Uncoupling Translocation from Translation: Implications for Transport of Proteins Across Membranes

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The segregation of secretory proteins into the cisternae of the endoplasmic reticulum (ER) is normally tightly coupled to their synthesis. This feature distinguishes their biogenesis from that of proteins targeted to many other organelles. In the examples presented, translocation across the ER membrane is dissociated from translation. Transport, which is normally cotranslational, may proceed in the absence of chain elongation. Moreover, translocation across the ER membrane does not proceed spontaneously since, even in the absence of protein synthesis, energy substrates are required for translocation. These conclusions have been extended to the cotranslational integration of newly synthesized transmembrane proteins.

THE ELUCIDATION OF THE SECRETORY PATHWAY IN HIGHER eukaryotes brought into focus the fundamental cell biological problem of translocation of proteins across membranes (*1*). How was it that certain classes of newly synthesized proteins were selectively transported into the lumen of the endoplasmic reticulum (ER) and once there, remained segregated from the cytosol throughout the course of intracellular transport and exocytosis? The impermeability of the lipid bilayer to bulky, charged macromolecules such as proteins could account for the maintenance of asymmetry and segregation, but only underscored the problem of generating the asymmetry in the first place (*2*). For this and other reasons, early workers searched for a specialized mechanism by which newly synthesized secretory proteins might be directed into the cisternal space of the ER (*3*).

A major breakthrough in these efforts came with the development of cell-free systems assembled from heterologous components, which were capable of reconstituting the early events of secretion (*4, 5*). For the past decade such systems have been a major source of insight into the translocation process (*6*), but the molecular mechanisms of these events remain in large measure undetermined (*7*).

One of the most striking features to emerge from the study of these systems was the tight coupling of translocation across the ER membrane to synthesis of the polypeptide chain (*8–10*). This phenomenon accounted for the lack of detectable precursors in tissues in vivo, since it was the nascent and not the completed chains that were translocated, with the signal sequence being cleaved from the still growing chain (*5*). It was also consistent with the in vitro demonstration of vectorial discharge of nascent polypeptides on isolated rough microsomes (*11*).

In contrast, translocation from the cytosol across the membranes of mitochondria and chloroplasts has been shown to be a posttranslational event (*12, 13*). In bacteria (*14, 15*) and at least for some proteins in yeast (*16*), translocation is not coupled to protein synthesis. Thus, the tight coupling of translocation to translation is a distinguishing feature of the ER membrane, but perhaps only in higher eukaryotic systems.

This "cotranslational" aspect of translocation in higher eukaryotic systems provided a sensitive and specific assay for early biosynthetic events. However, it posed a great obstacle to elucidation of mechanism, since the transport events could be analyzed only during the narrow window of time and under the fastidious conditions required for synthesis of the very molecule whose transport was being studied. Thus, for example, it has been unclear whether the functioning ribosome extrudes or "pushes" the growing chain across the bilayer or whether (proteinaceous) machinery in the membrane "pulls" the chain across, or even if translocation proceeds spontaneously (*7*).

Our laboratory has been engaged in molecular genetic manipulation of coding regions whose translocation we study in cell-free systems. We have defined certain features of the translocation event for secretory proteins (*17, 18*). We have shown that a signal sequence coding region engineered at the amino terminus is sufficient to achieve translocation of a normally cytoplasmic protein, globin, across microsomal membranes both in cell-free systems and in intact cells (*19, 20*).

The general approach involves cell-free transcription of coding regions that have been engineered behind an SP6 promoter (*21*). Such transcripts can be used to program cell-free protein-synthesizing systems. By adding membranes either before or after translation, transport across the membrane bilayer can be studied.

It occurred to us that we might be able to use a variation of this approach to investigate the coupling of translocation to translation. Messenger RNA (mRNA) could be transcribed from plasmid DNA which had been cleaved at a restriction site 5′ and close to the termination codon of a coding region of interest. Such truncated transcripts lack a termination codon. When they are used to program cell-free protein synthesis, the initial engaged ribosome should read to the truncated 3′ end of the transcript and be unable to release the nascent chain for lack of a termination codon. Since translocation is initiated well before completion of a protein's synthesis (*5*), that is, at a point well before a ribosome has reached the termination codon, it is likely that a nascent chain translated from such truncated mRNA would be translocation competent. The

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lack of further protein synthesis could be ensured by the addition of inhibitors of both initiation and elongation. Microsomal membranes could then be presented to such complexes of nascent chain and ribosome and translocation could be assayed. In this way the role of a chain's elongation in transport across the ER membrane could be studied in a normally cotranslational system.

The results of such experiments presented in this article demonstrate that translocation is, in fact, independent of ongoing protein synthesis, even in higher eukaryotes and for proteins where the apparent coupling of translocation to translation is strict. Because these transport events could now be studied in the absence of ongoing protein synthesis, we were able to determine that translocation does not proceed spontaneously, since energy substrates are still required. We have also determined conditions by which this posttranslational translocation can be abolished. Our results suggest that, at least for a period of time during chain growth, the ribosome may play a role in maintaining "translocation competence" of the emerged chain.

Co- and posttranslational translocation in a cell-free system. Several criteria have been established for translocation of proteins across microsomal membranes in cell-free systems: (i) Protection from exogenously added proteases except when detergents are present to solubilize the protecting lipid bilayer (*10*); (ii) glycosylation of newly synthesized chains (since oligosaccharidyltransferases are exclusively lumenally disposed enzymes) (*22, 23*); and (iii) cleavage of signal sequences [since signal peptidase of intact membranes appears accessible only to translocated chains (*24*)].

For these studies we chose pSPSG1, an SP6 expression plasmid that encodes a fusion protein consisting of the lactamase signal sequence fused to the amino terminus of chimpanzee alpha globin, and with a glycosylation site engineered into the globin sequence (Fig. 1). Expression of this coding region enables us to assay translocation by all three independent criteria described above. The restriction maps and coding regions of this construction (pSPSG1) and of a related construction (pSPSG3), in which the region encoding the signal sequence was deleted, are shown in Fig. 1A.

Plasmid pSPSG1 was expressed and was shown to program the synthesis of glycosylated and nonglycosylated globin-immunoreactive chains in the expected fashion: in the absence of membranes pSPSG1 encoded preSG1, a nonglycosylated precursor (Fig. 2, lane E). When membranes were present cotranslationally (Fig. 2, lanes A to D), nascent preSG1 was converted to a higher molecular weight form called SG1′. Endo H digestion converted SG1′ to a form of lower molecular weight than preSG1, demonstrating that SG1′ was processed (signal sequence cleaved) as well as glycosylated (Fig. 2, lane A). Protease digestion established that SG1′, but not residual preSG1, was translocated across microsomal membranes (Fig. 2, lane C). Addition of detergent abolished all protease resistance, an indication that protection was conferred by the lipid bilayer and was not a property intrinsic to the processed chain (Fig. 2, lane D). However, when membranes were added posttranslationally (Fig. 2, lanes F to H), preSG1 was the only species observed. From these results we conclude that only nascent preSG1 chains are transported across the ER membrane and hence that translocation of SG1 chains appears coupled to their translation.

Following the logic described in the introduction, we found a

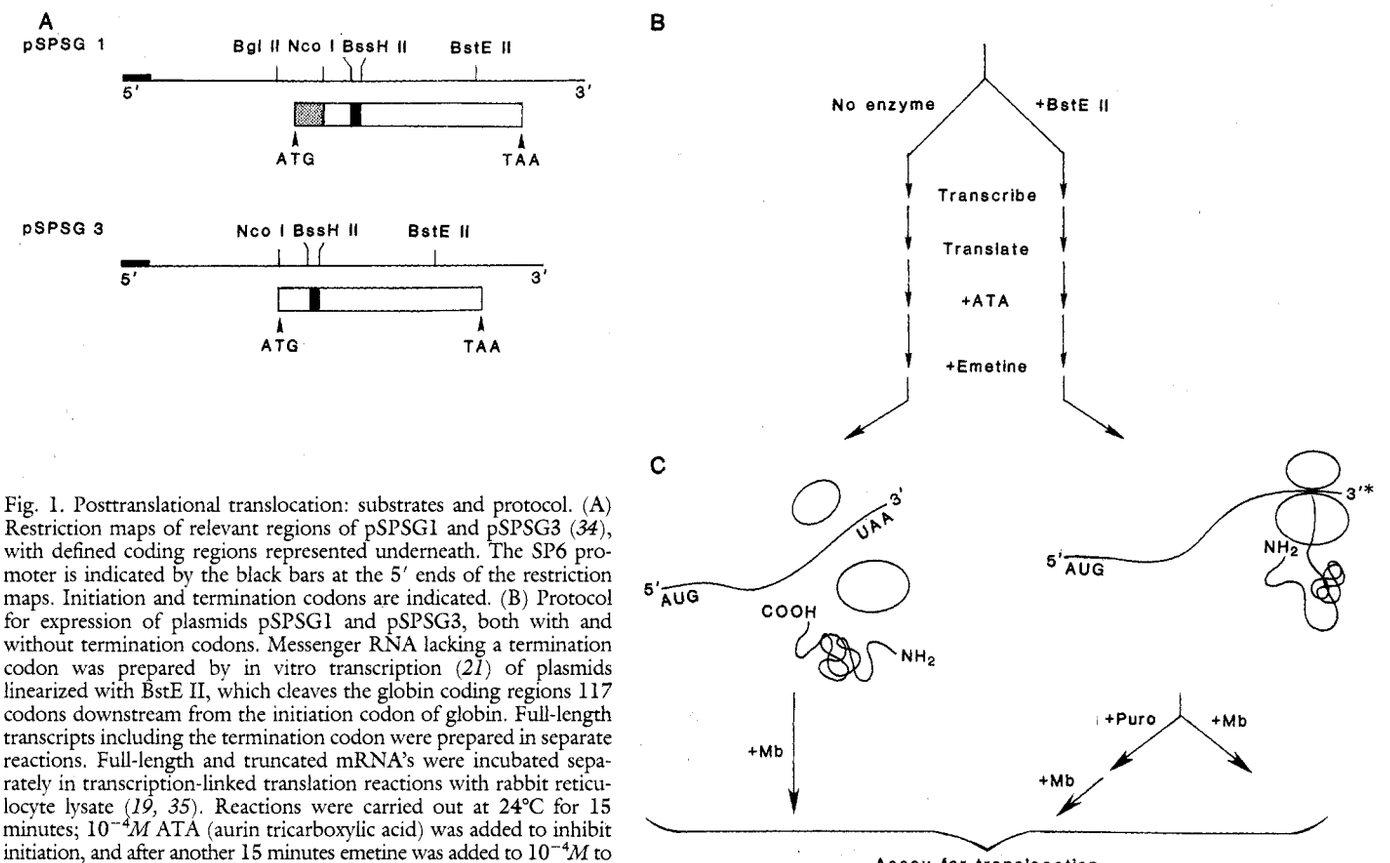


Fig. 1. Posttranslational translocation: substrates and protocol. (A) Restriction maps of relevant regions of pSPSG1 and pSPSG3 (*34*), with defined coding regions represented underneath. The SP6 promoter is indicated by the black bars at the 5′ ends of the restriction maps. Initiation and termination codons are indicated. (B) Protocol for expression of plasmids pSPSG1 and pSPSG3, both with and without termination codons. Messenger RNA lacking a termination codon was prepared by in vitro transcription (*21*) of plasmids linearized with BstE II, which cleaves the globin coding regions 117 codons downstream from the initiation codon of globin. Full-length transcripts including the termination codon were prepared in separate reactions. Full-length and truncated mRNA's were incubated separately in transcription-linked translation reactions with rabbit reticulocyte lysate (*19, 35*). Reactions were carried out at 24°C for 15 minutes; $10^{-4}M$ ATA (aurin tricarboxylic acid) was added to inhibit initiation, and after another 15 minutes emetine was added to $10^{-4}M$ to block elongation. (C) Substrates for posttranslational translocation. Expected translation products of full-length (left) and truncated (right) plasmids are schematically represented. Translation of full-length mRNA yields released full-length polypeptide, free ribosomes, and mRNA while translation of truncated transcripts produces intact polysomes with arrested nascent polypeptide chains emerging from the ribosomes. Polysomes were first incubated with or without 1 mM puromycin before addition of dog pancreas microsomal membranes [5.0 absorbancy units (at 260 nm) per milliliter (A_{260})] (*36*) and further incubation at 24°C for 20 minutes. Translation products from full-length plasmids were not treated with puromycin before incubation with membranes.

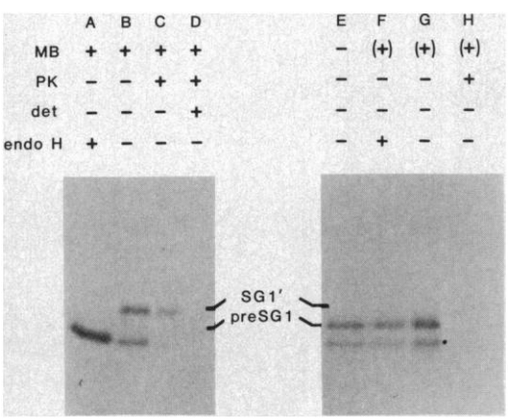
restriction site that would allow us to remove the termination codon by truncating the globin coding region. This site, for restriction endonuclease BstE II, was found 140 codons from the initiation AUG, and 34 codons from that of termination. Plasmid pSPSG1 was cleaved with BstE II and the resulting linear DNA was transcribed (Fig. 1B). This truncated transcript was called SG1/B. Its translation products were immunoreactive with antiserum to globin and migrated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at approximately 12 kD, 3 kD smaller than full-length pre-SG1, as expected for faithful expression of the molecules truncated at BstE II. In order to demonstrate that these polypeptide chains were maintained while emerging from ribosomes and were not released as ribosomes fell off truncated mRNA's, completed translation reactions were sedimented in sucrose density gradients. Analysis of gradient fractions by SDS-PAGE revealed that almost all full-length chains were sedimented under conditions which resulted in pelleted ribosomes (*25*).

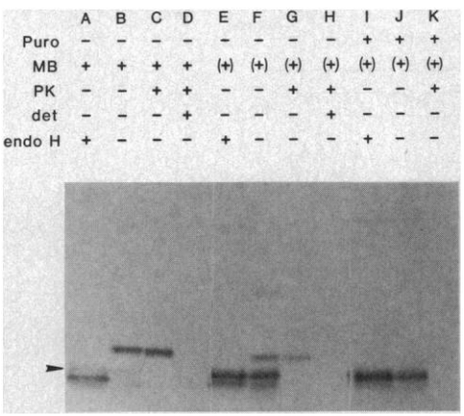
When SG1/B was translated with membranes present during translation (that is, cotranslationally), efficient translocation was demonstrated by all criteria, that is, protection from proteases (Fig. 3, lane C), addition of carbohydrate (Fig. 3, lanes A and B) and processing, as observed for SG1 (Fig. 2). The product is fully protease resistant, presumably because the ribosome protects the portion of the chain which it is anchoring on the cytoplasmic face of the ER membrane. In contrast to SG1, when membranes were added after translation of SG1/B was complete (posttranslationally), translocation was again observed by the same criteria (Fig. 3, lanes E to H), although with somewhat reduced efficiency. In the case of posttranslational membrane addition, either emetine (Fig. 3) or cycloheximide (*25*) were used to prevent further protein synthesis (*26*). Thus, translation of mRNA lacking 35 codons at the 3′ terminus (including the termination codon) permits transport across the ER membrane without ongoing chain elongation; that is, we have achieved an uncoupling of translocation from translation.

In order to probe the basis for uncoupling, we treated the translation reaction with puromycin, an aminoacyl transfer RNA analog that causes termination and release of nascent chains (*26, 27*). We found that puromycin treatment (Fig. 3, lanes I to K) abolished posttranslational translocation of chains encoded by SG1/B. These data suggest a role for the ribosome in transport across the ER membrane that is independent of its role in protein synthesis.

Translation products of SG1/B were filtered in an S-300 Sephacryl gel column, and a void fraction including all polysomes was collected. Incubation of this fraction with microsomal membranes and nucleoside triphosphates (Fig. 4, lane B), but not with membranes alone (Fig. 4, lane C), resulted in translocation. Moreover, the requirement for the energy supplement [consisting of adenosine triphosphate (ATP), guanosine triphosphate (GTP), and creatine phosphate with creatine kinase] indicated that translocation was not proceeding spontaneously but rather displayed an energy requirement independent of protein synthesis.

Plasmid pSPSG3 encoded a protein called SG3 whose mobility was identical in the presence or absence of membranes added either co- or posttranslationally (Fig. 5, lanes B–D), or after endo H





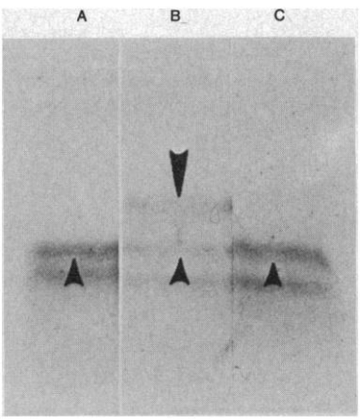


Fig. 2 (left). Cotranslational translocation of SG1 across microsomal membranes. Plasmid pSPSG1 was transcribed in vitro as described (*21*). Transcription-linked translation reactions were carried out (*19*) either in the presence (+ MB; lanes A to D) or absence (− MB; lane E) of microsomal membranes 2.5 A_{260}/ml or with membranes added posttranslationally ([+] MB; lanes F to H; see legend to Fig. 1). Some samples were treated with endoglycosidase H (+ endo H; A and F), others were treated with proteinase K at 0.1 mg/ml (+ PK) in the presence (+ det; lane D) or absence (− det; lanes C and H) of 1 percent Nikkol (a nonionic detergent). Proteolysis and endo H digestion have been described (*19*). The positions of the glycosylated species SG1′ and the precursor form, preSG1, are indicated between lanes D and E. The lower molecular weight band in lane G, indicated by a black dot, is an unidentified translation product of pSPSG1 which comigrates with the processed and unglycosylated form of SG1 (see lane A). It occurs in the presence and absence of membranes and is protease-sensitive. All samples were immunoprecipitated with rabbit antiserum to human hemoglobin (*19*) prepared as described (*10*) and subjected to SDS-PAGE. Gels were fluorographed, and labeled proteins were viewed by autoradiography. Fig. 3 (center). Co- and posttranslational translocation of SG1/B across microsomal membranes. Plasmid pSPSG1, linearized with BstE II, was transcribed to produce mRNA lacking a termination codon (in legend of Fig. 1). The transcript was translated in vitro, as described, either in the presence (+ MB; lanes A to D) or absence (lanes E to K; see legend to Fig. 1) of 2.5 A_{260}/ml of microsomal membranes. Some samples translated without membranes were first incubated in 1 m*M* puromycin (+ Puro; lanes I to K) for 20 minutes before the addition of emetine to 10^{-4}*M* with subsequent incubation with membranes at 5 A_{260}/ml ([+] MB). Other samples were treated with emetine alone (− Puro; lanes E to H), before incubation with membranes. Some samples were treated with endo H (+ endo H; lanes A, E, and I), some treated with proteinase K (0.1 mg/ml) (+ PK) in the presence (+ det; lanes D and H) or absence (− det; lanes C, G, and K) of 1 percent Nikkol. All samples were immunoprecipitated, subjected to electrophoresis on SDS-PAGE and viewed by autoradiography as described in the legend to Fig. 2. Fig. 4 (right). Reconstitution of translocation from S-300 fractionated cytoplasmic components. Plasmid pSPSG1, linearized with BstE II, was transcribed, translated, and treated with emetine as before. The translation mixture was applied to a 1-ml S-300 Sephacryl gel filtration column (*37*) and a void volume was collected. Portions of the void fraction, containing partially purified polysomes, were incubated with microsomal membranes at 2.5 A_{260}/ml at 25°C for 20 minutes either in the presence (lane B) or absence (lane C) of a solution of 1 m*M* ATP, 2 m*M* GTP, 12 m*M* creatine phosphate, and creatine phosphokinase at a final concentration of 40 μg/ml. The noncomplemented void fraction is shown in lane A. Samples were precipitated with trichloroacetic acid, subjected to SDS-PAGE, and viewed by autoradiography. The upward arrow indicates the unprocessed precursor, preSG1, the downward arrow indicates the glycosylated, processed species, SG1′.

digestion (*19*). None of the SG3 chains were protected from proteases (Fig. 5, lanes A, E, and F). Likewise, no translocation either co- or posttranslationally was observed for the similarly truncated form of pSPSG3, termed SG3/B (which lacks the signal sequence as well as the termination codon). This is consistent with the established role of the signal sequence in directing chain translocation.

We have extended our findings for a cleaved amino terminal signal sequence of a secretory protein to an uncleaved internal signal sequence of an integral membrane protein. Plasmid pSPRG95 encodes a fusion protein consisting of the amino terminal glycosylated domain of bovine rhodopsin and the first rhodopsin transmembrane helix, which includes both a signal and a stop transfer sequence (*28, 29*), followed by codons 19 to 143 of globin. This protein is integrated into membranes and glycosylation of its amino terminus reflects its translocation (*30*), as has been shown for secretory glycoproteins (*23*). Figure 6A shows that RG95, the protein encoded by pSPRG95, is glycosylated and, hence, integrated in an obligate cotranslational fashion when the termination codon is present. However, truncation of the coding region at the BstE II site of globin results in uncoupling of integration from translation: glycosylation can be achieved even when incubation with membranes is carried out after completion of protein synthesis and in the presence of elongation inhibitors (Fig. 6B). As in the case of SG1/B, posttranslational translocation was ribosome dependent because treatment with puromycin before incubation with membranes prevented glycosylation (Fig. 6B, lane F).

Implications for chain translocation. We have shown above that expression of certain in vitro transcribed mRNA's that lack termination codons permits the uncoupling of a protein's translocation across microsomal membranes from its synthesis. Furthermore, at least in the cases described here, we demonstrate that translocation, whether coupled or uncoupled to translation, is ribosome dependent. We demonstrated these findings for both a secretory protein (SG1) and for an integral membrane protein (RG95); and for both an amino terminal–cleaved signal (that of β-lactamase) and an internal, uncleaved signal (that of rhodopsin). We conclude that, in general, translocation across the ER membrane is not dependent on concurrent synthesis of the protein being transported. Our data have several implications, both theoretical as well as practical, for the problem of protein transport across the ER membrane.

The demonstration that puromycin treatment or deletion of the signal sequence abolishes translocation, even when it has been uncoupled from protein synthesis, suggests that both the ribosome and the signal sequence participate in maintaining a "translocation competent state" of nascent chains, at least in higher eukaryotes.

These findings, although general for many proteins, are limited to a particular period of time during the growth of any particular chain (*31*). For example, when truncation is carried out considerably more 5′ (78 codons after the signal sequence codons) than was done in the case of SG1/B described here (117 codons after the signal sequence codons), translocation proceeded posttranslationally, but in a ribosome independent fashion. When truncation was carried out considerably further 3′ (175 codons after the signal sequence codons) posttranslational translocation competence is greatly diminished. Thus it seems reasonable to suggest that the nascent chain passes through sequential stages of growth in which translocation competence is first independent of, and then subsequently maintained by, the ribosome. Finally, at a certain point in chain growth translocation competence is lost, presumably because of entanglement of the chain with the signal sequence (*8, 31*).

A large number of proteins studied in higher eukaryotic systems have displayed a strict cotranslational feature to their translocation. However, in bacteria (*14, 15*), translocation of newly synthesized

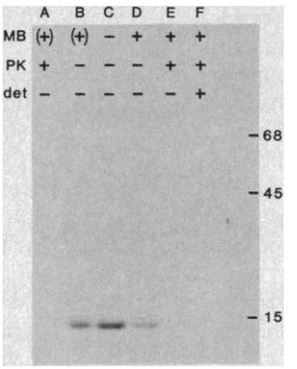


Fig. 5. Co- and posttranslational incubation of SG3 in the presence of microsomal membranes. Plasmid pSPSG3 (Fig. 1) was transcribed and translated. Translation reactions were carried out either in the presence (+MB; lanes D to F) or absence (−MB; lane C) of microsomal membranes (2.5 A_{260}/ml), or with membranes added posttranslationally ([+] MB; lanes A and B; 5 A_{260}/ml). Some reactions were then subjected to proteolysis with proteinase K at 0.1 mg/ml (+ PK; lanes A, E, and F) in the presence (+ det; lane F) or absence (− det; lanes A and E) of 1 percent Nikkol. All samples were immunoprecipitated, subjected to SDS-PAGE and bands viewed by autoradiography.

proteins was shown to lack the obligate coupling to protein synthesis observed in higher eukaryotic systems. Moreover, in a yeast cell-free system, completed and released chains of pre-pro-alpha factor can be demonstrated to cross microsomal membranes, even in the absence of any ribosomes (*16*). It seems likely that the ribosome-independent posttranslational translocation (*16, 31*) and the ribosome-dependent mode described here both reflect interactions of the chain with at least a subset of components in the ER membrane. Taken together with recent evidence suggesting that translocation proceeds through a proteinaceous tunnel (*19, 32*),

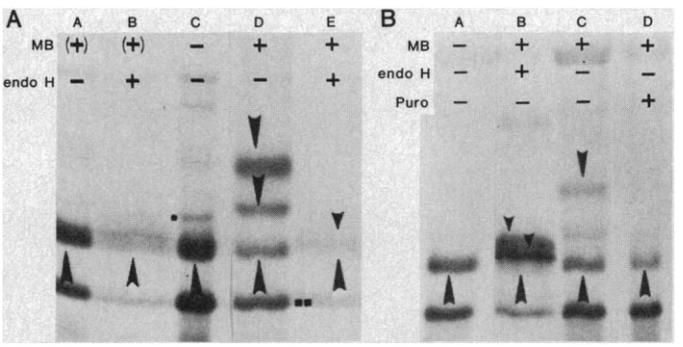


Fig. 6 (A). Co- and posttranslational translocation of RG95. Transcription-linked translation was carried out with plasmid pSPRG95 as described (*19*). Translation reactions were carried out either without microsomal membranes (lanes A to C) or with microsomal membranes present at a final concentration of 2.5 A_{260}/ml during translation (lanes D and E). Some portions were supplemented with microsomal membranes at a final concentration of 5 A_{260}/ml after completion of translation and addition of emetine to $10^{-4}M$ (lanes A and B). All samples were immunoprecipitated with antiserum to globin and some were treated with endo H (lane B, E), before SDS-PAGE. Upward pointing arrow indicates the precursor form of RG95. Downward pointing arrows in lane D, indicate position of glycosylated bands resulting from transmembrane integration of RG95. Downward pointing arrowhead of lane E indicates position of the endo H–digested form of glycosylated RG95. The dot to the left of lane C indicates the position of a minor artifact band, which nearly comigrates with one of two glycosylated forms of RG95. Double dots to the left of lane E indicate position of another artifact band presumed to result from initiation at an internal methionine. (B) Posttranslational translocation of RG95/B. Plasmid pSPRG95 was cleaved with BstE II and subjected to transcription-linked translation as before (*19*). The translation reaction in lane A contained no microsomal membranes. Lane C had microsomal membranes added to a final concentration of 5 A_{260}/ml, after 20 minutes of translation and after addition of emetine to $10^{-4}M$. Lane B is the same as for lane C except that products were treated with endo H. Lane D was the same as indicated for lane C except that sample was treated with puromycin to 1 mM for 20 minutes at 24°C before incubation with microsomal membranes. All incubations with membranes were for 20 minutes at 24°C.

these data lead us to suggest that translocation is driven by a proteinaceous machine in the microsomal membrane.

The energy-dependence of the uncoupled translocation reaction argues that translocation is not a spontaneous process, although it remains to be determined whether expenditure of energy is for translocation per se or rather for assembly and maintenance of the translocation competent state. Thus, as has been observed in both bacteria (*33*) and yeast (*16*), translocation in higher eukaryotes displays an energy requirement independent of that of protein synthesis.

Our results also have practical implications for addressing the problem of protein translocation across the ER membrane. Progress in defining the mechanism of translocation events as well as in isolating the putative membrane components has been hampered by the heretofore coupled nature of the assay: ongoing synthesis of the chain whose translocation was to be studied. The novel assay presented permits discrimination between requirements for these two events by separating the processes of transport from those of synthesis. It should now be possible to distinguish between steps in the transport process, a necessary prerequisite for fractionation and reconstitution of translocation components.

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34. pSPSG1 is an exact in-frame fusion of the signal sequence coding region of the β-lactamase gene (stippled bar) at the 5′ end of the chimpanzee α-globin gene (white regions) into which a synthetic oligonucleotide encoding His-*Asn-Gly-Ser*-Gly-Ser-Gly-Ala (vertical black bars in Fig. 1) has been inserted into a BssH II site. The inserted oligonucleotide encodes the concensus sequence for *N*-linked glycosylation (italicized) *Asn-Gly-Ser*. pSPSG3 was constructed from pSPSG1 by the deletion of the region encoding the β-lactamase signal sequence, leaving the initial ATG of the globin coding region intact (*19*).
35. Rabbit reticulocyte lysate was prepared as described by W. C. Merrick [*Methods Enzymol.* **101**, 606 (1983)].
36. Dog pancreas rough microsomes were prepared as described [P. Walter and G. Blobel, *Methods Enzymol.* **96**, 84 (1983)].
37. Columns were equilibrated in reticulocyte lysate translation buffer: 120 m*M* potassium, 3 m*M* magnesium acetate, 20 m*M* TEA, 1 m*M* dithiothreitol.

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