support this hypothesis is available from studies utilizing in vitro translation of cytochrome P-450 in a cell-free lysate.[53,55] In a preliminary study, Kumar found that the in vitro translation product of phenobarbital inducible cytochrome P-450 mRNA was identical in molecular weight to the mature protein.[53] More recently, using a combination of subcellular fractionation, in vitro elongation of nascent chains of

* More recent results[111] with sequence analysis of P-448 mRNA product demonstrate that it does *not* contain any signal sequence.

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polysomes, and mRNA in vitro translation, Sabatini and co-workers[55] have provided definitive results concerning P-450 biosynthesis. The NH_2-terminal sequences of mature rat liver P-450 and the immunoreactive product of translation of its mRNA were found to be identical. Unfortunately, these data did not prove that the NH_2-terminal methionine residue was an initiator methionine and the remote possibility exists that the complete product of translation of P-450 mRNA could be larger. As Coon had noted[53], this sequence bears a strong resemblance to those of signal peptides. Further results convincingly demonstrated that nascent chains of P-450 were co-translationally bound to RER membranes. After completion of these nascent chains in vitro, newly synthesized P-450 was found within the membrane (*not* in the lumen) of the ER as assayed by differential release with detergents. No evidence was presented to show that nascent P-450 shared with secretory protein translocation apparatus. These results provided strong evidence that P-450 is co-translationally incorporated into ER membranes, but is *not* synthesized with a cleavable signal sequence. Rather, the signal may be contained in the NH_2-terminal sequence of the mature protein. Similar results have been obtained for epoxide hydratase, another ER integral membrane protein.[56] The in vitro product appears to be identical to the mature protein and in vivo is synthesized on membrane-bound polysomes.

Bovine retinal opsin is an integral transmembrane glycoprotein (COOH terminus cytoplasmic) which is part of the visual pigment of photoreceptor cells.[57] It is initially made on RER-membrane-bound polysomes and travels via the Golgi zone and later vesicules to its final location in the disks of the outer rod segment. Papermaster and co-workers[57] determined the NH_2-terminal sequence of the complete product of opsin mRNA translation. Except for an NH_2-terminal methionine, this sequence was that of mature opsin. The methionine was documented to be an initiator methionine (by translational labeling with [^{35}S]-methionyl initiator tRNA). However, it is noteworthy that mature opsin contains a blocked NH_2-terminus, a modification which may be post-translational since the in vitro product is not blocked. Furthermore, unlike liver cytochrome P-450,[54] the NH_2-terminus of opsin does not resemble a signal peptide. Unfortunately, no in vitro reconstitution experiments with microsomal membranes were performed. It, therefore, remains unclear how nascent opsin chains bind to the RER. Nevertheless, these results unequivocally demonstrated that this integral membrane protein does not contain a cleavable signal sequence.

B. Two Integral Membrane Proteins are Post-translationally Incorporated into Membranes

All of the integral membrane proteins so far discussed are co-translationally incorporated into ER membranes (i.e., synthesized on bound ribosomes) although some are not synthesized with a cleavable signal sequence. In contrast, Bergeron et al.[58] demonstrated that rat liver cytochrome b_5 is synthesized on free polyribosomes. Moreover, they showed that the in vitro product of b_5 mRNA was identical in size to mature b_5. This result has been independently confirmed.[59] In view of the known structure of b_5 these results are, perhaps, expected. It contains a long hydrophilic domain comprising all of the NH_2-terminal portion of the molecule, which *in situ* is exposed to the cytoplasm. The 40 amino acid long hydrophobic portion of b_5, which anchors it to intracellular membranes, is localized at the $-$COOH terminus. Since purified b_5 can bind to natural or synthetic membranes, it is not surprising that the in vitro product is made on free polysomes. Finally, b_5 is found in many intracellular membranes, a finding which is explained if membrane binding occurred nonspecifically after translation. Similar conclusions have been reached concerning NADH-cytochrome b_5 reductase, which is also made on free polysomes and incorporated post-translationally into several intracelluar organellar membranes, where it is anchored by a $-$COOH terminal

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membrane binding segment.[60] While post-translational integration might prove to be unusual for monotopic, integral membrane proteins, these results show that compartmentalization of these proteins may proceed by several different mechanisms. Clearly, the concept of a specific signal peptide directing membrane proteins to the ER[2] does not apply in all cases.

C. Conclusions

These recent studies concerning the biosynthesis of integral membrane proteins allow the following conclusions:

Some are made with NH_2-terminal extensions ("signal peptides") which are removed during translation and which may function in binding of their nascent chains to the ER membranes. These proteins may share some elements of the ER translocation apparatus utilized by nascent pre-secretory proteins. However, the obvious difference is that some structural feature of the nascent chains of membrane proteins causes them to remain embedded in the ER membrane — the proposed "stop-transfer" signal.[42] A second group of integral membrane proteins are also co-translationally bound to ER membranes via their nascent chains. However, the "signal peptide" of these nascent chains is *not* removed during translation — rather this binding sequence appears to be internal. It remains unknown if this internal binding (signal) sequence utilizes the same binding site as the pre-protein signal peptides. A third category of integral membrane proteins are incorporated into membranes *after* translation. It would seem unlikely that these proteins share any of the pre-secretory protein translocation apparatus. Thus, several different mechanisms exist by which integral membrane proteins are directed to their final destinations. That is, compartmentalization occurs via different pathways even for proteins ultimately localized to the same organelle.

III. COMPARTMENTALIZATION OF ORGANELLAR PROTEINS

Most of the proteins destined to be sequestered within other organelles are encoded by cytoplasmic mRNAs. A basic question concerning organelle biosynthesis thus relates to the mechanism by which these proteins can be translocated from the cytoplasm across or into organellar membranes to their ultimate sites within mitochondria, chloroplasts, peroxisomes, glyoxysomes, and other intracellular compartments. Furthermore, since various proteins synthesized in the cytoplasm end up in different organelles and suborganellar compartments, it is imperative to determine how this discrimination is specified.

Recent progress in this area was undoubtedly prompted by the results of investigations into the mechanism by which secretory proteins cross the RER membrane, reviewed above. Initially the signal hypothesis[8,10,15] was based on experimental data relevant only to secretory proteins. However, the possibility that NH_2-terminal extensions might act as signals directing translocation of other organellar proteins was raised. It was suggested, for instance, that all cytoplasmically synthesized mitochondrial proteins might contain identical NH_2-terminal signal sequences which would specifically direct them to mitochondria. An explicitly stated corollary to this proposal was that the NH_2-terminal signal sequences would usually be removed by organelle-specific proteases and that translocation would occur co-translationally.

Investigation of organellar protein translocation was also facilitated by adaptation of the techniques utilized for studying the biosynthesis of secretory proteins. The isolation of highly enriched organelles, purification of specific mRNAs with the subsequent sequencing of their cDNAs or genes, and reconstitution in vitro of translocation events have been crucial to the study of nonsecretory protein compartmentalization.

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Subsequent sections will review recent experimental data concerning compartmentalization of cytoplasmically synthesized proteins translocated into chloroplasts, mitochondria, peroxisomes and other intracellular organelles. While this subject has been extensively reviewed,[61-64] significant advances in the last three years have occurred. Because many of these results are preliminary, only tentative conclusions can be reached. However, it is clear that translocation and compartmentalization of these organelle proteins can occur *after* translation. Moreover, it appears that this *post-translational compartmentalization* may occur by several different mechanisms, even for proteins destined for the same organelle.

It is worth noting that two different types of investigation have been necessary and complementary in arriving at these conclusions, as was the case with secretory proteins. First, structural characterization of the primary translation product of a given mRNA was essential. Second, addition of intact, purified organelles to the in vitro translation reactions has documented translocation and sequestration of the protein. In vitro reconstitution has also demonstrated that, once within the organelle, the mature form of the protein is correctly assembled with other components of the organelle. It is crucial that both structural and in vitro biological studies be performed in the study of compartmentalization. As will be shown in this review, unless both aspects are investigated, ambiguous and conflicting results may be obtained.

A. Compartmentalization of Cytoplasmically Synthesized Algal and Plant Organellar Proteins

Plant or algal chloroplasts and glyoxysomes are intracellular organelles with many features in common with mitochondria and peroxisomes, respectively. Compartmentalization of cytoplasmically synthesized chloroplast proteins is reviewed here because these studies have been historically important in establishing the concept of post-translational translocation and because structural studies of the primary translation products are more definitive than for other organellar proteins. Moreover, the organellar structure is sufficiently different from mitochondria to raise questions concerning how suborganellar localization of proteins occurs.

The structure of chloroplasts has been detailed.[61] We need only reiterate that the chloroplast contains three membranes — the outer envelope membrane, the inner envelope membrane, and the thylakoid membrane — which separate three aqueous compartments (the intermembrane space, the stromal space, and the thylakoid space). Cytoplasmically synthesized chloroplast proteins become localized in all six suborganellar parts.

1. Characterization of the Initial Translational Products of Cytoplasmically Synthesized Translocatable Chloroplast Proteins

a. Ribulose-1,5-bisphosphate Carboxylase Small Subunit (S)

This enzyme is located in the chloroplast stroma and is an oligomer of eight identical large subunits (~55,000 daltons) which are made in the chloroplast, and eight identical small subunits (~14,000 daltons) synthesized in the cytoplasm. The enzyme is very abundant in photosynthesizing cells and is an obvious candidate for examining organellar protein biosynthesis. It is of interest, historically, that during studies of light-driven synthesis of proteins by intact pea chloroplasts, Blair and Ellis[65] demonstrated that the large subunit of ribulose-1,5-bisphosphate carboxylase was encoded by the chloroplast genome. They did not observe synthesis of S and thus assumed it was made by cytoplasmic polyribosomes. They then speculated that a membrane protein existed in the outer envelope which would recognize a site common to those proteins destined to be translocated from cytoplasm into the plastid. However, no data were presented to

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support this *"envelope carrier hypothesis."* Roy et al.[66] were among the first to study synthesis of S in cell-free lysates. Elongation of nascent chains of wheat leaf cytoplasmic ribosomes in the presence of a wheat germ supernatant fraction resulted in substantial incorporation of radioactivity into completed polypeptides. Following immunoprecipitation with anti-S, these investigators observed two major radioactive peaks of M_r 20,000 and 12,000. The latter peak comigrated with mature S. Unfortunately, a great deal of nonspecific trapping of labeled polypeptides occurred so that the significance of the 20,000 dalton peak was not appreciated. However, it was suggested that this might represent a larger precursor to the small subunit.

These early studies raised some interesting possibilities concerning translocation of cytoplasmically synthesized organellar polypeptides, but only with translation of their mRNAs in cell-free lysates were more definitive results obtained. Dobberstein et al.[67] were the first to demonstrate unequivocally that the initial translocation product of S mRNA was a larger precursor. Poly(A)-containing RNA was isolated from *Chlamydomonas reinhardtii* and translated in a wheat germ lysate. A major product of translation (pre-S) was immunoprecipitable with anti-S IgG and migrated on SDS-PAGE with an apparent molecular weight of 20,000. Thus, in the in vitro product of S mRNA was larger than mature S by 4000 daltons. No further structural characterization was reported. These authors further demonstrated that free polysomes contained the mRNA encoding S. However, since the mutant algae which they used contained few membrane-bound ribosomes, it is difficult to be certain that S mRNA was associated only with free polysomes.

Several other investigators also demonstrated that pS was the initial product of translation of SmRNA. Pea plant *(Pisum sativam)* seedlings,[68,69] tobacco plants,[70] and *Lemna gibba*,[70] have been used as sources of S mRNA. In all cases, pS was identified and it was about 4000 daltons larger than mature S.

As described above, structural analysis of the presumed precursor in earlier papers was limited and the location of the 4000 dalton peptide extension was not determined (i.e., NH_2- or COOH-terminal). As with secretory protein preproteins, definitive evidence could be obtained by microsequencing of in vitro synthesized pS radiolabeled with various amino acids. Schmidt et al.[71] sequenced pS encoded by mRNA from *Chlamydomonas reinhardtii*. They showed that the NH_2-terminus of pS was blocked by an acetyl group which was eliminated by trapping endogenous acetyl CoA[72] during in vitro translation. The NH_2-terminal amino acid sequence of pS was then determined and is shown in Figure 2.

The NH_2-terminal extension was termed the "transit peptide" because of its presumed role in translocation. Overlap into the authentic sequence of mature S was demonstrated. Methionine residues occupied positions 45 and 46 of pS and mature S contained methionines at residues 1 and 2. Moreover, possible overlap also occurred at cycles 50 (Pro-6 of mature pS), 52 and 53 (Asn-8 and 9), and 55 (Met-11). Unfortunately, because the peaks of radioactivity at these cycles were small, assignment of these residues must be considered tentative. This suggested that pS was 44 residues larger than S; an $-NH_2$ terminal extension of sufficient length to explain the size difference between pS and S. While the sequence data were generally clear, as in many such analyses, some ambiguity in assignment of residues was present, particularly in positions 1, 4, 37. Results of several sequencer runs were equivocal because of a high and variable background (alanine, serine, arginine, valine, asparagine). The authors concluded that seven amino acids were not present in the transit peptide because no radioactivity over background was found at any cycle. This conclusion must be considered tentative since at least two of these amino acids which should have been found at residues 48 and 49 (Trp-4 and Thr-5, respectively) were not seen. An omission from the data is the percent of theoretical yields.[73] Finally, it

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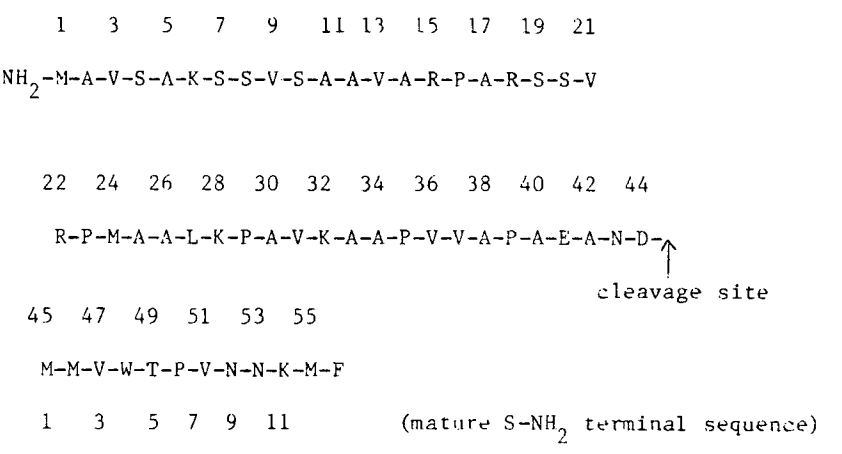

FIGURE 2. NH_2-terminal sequence of the precursor to the small subunit of ribulose-1,5-biphosphate carboxylase.

was not demonstrated that the methionine residue at position 1 was an initiator methionine. This is of some concern because in wheat germ lysates, the initiator methionine is frequently removed if the following residue has a small side chain, such as alanine.[73] Thus, it is uncertain whether the complete product of translation has been sequenced.

The primary structure of the transit peptide of pS is of great interest because this is the first chloroplast or mitochondrial precursor protein to be studied by sequence analysis. Since this precursor is incorporated post-translationally into chloroplasts (see below), comparison of the structure of the transit peptide with the structure of various signal peptides of secretory proteins is of value. Most obviously, the pS transit peptide is longer than any signal peptide yet found (44 residues vs. 15 to 32 residues). Second, many charged residues are present (10/44 = 23%) and are distributed throughout the transit peptide. The transit peptide is also rich in proline (5/44 = 11%). Since pS is almost certainly soluble in the algal cytoplasm, it is likely that these residues cause much of the peptide to be a random coil flanked by β-turns. Moreover, the sequence does not contain an 8 to 15 amino acid long clustering of hydrophobic residues as seen in signal peptides.[16] The transit peptide is clearly different than signal peptides and it probably would not fit the models which have been proposed for translocation of secretory and integral membrane protein precursors — i.e., the loop model, the signal hypothesis or the membrane trigger hypothesis.

b. Precursors of Other Chloroplast Proteins

Several other cytoplasmically synthesized plant or algal organellar proteins (or subunits) have been studied in vitro (see below). These results suggest that most translocated organellar proteins are initially made in the cytoplasm as larger precursors with hydrophilic transit peptides.

mRNAs encoding *ferredoxin* were translated in vitro after isolation from *Nicotiana tabacum, Phaseolus vulgaris* and *Chlamydomonas reinhardtii.*[74] The in vitro product was larger than mature ferredoxin (M_r 20,000 vs. 17,000). No further structural studies were reported. However, it seems likely that this iron-storing protein also contains a transit peptide.

The mRNAs encoding the apoproteins of the light-harvesting *chlorophyll a/b protein (CP II) complex* have also been translated in vitro.[68,75,76] The products of translation were

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peptides of M_r 33,000 and 32,000 (vs. the pea and lettuce apoproteins M_r 29,000);[75] M_r 29,500 (vs. mature barley apoprotein of M_r 25,000);[76] or M_r 33,000 (vs. mature pealeaf apoprotein of M_r 29,000).[68] That these CP II apoproteins are initially synthesized with a transit peptide is of interest since they are integral membrane components of the thylakoid membrane of chloroplasts. The cytoplasmic precursor must thus be translocated across two membranes and two aqueous compartments to reach its ultimate location.

Nelson and Schatz[77] utilized translation of spinach leaf RNA to study biosynthesis of various nonidentical subunits of the proton-translocating ATPase complex. Their results clearly demonstrated that at least one of the subunits (δ) was made by cytoplasmic RNA. The translation product of the δ subunit mRNA was 8000 daltons larger than the mature δ subunit. They also proposed that the II subunit of this complex was cytoplasmically synthesized. This protein complex is anchored in the thylakoid membrane (subunits I-III) and extends into the stromal space (subunits α-ϵ). It thus appears that at least one of the stromal components (δ) is synthesized as a cytoplasmic subunit precursor with a very large, and as yet poorly characterized, transit peptide.

c. Glyoxysomal Malate Dehydrogenase (MDH)

Malate dehydrogenase is an ubiquitous enzyme present as different isozymes in the cytoplasm, mitochondria and glyoxysomes of both plants and animals. Of the many different structural forms of this enzyme, several are located in organelles, but are synthesized in the cytoplasm. While some reports[61,78] have suggested that MDH is synthesized on microsomal membrane-bound polysomes, definitive results concerning the site of synthesis and mechanism of translocation are lacking.[61]

In plants, it is clear that glyoxysomal MDH (gMDH) (M_r 33,000) is made on free ribosomes.[79] Moreover, the initial product of translation of gMDH has been evaluated.[79] gMDH mRNA from watermelon seeds *(Citrullis vulgaris)* was translated in vitro. Following immunoprecipitation and SDS-PAGE, the gMDH mRNA products migrated with M_r ranging from 33,000 to 38,000. The major radiolabeled band was the highest in molecular weight. While no further structural analysis was performed, this result strongly suggested that gMDH in this plant was made as a cytoplasmic precursor with a transit peptide of about 45 amino acids. The smaller immunoprecipitable bands probably represented nonspecific proteolysis, although the wheat germ cell-free lysate used may have been contaminated with organelle-specific proteases capable of removing the transit peptide.

Taken together, these results demonstrated that at least four cytoplasmically synthesized polypeptides which must be translocated into algal or plant organelles are initially made as larger precursors. An exception to this rule has not yet been seen. It appears that translocation of these cytoplasmic precursors occurs with removal of a 4- to 8000-dalton transit peptide. Further, the transit peptide for pS has been characterized and is a highly basic peptide differing in many aspects from the signal peptides found on the precursors of secretory proteins.

2. In Vitro Translocation of Plant Organellar Precursor Proteins is Post-translational

Because the complete translational products of mRNA for organellar proteins to be translocated were found to be larger than the mature peptides and because some of these precursors were made on free-cytoplasmic polysomes, Chua[67] speculated that translocation occurred post-translationally.

a. Post-translational Translocation

Highfield and Ellis[69] synthesized radiolabeled precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pS) in vitro in a cell-free lysate. Following

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translation, they added intact pea chloroplasts to the reaction for 1 hr. Analysis of the products by SDS-PAGE revealed a reduction in the amount of labeled pS and an increase in the amount of mature S. This processing occurred in the presence of chloramphenicol and cycloheximide, proving that neither plastid nor cytoplasmic protein synthesis were required. It was further demonstrated that the labeled, mature S was resistant to trypsin added after incubation of pS with chloroplasts. This was the first direct evidence supporting the concept that translocation of chloroplast precursor polypeptides was a post-translational event.

These initial results have been confirmed for pS in other instances.[61,80] pS translated from spinach and pea mRNA present in the high-speed supernatant of the translation reaction was incubated with pea chloroplasts in the dark. After chloroplast lysis and centrifugation, a radioactive protein migrating with S was found in the supernatant fluid. Moreover, this protein sedimented in sucrose density gradients exactly with ribulose-1,5-bisphosphate carboxylase holoenzyme. This result strongly suggested that processed radiolabeled S had been assembled into the enzyme complex. It is noteworthy that pea chloroplasts could not incorporate algal pS, but would incorporate and process spinach pS, suggesting recognition by specific receptors. Post-translational transport of the precursors of the two apoproteins of pea light-harvesting chlorophyll a/b protein complex has also been demonstrated.[81]

These biological studies are very suggestive that post-translational processing of chloroplast cytoplasmic precursors or peptides is a physiological event. However, these studies must be interpreted with caution. First, little data were given to prove that the chloroplasts used in the reconstitution studies were intact. Second, no adequate structural study of the incorporated, processed radiolabeled peptides was performed. In view of the proteases known to be present in translation systems and in isolated organelles, it is possible the proteolytic processing demonstrated is nonphysiologic. Because the transport assay utilizes the complex mixture of enzymes and cofactors present in the translation reaction, the minimal requirements for the process are obscure. It would be of interest to see if purified precursors are incorporated into organelles and to determine which, if any, "cofactors" are required.

b. Characterization of Elements Involved in Post-translational Translocation

By analogy with co-translational translocation of secretory precursors or receptor-mediated uptake of extracellular polypeptides, it might be predicted that post-translational translocation of precursors involves several different events. First, binding of the precursor to a specific site in the chloroplast outer envelope seems likely. At least in plants or algae, this binding could involve recognition between protein receptors and "transit peptides." All proteins destined to be sequestered within a given compartment-specific receptor might share an identical transit peptide. A compartment-specific receptor might then recognize only proteins containing this transit peptide and direct them to the appropriate sub-organellar compartment. Proteolytic removal of the transit peptide must occur. This processing could involve a single endoproteolytic event or might involve several hydrolytic steps catalyzed by different proteases, localized to different compartments of the organelle. Some alteration in protein conformation is likely to occur during translocation as a result of the removal of the transit peptide. Post-translational modifications may take place which could also change the conformation of the protein. However, transit peptides are not present on all mitochondrial proteins synthesized in the cytoplasm and subsequently incorporated into the organelle (see Section B, below). In any event, we wish to review the available information which characterizes the post-translational translocation process.

The binding site — Grossman et al.[81] have used the reconstitution system for chloroplast uptake of pea pS to demonstrate that the pH optimum for the process is 7.8 to

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8.5. pS bound to chloroplasts at 4° C, but uptake and processing of pS to S occurred only after the incubation reaction temperature was raised to 25° C for 30 min. Finally, uptake of pS appeared to be saturable. Analogous to other receptor-mediated processes, these results suggest that a precursor protein-receptor interaction is required for translocation of pS into chloroplasts. It has been suggested[61] that prior treatment of intact chloroplasts with proteases abolishes uptake and processing activity. However, no data to prove this have been published. At least, by inference, some specificity of the proposed binding site may exist since heterologous pea or spinach chloroplasts do not translocate pS derived from *C. reinhardtii*.[81] Unfortunately, competition experiments with various precursors such as pS and the precursor C II apoproteins have not been performed. It is thus unknown whether or not translocation of these precursors is mediated by the same receptor complex.

The transit peptide cleavage enzyme-characterization and location — Originally, it was found that pS could be processed to S by a protease which was soluble after cell breakage. Chua[71] has proven that incubations of pS with a post-ribosomal supernatant of *C. reinhardtii* precisely and correctly cleaves pS to S. This was shown by NH_2-terminal sequence analysis of the cleaved product. Moreover, it was stated that the cleavage process was inhibited by iodoacetamide, N-ethyl maleimide, and by heating of the post-ribosomal supernatant to 95° C. Unfortunately, a very crude mixture of proteins was present during the cleavage assay. Thus, it is possible that those sulfhydryl blockers altered some other proteins such that cleavage did not occur. It is unclear that the cleavage protease is actually a sulfhydryl protease. Highfield and Ellis[69] have attempted to localize pS cleaving enzyme by incubating pS with various chloroplast fractions and determining the extent of formation of S. Their initial results suggested that the protease was present in the crude envelope fraction. Processing activity was not found in a high-speed supernatant (stromal fraction) or in thylakoid membranes. A later publication,[82] however, revealed that high-speed supernatants of lysed chloroplasts (the stromal fraction) *did* contain processing activity. No activity which converted pS to S was found in thylakoid or envelope membranes. However, significant "nonspecific" proteolytic activity was present in these membrane fractions. Unfortunately, proof that the radioactive S contained the authentic sequence of S was not given and the conclusion that the pS processing enzyme is stromal in location must remain tentative.

Translocation efficiency is increased by ATP — The translocation event is complex and might involve many different receptors, proteases and other proteins. It had been suggested[67] that the overall event did not require light. However, in a recent paper,[83] it was found that incubation of pS with chloroplasts in the presence of light increased the rate of translocation of pS by 2- to 10-fold. This effect was not altered in the presence of chloramphenicol or the electron transport inhibitor, dichlorophenyldimethyl urea. The stimulating effect of light could be partially reproduced in the dark by the addition of exogenous ATP. The effect of ATP was not abolished by the addition of an uncoupler of chemical or electrical gradients. While the ATP effect was significant, it increased translocation to only one-half that produced by light. While these results showed that an energy-rich phosphate augmented translocation, the mechanism by which it does so or even if it is absolutely required remain unknown. Until further purification of the components involved in translocation is obtained, definitive conclusions concerning the energy requirements for this process will be difficult.

Fate of the transit peptide — As with the transient signal peptides which occur at the NH_2-termini of most nascent secretory proteins, the fate of the transit peptides of the larger precursors of chloroplast polypeptides is unknown. In one experiment,[67] a radioactive band of appropriate size was found after purified precursor to the ribulose-1,5-biphosphate carboxylase small subunit was cleaved in vitro. However, no structural

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analysis of the peptide was performed and its identity as a transit peptide remains in doubt.

3. Conclusions

The results of these studies concerning compartmentalization of algal and plant organellar proteins allow the following conclusions:

1. Cytoplasmically synthesized subunits of chloroplast or glyoxysomal proteins are translated on free polyribosomes and released into the cytoplasm
2. The complete products of translation contain extensions (transit peptides) of 2 to 6000 daltons which are rich in hydrophilic amino acid residues
3. The larger cytoplasmic precursor polypeptides are post-translationally incorporated into chloroplasts — the translocation event — and the transit peptide is proteolytically removed
4. Translocation may involve a species-specific receptor, requires energy, and the transit peptide cleavage may involve a sulfhydryl protease

The translocation event for chloroplast proteins is clearly very different from that involved for pre-secretory proteins.

B. Post-translational Compartmentalization of Cytoplasmically Synthesized, Translocatable Mitochondrial Polypeptides

Compartmentalization of polypeptides which are made on cytoplasmic ribosomes and must be translocated into mitochondria has also been under investigation for many years and the subject has recently been reviewed.[61,63] Our discussion will be limited to recent translation studies of cytoplasmic mRNAs encoding various mitochondrial polypeptides and the demonstration that the incorporated peptides are correctly assembled into complexes within various mitochondrial compartments. Of necessity, we will also review some in vivo pulse-chase studies which support the conclusions derived from in vitro experiments.

Similar to chloroplast protein biosynthesis (Section III.A.) most of the cytoplasmically synthesized polypeptides are initially made as larger precursors. The precursors are then post-translationally translocated into mitochondria with concomitant proteolytic removal of a transit peptide. However, in several cases, a larger cytoplasmic precursor is not made, rather the initial product of translation is identical in size to the mature polypeptide. These exceptions make it difficult to conceive a general, shared mechanism by which translocation of proteins into mitochondria might take place.

The structure of mitochondria is well known and has recently been reviewed.[61] The localization, as far as is known, of mitochondrial enzymes within the various suborganellar compartments has been detailed.[63,84] Cytoplasmically synthesized polypeptides occur in all four mitochondrial compartments — outer membrane, intermembranous space, inner membrane (and its invaginations — the cristae), and matrix space. Mitochondria may differ from chloroplasts in that apposition of inner and outer membranes is fairly well documented, suggesting the possibility that translocation of all mitochondrial proteins might require passage through only one membrane barrier.

It is of historical interest to mention the controversy concerning translocation of cytoplasmically synthesized mitochondrial polypeptides which existed until very recently. Various investigators have suggested that translocatable mitochondrial proteins were made on rough endoplasmic reticulum (membrane-bound polysomes) and transported into mitochondria either directly (i.e., through continuity of the RER lumen

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with mitochondria, analogous to the secretory protein pathway) or in vesicles which break off from the RER and then fuse with the mitochondria. Butow[85] suggested that translocation occurred as a co-translational event because some polysomes were bound directly to the outer membranes of mitochondria. A third hypothesis was that synthesis of translocatable mitochondrial proteins was on free polyribosomes and that incorporation was a post-translational event.[61,86] Present evidence, reviewed below, strongly supports the last mechanism, at least for many cytoplasmically synthesized translocatable mitochondrial proteins. While the other two mechanisms might exist for some polypeptides, little definitive evidence for their occurrence is presently available.[61]

1. Cytochrome c is Not Made as a Larger Precursor

Yeast cytochrome c is a peripheral membrane protein located on the cytoplasmic side of the inner mitochondrial membrane. The enzyme is made on cytoplasmic polyribosomes and, at some point, a heme group is covalently linked to the apoprotein.[84,87]

Zitomer and Hall[87] prepared poly(A)-containing RNA from yeast and allowed it to translate in a wheat germ cell-free lysate. Following immunoprecipitation with antibody to cytochrome c (c), they found a single band of radioactivity which comigrated exactly with mature cytochrome c marker in SDS-PAGE gels. This result strongly suggested that the complete product of translation of yeast cytochrome c was *not* a larger precursor. It was unclear from this early study whether the apoenzyme or the holoenzyme was immunoprecipitable. Moreover, neither structural studies to prove that this was the complete product of translation, nor in vitro reconstitution of translocation were performed.

Korb and Neupert[88] prepared antibodies to *Neurospora* apo- and holo-c. They prepared a cell-free homogenate which was then incubated with [^{3}H]-leucine and, after 10 min, further protein synthesis was prevented with cycloheximide. Holo- or apo-enzymes were then precipitated with specific antibodies. They found that ^{3}H was present in the apo-c to a much greater extent than in holo-c. However, if the incubation were continued for 30 min after addition of cycloheximide, the ^{3}H label was chased into holo-c. When the homogenate was fractionated, apo-c could not be found in mitochondria. Incubation of [^{3}H]-leucine-labeled peptides in a cycloheximide-containing post-ribosomal supernatant fraction with unlabeled mitochondria for 30 min at 25°C demonstrated that labeled apo-c was translocated into the mitochondria and converted to holo-c. This uptake and conversion to holo-c did not occur at 0°C or if detergent-lysed mitochondria were used. These results clearly demonstrated that newly synthesized apo-c present in the yeast cytoplasm could be incorporated post-translationally into mitochondria and the heme group attached during or following this uptake. The results of this study must be qualified since: (1) the mitochondria probably lacked outer membranes, (2) apo-c was not labeled in a completely organelle-free lysate, and (3) structural characterization of the cytoplasmic apo-c was not performed.

Smith et al.[30] proved that the complete product of translation of yeast iso-1-cytochrome c is not a larger precursor. They determined the complete nucleotide sequence of the c gene coding region. Their results were well documented and unambiguous. The initiation codon was found immediately adjacent to the first amino acid codon of the mature protein. No other initiation codons prior to this one could possibly be utilized in translation (because of intervening termination codons in each conceivable reading frame). This result firmly established that iso-1-cytochrome c, was not synthesized via a larger cytoplasmic precursor.

Further confirmation of this conclusion was provided by the in vitro biological studies of Zimmerman et al.[90] *Neurospora* poly(A)-containing RNA was translated in a

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reticulocyte lysate. Antibody to apo-c precipitated a labeled protein that comigrated in SDS-PAGE exactly with unlabeled apo-c. Formyl-[^{35}S]-methionyl $tRNA_f$ labeling indicated that the apo-c precipitated was, indeed, the complete product of translation. Partial NH_2-terminal sequence analysis suggested that the amino acid sequence of the in vitro synthesized apo-c was f-Met - ? - Phe - . . . . A phenylalanine residue occupies the second position in mature apo-c. This result suggested that the complete translation product was identical with apo-c, except for the initiator methionyl residue, but was somewhat ambiguous because of poor yields of ^{35}S at cycle 1. Post-translational translocation was demonstrated by incubation of mitochondria with a post-ribosomal supernatant of the translation reaction. Labeled holo-c was found in the mitochondria.

The results of these several studies provide fairly strong evidence that (1) apocytochrome c is the initial product of translation of its mRNA, (2) the apo protein is released into the cytosol, and (3) post-translational translocation into mitochondria occurs concomitantly with formation of the heme-containing holo enzyme. These results have been discussed in some detail because most other cytoplasmically synthesized translocatable mitochondrial polypeptides are made as larger precursors.

2. Some ATPase Subunits are Made as Individual, Larger Precursors in the Cytoplasm

Saccharomyces mitochondrial ATPase[91] is an oligomeric enzyme which is located partly within the inner membrane and partly on the matrix side of the membrane. At least five of its nonidentical subunits are synthesized in the cytoplasm, while the other subunits are encoded on the mitochondrial genome.

Maccecchini et al.[92] provided evidence that three of the ATPase subunits are initially made in the cytoplasm as individual, larger precursors which are post-translationally translocated into mitochondria and converted to their mature forms. Yeast RNA was translated in a cell-free lysate and specific antisera against F_1-ATPase subunits α, β, or γ were used in separate immunoprecipitation reactions. The translation products were compared by SDS-PAGE with in vivo labeled mature subunits also isolated by immunoprecipitation. The in vitro products migrated with apparent M_r of 6000, 2000, and 6000 daltons greater than the corresponding mature subunits. However, no experiments were presented to prove that these precursors were the complete products of translation of *separate* subunit mRNAs. To assess whether these precursors also could be found in vivo in the cytoplasm, spheroplasts were pulsed with [^{35}S]-methionine for 5 min, lysed, and analyzed by immunoprecipitation. Although greater than 90% of the labeled products formed were mature subunits, a small amount of precursor comigrating with the in vitro products were also demonstrated. If the spheroplasts were chased with cold methionine prior to lysis, no subunit precursors were detected. Together, these results suggested that in vivo completed chains of the subunit precursors were released into the cytoplasm, subsequently incorporated into mitochondria and cleaved to their mature forms. Unfortunately, it was not demonstrated quantitatively that the in vivo precursors were incorporated into the mitochondrial mature subunits. An alternative explanation would be that the in vivo precursors were not incorporated, but simply degraded during the chase.

Labeled precursors synthesized in vitro were then incubated with mitochondria and the mitochondria reisolated by centrifugation. The result showed that, while some of the subunit precursors remained in the cytoplasm, some were bound to the mitochondria. Significantly, labeled mature subunits were found in the mitochondria. Addition of exogenous protease to the incubation prior to isolation of the mitochondria demonstrated that all the precursors were completely degraded, but the mature subunits were protected. Although the authors attempted to show that mature labeled subunits could not be incorporated into mitochondria, this experiment was not convincing. An

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attempt to demonstrate structural similarity of mature γ subunit with its precursor was equivocal because the one-dimensional proteolytic map revealed identity of only two of the six or more labeled bands. Despite these limitations, these data do suggest that these subunits were initially made as individual, larger precursors which were post-translationally translocated into mitochondria and cleaved to their mature size.

Further evidence to support this concept has been presented.[93] Yeast RNA was translated in a reticulocyte lysate as above except that the label was N-formyl-[^{35}S]-methionyl-tRNA$_f$. Labeled F_1-ATPase subunit precursors were then immunoprecipitated and were shown by SDS-PAGE to be identical with those observed above. The result proved that the immunoprecipitable precursors of each subunit were indeed the product of translation of separate, subunit-specific mRNAs, each with its own initiator codon.

Independent evidence for synthesis of ATPase subunits as individual, cytoplasmic, larger precursors was provided by in vitro translation of *Neurospora* mRNA encoding another subunit.[94] The cytoplasmically synthesized proteolipid subunit* apoprotein is an integral inner membrane component of the complex with a M_r of 8000. The in vitro product had an apparent M_r = 12,000. Partial sequence analysis of a COOH terminal fragment of the 12,000 dalton precursor demonstrated identity with this fragment of the mature subunit. These results leave little doubt that some ATPase subunits are initially synthesized as individual cytoplasmic precursors which are post-translationally incorporated into mitochondria and cleaved to their mature sizes.

3. Other Mitochondrial Polypeptides

Similar experiments to those outlined above have subsequently been performed utilizing mRNAs for other cytoplasmically synthesized mitochondrial proteins and identification of the in vitro translated cell-free product following immunoprecipitation (Table 1).

The complete products of translation of the mRNA encoding the subunits of rat liver *carbamyl phosphate synthetase*[95-97] and *Neurospora citrate synthetase,*[98] two mitochondrial matrix enzymes, were found to be larger precursors of the mature subunits. In vivo pulse-chase experiments suggested that the cytoplasmic precursors could be post-translationally translocated into mitochondria. Similar conclusions concerning biosynthesis of subunit II of yeast *cytochrome bc_1 complex,*[99] an inner membrane protein, have been reported. The lack of convincing structural studies, and the absence of data dealing with translocation, made some of these conclusions tentative. However, these results were consistent with the cytoplasmic precursor, post-translational uptake mechanism.

Cytochrome c peroxidase (CcP) is localized within the intermembranous space of yeast mitochondria and is encoded by the nuclear genome. Thus, it is similar to cytochrome c in its suborganellar location. Based on similar experiments to those discussed above, Meccecchini and colleagues[100] found that CcP is made as a cytoplasmic precursor (M_r = 39,500) both in vivo and in vitro, which could be chased to the mature form (M_r = 33,500) in vivo. The in vivo pulse-chase experiments showed quantitative conversion of pre-CcP to CcP. One-dimensional fingerprints with two proteases showed convincing similarities in the labeled fragments. Post-translational uptake of pre-CcP into mitochondria with processing to the mature form was also demonstrated. The results are the most convincing relative to mitochondrial precursors of those reviewed. It is noteworthy that CcP and cytochrome c are both localized in the intermembranous

* In *Saccharomyces* this subunit is encoded on the mitochondrial genome.

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Table 1
CYTOPLASMICALLY SYNTHESIZED TRANSLOCATABLE MITOCHONDRIAL POLYPEPTIDES

Polypeptide	Localization	Mature subunit M_r	Cytoplasmic precursor M_r	In vitro translocation	In vivo pulse-chase	Ref.
Carbamyl phosphate synthetase	Matrix	160,000 160,000	165,500 160,000	Not tested Not tested	Yes Yes	95—97
Cytochrome c	Inner membrane (cytoplasmic side-peripheral)	13,000 + heme	13,000 − heme	Yes	Yes	87—90
Cytochrome c peroxidase	Intermembranous space	33,500	39,500	Yes	Yes	100
ADP/ATP carrier	Inner membrane (integral)	32,000	32,000	Not tested	Yes	101
ATPase						
Subunits						
α	Inner membrane	58,000	64,000	Yes	Yes	92, 93
β	(matrix side-	54,000	56,000	Yes	Yes	
γ	peripheral)	34,000	40,000	Yes	Yes	
Proteolipid	Inner membrane (integral)	8,000	12,000			94
Cytochrome bc_1 subunit V	Inner membrane (integral)	25,000	27,000	Not tested	Yes	99
Citrate synthetase	Matrix	45,000	47,000	Not tested	Maybe	98
Cytochrome c oxidase subunits IV—VII	Inner membrane (transmembrane)	Total 47,000	55,000	Yes	Yes	102, 103
subunits						
IV		17,000	20,000			
V		16,000	18,000	Not tested	Yes	93, 104
VI		12,500	19,000			
VII		~5,000	~5,000			

space, and yet CcP is made as a larger cytoplasmic precursor, while cytochrome c is not (see above).

4. A Possible Exception

ADP/ATP carrier protein is an integral membrane protein of the inner mitochondrial membrane which is translated on cytoplasmic ribosomes.[101] Translation of *Neurospora* RNA in cell-free lysates and immunoprecipitation demonstrated that the subunit carrier protein product was identical in size to the mature subunit. Labeling of the in vitro product with formyl-[^{35}S]-methionyl $tRNA_f$ provided further evidence that this protein was the initial product of translation. Cyanogen bromide fragments of mature subunit and the product formed by elongation of the nascent chains in vitro were compared. Unfortunately, since the product of elongation was not uniformly labeled (i.e., poorly labeled at the NH_2 terminus) this experiment might not have detected differences at the NH_2 terminus. Moreover, because of the anomalous behavior of small, hydrophobic proteins in SDS-PAGE, it is possible that the in vitro synthesized product and the mature protein do differ significantly in molecular weight. These considerations are important because the mature subunit is soluble only in detergents while the in vitro product was quite soluble in aqueous solutions. This difference remains unexplained. Proof that the translation product and the mature form are identical will require more complete structural characterization.

5. A Controversy — Saccharomyces Cytochrome c Oxidase

Cytochrome c oxidase is composed of three (I-III) subunits which are made within the mitochondria and localized within the inner membrane and four (IV-VII) cytoplasmically synthesized subunits. The enzyme complex is associated with heme, copper and cardiolipin. Biosynthesis of subunits IV-VII has been studied by Poyton and McKemmie[102,103] and by Schatz and colleagues.[93] Poyton concluded that all four subunits are made in the cytoplasm as a single polypeptide precursor which is post-translationally translocated to the inner membrane and proteolytically processed there to the four mature subunits. More recently, Schatz[93] and later Blobel[104] concluded that subunits V and VI are initially synthesized in the cytoplasm as separate, larger, individual precursors. The data which led to these conflicting conclusions are described below.

Poyton's work[102,103] was performed utilizing whole cell in vivo labeling. No in vitro translation experiments were reported. Yeast cells were labeled, a 100,000 × g supernatant prepared, and direct immunoprecipitation with anti-holoenzyme antisera performed. The major radioactive band precipitated was of $M_r = 55{,}000$ as determined by SDS-PAGE. This protein was named Pr IV-VII because it was assumed to be the cytoplasmic precursor of these four subunits of cytochrome c oxidase. Subunit IV or VI specific antisera were also stated to precipitate Pr IV-VII, although the data were not shown. All further studies to characterize Pr IV-VII were performed on this protein following purification with holoenzyme antiserum and preparative SDS-PAGE. Ouchterlony double diffusion analysis vs. subunit specific antibodies revealed cross reaction with anti-IV and anti-VI, but *not* anti-(V + VII). [^{14}C]-leucine-labeled tryptic peptides of Pr IV-VII were compared to those of [^{3}H]-leucine-labeled mixed subunits IV-VII and to each individual subunit by isoelectric focusing in polyacrylamide gels. Greater than 20 labeled peaks were compared in 80 gel slices. While close similarity seemed apparent, ratios of ^{14}C to ^{3}H were not identical for all peptides, and resolution of the various peaks was poor. Based on these data, and on dual-label pulse and pulse-chase experiments, it was concluded that Pr IV-VII was a cytoplasmic, biosynthetic, polyprotein precursor to subunits IV-VII.

Poyton further showed that Pr IV-VII could be found in the inner membrane fraction

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of sub-fractionated mitochondria and that this mitochondrial Pr IV-VII was similar to the cytoplasmic form by isoelectric focusing of tryptic peptides. Dual-label pulse-chase experiments followed by subcellular fractionation purported to show a precursor-product relationship between Pr IV-VII and the individual subunits. However, the amount of label in this experiment was insufficient to allow this conclusion. In an attempt to demonstrate translocation, [^{35}S]-labeled cytosol was incubated with purified mitochondria. Only labeled Pr IV-VII was found in the cytosol, while several protein bands including subunit IV were recognized in the inner membrane. It was concluded that Pr IV-VII was taken up by the mitochondria in vitro and processed via several intermediates to mature subunits.

Schatz[93] explored the biosynthesis of cytochrome c oxidase subunits using in vivo labeling and in vitro translation of RNA, both followed by indirect immunoprecipitation with subunit specific antibody and *S. aureus* protein A. The product of translation precipitated with anti-subunit VI antibody, as determined following SDS-PAGE, was of M_r = 19,000 vs. mature subunit VI M_r = 12,500. This result was found whether the label was [^{35}S]-methionine or N-formyl-[^{35}S]methionyl $tRNA_f$. Notably, no other radioactive protein bands were visualized, suggesting that the immunoprecipitation reactions were specific. Similar experiments using subunit V antiserum revealed one major labeled protein band following in vitro translation, (M_r = 18,000 vs. mature subunit M_r = 16,000) and several minor labeled bands of larger sizes (one of which comigrated with presubunit VI). Two dimensional fingerprint comparisons of pre-VI and mature VI (leucine-labeled) or pre-V and mature V (methionine labeled) provided convincing evidence that significant sequence homology existed. No amino acid sequencing comparisons were reported for any of the polypeptides. These in vitro experiments, particularly the initiator labeling studies and the two-dimensional maps, prove that for these two subunits, the initial (and complete) products of translation are separate, larger precursors.

Some in vivo pulse-labeling experiments were also reported.[93] Pre-V and pre-VI were found in pulse-labeled spheroplasts only if the uncoupler carbonyl cyanide *m*-chlorophenylhydrozone (CCCP), was included (28° C). However, if spheroplasts were pulse-labeled at 12°, pre-V was found. If a chase with cold methionine was then performed, labeled pre-V was quantitatively converted to mature V. As noted above, immunoprecipitation with anti-V also contained several larger bands, one of which appeared to be of M_r = 50,000. In this manuscript, no subcellular fractionation experiments were performed, no in vitro reconstitution of translocation was reported, and no studies with subunits IV or VII were available.

Blobel[104] also utilized in vitro translation, pulse-labeling of spheroplasts, and indirect immunoprecipitation with subunit-specific antibodies to study biosynthesis of cytochrome c oxidase subunits. The results were identical to those of Schatz[93] in that the complete products of translation of subunit IV, V and VI mRNAs were larger precursors. However, subunit VII mRNA translation showed a labeled protein band identical in size to the mature subunit, although the gel system may not have resolved proteins of slightly differing molecular weights in this range.

More recently, Poyton has also used translation of yeast mRNA to study the biosynthesis of cytochrome c oxidase subunits.[112] In vitro products labeled with [^{35}S]-methionine or N-formyl-[^{35}S]-methionyl $tRNA_f$ were immunoprecipitated with subunit specific antisera. The labeled protein precipitated was identical in size to Pr IV-VII and no smaller labeled peptides were found.

Clearly, the conclusions of these investigators concerning the biosynthesis of the cytoplasmically synthesized subunits of cytochrome c oxidase are conflicting. However, translocation of these subunit precursors occurs post-translationally and is accompanied by proteolytic processing. More definitive structural studies of the gene(s) encoding the subunits or of the in vitro translation products should resolve this controversy.

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6. Characterization of the Elements Involved in Post-translational Translocation of Mitochondrial Proteins

No evidence is presently available concerning the mechanism by which post-translational translocation of cytoplasmically synthesized mitochondrial polypeptides occurs. However, a few facts relative to this process are known. Concomitant protein synthesis, either by mitochondrial or cytoplasmic polysomes, is not necessary for translocation. As with chloroplasts, ATP appears to be required for the overall process.[105] Pulse-labeling of these energy-depleted spheroplasts indicated that translocation and processing of cytoplasmic precursors of several mitochondrial proteins were partially or completely inhibited. These mature proteins are all located in the inner membrane or on the matrix side of it.[84] However, processing of the cytochrome c peroxidase precursor, which is destined for the intermembranous space, was not altered. Since no subcellular fraction was performed, it is not clear if the precursors were incorporated into mitochondria. It was concluded that ATP was required for proteolytic processing of inner membrane or matrix membrane protein precursors, but not for the precursor of the intermembranous cytochrome c peroxidase. Whether or not this ATP requirement was direct, or mediated through some indirect effect, such as phosphorylation or induction of a conformation change in a putative receptor, is not known.

The nature of the mitochondrial protease which might process translocatable precursors is unknown. In fact, it is as yet unproven that processing even occurs within the mitochondria. However, neutral and sulfhydryl proteases have been shown to exist in mitochondrial inner membranes.[106,107] Table 1 provides a summary of the published results concerning post-translational translocation of mitochondrial polypeptides (see also Reference 108).

7. Conclusions

It is clear upon review of these results that most but not all cytoplasmically synthesized mitochondrial proteins are made as separate larger precursors of proteins or their individual subunits. These larger precursors are released into the cytoplasm, post-translationally translocated into mitochondria, and the transit peptide proteolytically removed. This sequence of events occurs for proteins ultimately destined for any of the submitochondrial compartments — matrix, inner membrane, or intermembranous space. The only obvious difference yet demonstrated during translocation of these preproteins is that translocation of cytochrome c peroxidase precursor was not inhibited by ATP depletion of mitochondria.[105] This result suggests that two translocation sites may exist in mitochondria, one for crossing the outer membrane (ATP independent) and a second for crossing to or through the inner membrane (ATP dependent). However, no larger precursor exists for cytochrome c, the ADP/ATP carrier protein and cytochrome c oxidase subunit VII. Rather, post-translational translocation occurs without proteolytic processing — a situation similar to ovalbumin and several integral membrane proteins of the ER. It is unclear from the results presented whether a common translocation mechanism is shared by these proteins and those with transit peptides, or whether separate mechanisms exist. Although it has been postulated[42] that translocation of mitochondrial proteins involves binding to one or more specific receptor proteins in mitochondrial membranes, this remains unproved.

C. Compartmentalization of Peroxisomal Proteins

Peroxisomes are membrane-enclosed organelles which contain oxidative enzymes, including catalase and uricase. Morphological studies have suggested that liver peroxisomes form by budding off from the rough endoplasmic reticulum. Robbi and Lazarow[109] and Blobel[110] studied the biosynthesis of catalase and uricase by in vitro

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translation of rat liver RNA, in vitro elongation of free and membrane-bound polysomes and in vivo pulse-labeling of proteins followed by subcellular fractionation and localization of the labeled enzymes. Both uricase and catalase were synthesized by free polysomes, not by membrane-bound polysomes. In both cases, the in vitro products were identical in size to the mature enzymes. If microsomal membranes were added during translation, newly synthesized uricase was not sequestered within the membranous vesicles, as assayed by its susceptibility to exogenously added protease. In vivo labeling demonstrated that significant amounts of labeled catalase subunit were present in the cytosolic fraction. It thus appears that these two peroxisomal enzymes are *not* made as larger precursors and that they are translocated post-translationally. However, amino acid sequence comparisons of in vitro products and the mature enzymes were not performed and the translational products were not labeled with initiator methionine, so that the possibility remains that a larger precursor exists. Moreover, in vitro reconstitution of translocation into peroxisomes was not demonstrated. In spite of these qualifications, it seems likely that these peroxisomal enzymes differ in the mechanism of translocation compared to plant glyoxysomal malate dehydrogenase[79] which does seem to be initially made as a larger precursor.

IV. SUMMARY

In the ten years since the proposal[8] and evidence[10] that translocation of secretory proteins across the endoplasmic reticular membrane depended upon an amino acid sequence "signal", our understanding of the events which allow compartmentation of eukaryotic proteins into different organelles has progressed rapidly. During this time, segregation of bacterial proteins has also been found to share many similar features.[2] While the proposal that all proteins destined to cross organellar[15] membranes might utilize an identical mechanism for translocation has been disproved, the impetus provided by the "signal hypothesis" greatly accelerated investigation in this area.

Present evidence indicates that intracellular translocation across membranes, i.e., compartmentation, can occur by four mechanisms:

1. Removal of NH_2-terminal signal (pre) peptide of 15 to 30 amino acids from secretory, some viral envelope, integral plasma membrane and lysosomal proteins occur when they cross the ER membrane during translation. Further co-translational and post-translational modifications including glycosylation, processing of oligosaccharide side chains, phosphorylation, further proteolytic processing, acetylation, amidation and fatty acylation also play a role in determining the ultimate localization of these newly synthesized proteins
2. Alternatively, for ovalbumin and some ER integral membrane proteins, compartmentation occurs during translation but without the removal of any peptide
3. For most chloroplast and mitochondrial proteins, translocation from the cytoplasm into the organelle occurs *after* translation is completed and involves the removal of an NH_2-terminal peptide. In contrast to secretory proteins, however, this transit peptide is larger and probably hydrophilic
4. Finally, a few proteins destined for either the ER membrane, mitochondria, or peroxisomes are incorporated post-translationally but without the removal of any peptide

While compartmentation has been shown to occur by these mechanisms, the component parts of the translocation apparatus are not well characterized. One of the major directions of current investigations is to separate, purify and characterize these

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components and, in most cases, proteases. In vitro reconstitution of the translocation event is essential for elucidating the mechanism of this reaction. A second approach, which has already provided useful information, is to construct genes with modifications in the coding regions of translocated proteins and to insert such genes into the bacterial genome. An alternative method is to introduce amino acid analogs into the proteins and assess alterations in translocation. In conjunction with these studies, it is clear that further structural analysis of larger precursors, particularly of mitochondrial protein subunits, will be essential.

When the above information is available, questions concerning the unique structural features of proteins which direct them to a specific organelle, and the components of the translocation apparatus which are common among different organelles can then be answered. Finally, the importance of compartmentation as a regulatory event in modifying localization of active proteins can then be addressed.

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REFERENCES

1. **Silhavy, T., Bassford, P., and Beckwith, J.,** in *Bacterial Outer Membranes: Biosynthesis Assembly and Functions,* Inouye, M., Ed. John Wiley & Sons, New York, 1980, 203.
2. **DiRienzo, J., Nakamura, K., and Inouye, M.,** *Annu. Rev. Biochem.,* 47, 481, 1978.
3. **Chefurka, W. and Hayashi, Y.,** *Biochem. Biophys. Res. Commun.,* 24, 633, 1966.
4. **Redman, C. and Sabatini, D.,** *Proc. Natl. Acad. Sci.,* 56, 608, 1966.
5. **Aronson, A.,** *J. Mol. Biol.,* 15, 505, 1966.
6. **Redman, C., Siekevitz, P., and Palade, G.,** *J. Biol. Chem.,* 241, 1150, 1966.
7. **Redman, C.,** *J. Biol. Chem.,* 242, 761, 1967.
8. **Blobel, G. and Sabatini, D.,** *Biomembranes,* 2, 193, 1971.
9. **Swan, D., Aviv, H., and Leder, P.,** *Proc. Natl. Acad. Sci.,* 69, 1967, 1972.
10. **Milstein, C., Brownlee, G., Harrison, T., and Mathews, M.,** *Nat. New Biol.,* 239, 117, 1972.
11. **Schechter, I., McKean, D., Guyer, R., and Terry, W.,** *Science,* 188, 160, 1975.
12. **Kemper, B., Habener, J., Mulligan, R., Potts, J., and Rich, A.,** *Proc. Natl. Acad. Sci.* 71, 3731, 1974.
13. **Suchanek, G., Kindas-Mugge, I., Kreil, G., Schreier, M.,** *Eur. J. Biochem.,* 60, 309, 1975.
14. **Szczesna, E. and Boime, I.,** *Proc. Natl. Acad. Sci.,* 73, 1179, 1976.
15. **Blobel, G. and Dobberstein, B.,** *J. Cell Biol.,* 67, 852, 1975.
16. **Austen, B.,** *FEBS Lett.,* 103, 308, 1979.
17. **Jacobson, M., Asso, J., and Baltimore, D.,** *J. Mol. Biol.,* 49, 657, 1970.
18. **Hortin, G. and Boime, I.,** *Proc. Natl. Acad. Sci.,* 77, 1356, 1980.
19. **Thibodeau, S. and Walsh, K.,** *Ann. N.Y. Acad. Sci.,* 343, 180, 1980.
20. **Walter, P., Jackson, R., Marcus, M., Lingappa, V., and Blobel, G.,** *Proc. Natl. Acad. Sci.,* 76, 1795, 1979.
21. **Jackson, R., Walter, P., and Blobel, G.,** *Nature,*
22. **Warren, G. and Dobberstein, B.,** *Nature,* 273, 569, 1978.
23. **Birken, S., Smith, D., Canfield, R., and Boime, I.,** 74, 106, 1977.
24. **Lingappa, V., DeVillers-Thiery, A., and Blobel, G.,** *Proc. Natl. Acad. Sci.,* 74, 2432, 1977.
25. **Strauss, A., Bennett, Donahue, A., Rodkey, J., Boime, I., and Alberts, A.,** *J. Biol. Chem.,* 253, 6270, 1978.
26. **Strauss, A., Zimmerman, M., Boime, I., Ashe, B., Mumford, R., and Alberts, A.,** *Proc. Natl. Acad. Sci.,* 76, 4225, 1979.
27. **Jackson, R. and Blobel, G.,** *Proc. Natl. Acad. Sci.,* 74, 5598, 1977.
28. **Smith, D. and Boime, I.,** *FEBS Lett.,* 84, 115, 1977.
29. **Palmiter, R., Gagnon, J., and Walsh, K.,** *Proc. Natl. Acad. Sci.,* 75, 94, 1978.
30. **Chambon, P., Perrin, F., O'Hare, K., Mandel, J., LePennec, J., LeMeur, M., Krust, A., Hellig, R., Gerlinger, P., Gannon, F., Cochet, M., Breathnach, R., and Benoist, C.,** Eucaryotic gene regulation, in *ICN-UCLA Symposia on Molecular and Cellular Biology,* Academic Press, 1979, 259.
31. **Lingappa, V., Shields, D., Woo, S., and Blobel, G.,** *J. Cell Biol.,* 79, 567, 1978.
32. **Palmiter, R., Thibodeau, S., Rogers, G., and Boime, I.,** *Ann. N.Y. Acad. Sci.,* 343, 192, 1980.
33. **Lingappa, V., Lingappa, J., and Blobel, G.,** *Nature,* 281, 117, 1979.
34a. **Meek, R., Walsh, K., and Palmiter, R.,** *Fed. Proc.,* 71, (Abstr.) 1370, 1980.
34b. **Boime, I., Szczesna, E., and Smith, D.,** *Eur. J. Biochem.,* 73, 515, 1977.

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35. **Kreibich, G., Urlrich, B., and Sabatini, D.,** *J. Cell Biol.,* 77, 464, 1978.
36. **Bielinska, M., Rogers, G., Rucinsky, T., and Boime, I.,** 76, 6152, 1979.
37. **Dallner, G., Siekevitz, P., and Palade, G.,** *J. Cell Biol.,* 39, 97, 1966.
38. **Depierre, J. and Dallner, G.,** *Biochim. Biophys. Acta,* 415, 411, 1976.
39. **Mechler, B. and Vassalli, P.,** *J. Cell Biol.,* 67, 16, 1975.
40. **Singer, J. J.,** *Ann. Rev. Biochem.,* 43, 805, 1974.
41. **Rothman, J. E. and Lenard, J.,** *Science,* 195, 743, 1977.
42. **Blobel, G.,** *Proc. Natl. Acad. Sci. U.S.A.,* 77, 1496, 1980.
43. **Lingappa, V. R., Cunningham, B., Jazwinski, S., Hopp, T. A., Blobel, G., and Edelman, G.,** *Proc. Natl. Acad. Sci. U.S.A.,* 76, 3651, 1979.
44. **Palade, G.,** *Science,* 189, 347, 1975.
45. **Marchesi, V. T., Furthmayr, H., and Tomita, M. A.,** *Ann. Rev. Biochem.,* 45, 667, 1976.
46. **Lingappa, V. R., Katz, F. N., Lodish, H. F. and Blobel, G.,** *J. Biol. Chem.,* 253, 8667, 1978.
47. **Irving, R. A., Toneguzzo, F., Rhee, S. H., Hottman, T., and Ghosh, H. P.,** *Proc. Natl. Acad. Sci. U.S.A.,* 76, 570, 1979.
48. **Rose, J. K., Welch, W. J., Sefton, B. M., Esch, I. S., and Ling, N. C.,** *Proc. Natl. Acad. Sci. U.S.A.,* 77, 3884, 1980.
49. **McCauley, J., Bye, J., Elder, K., Gething, M. J., Skehel, J. J., Smith, A., and Waterfield, M. D.,** *FEBS Lett.,* 108, 422, 1979.
50. **Ploegh, H. L., Cannon, L. E., and Strominger, J. L.,** *Proc. Natl. Acad. Sci. U.S.A.,* 76, 2273, 1979.
51. **Jokinen, M., Gahmberg, C. G., and Andersson, L. C.,** *Nature,* 279, 604, 1979.
52. **Chyn, T., Martonosi, A. N., Morimoto, T., and Sabatini, D.,** *Proc. Natl. Acad. Sci. U.S.A.,* 76, 1241, 1979.
53. **Kumar, A. and Padmanaban, G.,** *J. Biol. Chem.,* 255, 522, 1980.
54. **Haugen, D. A., Armes, L. G., Yasanobu, K. T., and Coon, M. J.,** *Biochem. Biophys. Res. Commun.,* 77, 967, 1977.
55. **Bar-Nun, S., Kreibich, G., Adesnik, M., Alterman, L., Negishi, M., and Sabatini, D.,** *Proc. Natl. Acad. Sci. U.S.A.,* 77, 965, 1980.
56. **Gonzalez, F., and Kasper, C.,** *Biochem. Biophys. Res. Commun.,* 93, 1254, 1980.
57. **Schechter, I., Burstein, Y., Zemmell, R., Ziv, E., Kantor, F., and Papermaster, D.,** *PNAS,* 76, 2654, 1979.
58. **Rachubinski, R., Verma, D., and Bergeron, J.,** *J. Cell Biol.,* 84, 705, 1980.
59. **Kreiter, P. A., and Shires, T. K.,** *Biochem. Biophys. Res. Commun.,* 94, 606, 1980.
60. **Borghese, N., and Gaetani, S.,** *FEBS Lett.,* 112, 216, 1980.
61. **Chua, N.-H. and Schmidt, G. W.,** *J. Cell Biol.,* 81, 461, 1979.
62. **Ellis, R. J.,** *Biochim. Biophys. Acta,* 463, 185, 1977.
63. **Schatz, G. and Mason, T. L.,** *Ann. Rev. Biochem.,* 43, 51, 1974.
64. **Wickner, W.,** *Ann. Rev. Biochem.,* 48, 23, 1979.
65. **Blair, G. E. and Ellis, R. J.,** *Biochim. Biophys. Acta,* 319, 223, 1973.
66. **Roy, H., Patterson, R., and Jugendorf, A. T.,** *Arch. Biochem. Biophys.,* 172, 64, 1976.
67. **Dobberstein, B., Blobel, G., and Chua, N.-H.,** *Proc. Natl., Acad. Sci. U.S.A.,* 74, 1082, 1977.
68. **Cashmore, A. R., Broadhurst, M. K., and Gray, R. E.,** *Proc. Natl. Acad. Sci. U.S.A.,* 75, 655, 1978.
69. **Highfield, P. E. and Ellis, R. J.,** *Nature,* 271, 420, 1978.
70. **Tobin, E. M.,** *Proc. Natl. Acad. Sci. U.S.A.,* 75, 4749, 1978.
71. **Schmidt, G. W., Devillers-Thiery, A., Desruisseaux, H., Blobel, G., and Chua, N.-H.,** *J. Cell Biol.,* 83, 615, 1979.
72. **Palmiter, R. D.,** *J. Biol. Chem.,* 252, 8781, 1977.
73. **Strauss, A., Bennett, C. D., Donohue, A. M., Rodkey, J. A., and Alberts, A. W.,** *J. Biol. Chem.,* 252, 6846, 1977.
74. **Huisman, J. G., Moorman, A. F. M., and Verkley, F. N.,** *Biochem. Biophys. Res. Commun.,* 82, 1121, 1978.
75. **Schmidt, G. W., Bartlett, S., Grossman, A. R., Cashmore, A. R., and Chua, N.-H.,** *Genome Organization and Expression in Plants,* C. J. Leavan, Ed. Plenum Press, New York, 1979, 337.
76. **Apel, K. and Kloppstech, K.,** *Eur. J. Biochem.,* 85, 581, 1978.
77. **Nelson, N., Nelson, H., and Schatz, G.,** *Proc. Natl. Acad. Sci. U.S.A.,* 77, 1361, 1980.
78. **Kawajiri, K., Harano, T., and Omura, T.,** *J. Biochem. (Tokyo),* 82, 1417, 1972.
79. **Walk, R.-A. and Hock, R.,** *Biochem. Biophys. Res. Commun.,* 81, 636, 1978.
80. **Chua, N.-H. and Schmidt, G. W.,** *Proc. Natl. Acad. Sci. U.S.A.,* 75, 6110, 1978.
81. **Grossman, A. E., Bartlett, S. G., Schmidt, G. W., and Chua, N.-H.,** *Ann. N.Y. Acad. Sci.,* 343, 266, 1980.

Critical Reviews in Biochemistry and Molecular Biology Downloaded from informahealthcare.com by 89.163.34.136 on 01/06/12
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RIGHTSLINK

82. **Smith, S. M. and Ellis, R. J.,** *Nature,* 278, 662, 1979.
83. **Grossman, A., Bartlett, S., and Chua, N.-H.,** 1980 *Nature,* submitted.
84. **DePierre, J. W. and Ernster, L.,** *Ann. Rev. Biochem.,* 46, 201, 1977.
85. **Kellems, R. E., Allison, V. F., and Butow, R. A.,** *J. Biol. Chem.,* 249, 3297, 1974.
86. **Hallermayer, G., Zimmermann, R., and Neupert, W.,** *Eur. J. Biochem.,* 81, 523, 1977.
87. **Zitomer, R. S. and Hall, B. D.,** *J. Biol. Chem.,* 251, 6320, 1976.
88. **Korb, H. and Neupert, W.,** *Eur. J. Biochem.,* 91, 609, 1977.
89. **Smith, M., Leung, D. W., Gillam, S., Astell, C. R., Montgomery, D. L., and Hall, B. D.,** *Cell,* 16, 753, 1979.
90. **Zimmermann, R., Paluch, U., and Neupert, W.,** *FEBS Lett.,* 108, 141, 1979.
91. **Sebald, W.,** *Biochim. Biophys. Acta* 463, 1, 1977.
92. **Maccecchini, M.-L., Rudin, Y., Blobel, G., and Schatz, G.,** *Proc. Natl. Acad. Sci. U.S.A.,* 76, 343, 1979.
93. **Lewin, A. S., Gregor, I., Mason, T. L., Nelson, N., and Schatz, G.,** *Proc. Natl. Acad. Sci., U.S.A.,* 77, 3998, 1980.
94. **Michel, R., Wachter, E., and Sebald, W.,** *FEBS Lett.,* 101, 373, 1979.
95. **Shore, G. C., Carignan, P., and Raymond, Y.,** *J. Biol. Chem.,* 254, 3141, 1979.
96. **Raymond, Y. and Shore, G. C.,** *J. Biol. Chem.,* 254, 9335, 1979.
97. **Mori, M., Morris, S. M., Jr., and Cohen, P. P.,** *Proc. Natl. Acad. Sci. U.S.A.,* 76, 3179, 1979.
98. **Harmey, M. A. and Neupert, W.,** *FEBS Lett.,* 108, 385, 1979.
99. **Cote, C., Solioz, M., and Schatz, G.,** *J. Biol. Chem.,* 254, 1437, 1979.
100. **Maccecchini, M.-L., Rudin, Y., and Schatz, G.,** *J. Biol. Chem.,* 254, 7468, 1979.
101. **Zimmerman, R., Paluch, U., Sprinzl, M., and Neupert, W.,** *Eur. J. Biochem.,* 99, 247, 1979.
102. **Poyton, R. O. and McKemmie, E.,** *J. Biol. Chem.,* 254, 6763, 1979.
103. **Poyton, R. O. and McKemmie, E.,** *J. Biol. Chem.,* 254, 6772, 1979.
104. **Mihara, K. and Blobel, G.,** *Proc. Natl. Acad, Sci. U.S.A.,* 77, 4160, 1980.
105. **Nelson, N. and Schatz, G.,** *Proc. Natl. Acad. Sci. U.S.A.,* 76, 4365, 1979.
106. **Haas, R. and Heinrich, P. C.,** *Eur. J. Biochem.,* 96, 9, 1979.
107. **Duque-Magalhaes, M. C.,** *FEBS Lett.,* 105, 317, 1979.
108. **Schatz, G.,** *FEBS Lett.,* 103, 203, 1979.
109. **Robbi, M. and Lazarow, P. B.,** *Proc. Natl. Acad. Sci. U.S.A.,* 75, 4344, 1978.
110. **Goldman, B. M. and Blobel, G.,** *Proc. Natl. Acad. Sci. U.S.A.,* 75, 5066, 1978.
111. **Pickett, C. and Waterman, M. R.,** personal communications.
112. **Poyton, R.,** personal communications.

NOTE ADDED IN PROOF

Since the preparation of this review, several related manuscripts and reviews have been published and are referenced below.

Reviews

1. **Neupert, W. and Schatz, G.,** *TIBS,* 6, 1, 1981.
2. **Lord, J. M. and Roberts, L. M.,** *TIBS,* 5, 271, 1980.
3. **Engleman, D. M. and Steitz, T. A.,** *Cell,* 23, 411, 1981.
4. **Waksman, A., Hubert, P., Cremel, G., Rendon, A., and Burguin, C.,** *Biochem. Biophys. Acta.,* 604, 249, 1980.
5. **Von Heijne, G.,** *Eur. J. Biochem.,* 116, 419, 1981.

Section I

Zwizinski, C. and Wickner, W., *J. Biol. Chem.,* 255, 7973, 1980.

Section II

Rice, C. M. and Strauss, J. H., *Proc. Natl. Acad. Sci. U.S.A.,* 78, 2062, 1981.
Bergmann, J. E., Tokuyasa, K. T., and Singer, S. J., *Proc. Natl. Acad. Sci. U.S.A.,* 78, 1746, 1981.
Mostov, K. E., Kraehenbuhl, J. P. and Blobel, G., *Proc. Natl. Acad. Sci. U.S.A.,* 77, 7257, 1980.
Schlesinger, M. J., Magee, A. I., and Schmidt, M. F. G., *J. Biol. Chem.,* 255, 10021, 1980.
Reithmeier, R. A. F., deLeon, S., and MacLennan, D. H., *J. Biol. Chem.,* 255, 11839, 1980.
Rogeus, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L., and Wall, R., *Cell,* 20, 303, 1980.
Negishi, M. and Nebert, D. W., *J. Biol. Chem.,* 256, 3085, 1981.

Critical Reviews in Biochemistry and Molecular Biology Downloaded from informahealthcare.com by 89.163.34.136 on 01/06/12
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Section III

Scarpulla, R. C., Agne, K. M., and Wu, R., *J. Biol. Chem.,* 256, 6480, 1981.
Schmelzer, E. and Heinrich, P. C., *J. Biol. Chem.,* 255, 7503, 1980.
Raymond, Y. and Shore, G. C., *J. Biol. Chem.,* 256, 2087, 1981.
Pickett, C. B., Rosenstein, N. R., Jeter, R. L., Montisano, D., and Cascarano, J., *Biochem. Biophys. Acta.,* 501, 441, 1980.
Ades, I. F. and Bukow, R. A., *J. Biol. Chem.,* 255, 9918, 1980.
Dubois, R. N., Simpson, E. R., Tuckey, J., Lambeth, J. D., and Waterman, M. R., *Proc. Natl. Acad. Sci. U.S.A.,* 78, 1028, 1981.
Conboy, J. G. and Rosenberg, L. E., *Proc. Natl. Acad. Sci. U.S.A.,* 78, 3073, 1981.
Anderson, L., *Proc. Natl. Acad. Sci. U.S.A.,* 78, 2407, 1981.
Morita, T., Mori, M., Tatibana, M. and Cohen, P. P., *Biochem. Biophys. Res. Commun.,* 99, 623, 1981.
Sakakibara, R., Huynh, Q. K., Nishida, Y., Watanabe, T. and Wada, H., *Biochem. Biophys. Res. Commun.,* 95, 1781, 1980.

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