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Signal Recognition Particle-dependent Insertion of Coronavirus E1, an Intracellular Membrane Glycoprotein*

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The membrane insertion of the E1 protein of a coronavirus, mouse hepatitis virus A59, was studied in a wheat germ cell-free translation system. E1 is a transmembrane protein spanning the lipid bilayer several times. It is synthesized without a cleavable signal sequence, localized intracellularly, and not transported to the cell surface. It thus represents a model intracellular protein. We found that the synthesis of E1 is specifically and stably blocked by the addition of signal recognition particle to the wheat germ system. Subsequent addition of salt-extracted pancreatic microsomes resulted in the full release of this arrest as well as the completion and the correct membrane integration of E1. Such signal recognition particle-induced arrests failed to produce shorter peptides of a defined length. Addition of signal recognition particle to a synchronized translation at any time during the synthesis of about the first two thirds of E1 (150 amino acids) blocked further translation, suggesting that the most C-terminal of the three internal hydrophobic domains of E1 could function as its signal sequence.

Signal recognition particle (SRP[1]) has been shown to be required for the proper membrane insertion or translocation of a number of membrane and secretory proteins (1, 2). It is generally accepted that SRP binds to the signal peptide as it emerges from the ribosome, specifically arresting translation (2). The blocked translation complex migrates to the membrane of the rough endoplasmic reticulum, where an interaction with docking protein (also called SRP receptor) causes the release of the translation block; the nascent polypeptide is then translocated across the endoplasmic reticulum membrane concomitant with its continued synthesis (3, 4). SRP has been shown to be involved in the recognition of secretory (3, 4), lysosomal (5) as well as plasma membrane (6, 7) proteins.

Several recent attempts have been made to determine if this sequence of events is also a characteristic of proteins which remain in an intracellular location, principally the endoplasmic reticulum. Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum (8), baby hamster kidney cell hydroxymethylglutaryl-CoA reductase (9) and rabbit liver cytochrome P-450 (10) have all been examined with regards to the role of SRP in their membrane insertion. However, a clear picture has yet to emerge. It was initially postulated (9) that endoplasmic reticulum proteins lack a cleavable signal sequence. Recently, Rosenfeld *et al.* (11) have shown that the endoplasmic reticulum-specific ribophorins possess a transient signal sequence, cleaved cotranslationally. Whether SRP was required for mediating this insertion was not studied. Of the remaining proteins, all were found to require SRP for integration, yet an SRP-mediated translation arrest was observed only for cytochrome P-450 (10). In this case, an arrested peptide, indicative of a translation block (3), could not be observed, possibly due to lack of an antibody capable of recognizing such a species (10).

Mouse hepatitis virus A59, a coronavirus, has the unusual property of acquiring its envelope by budding at membranes inside the cell (12, 13) rather than at the plasma membrane. This behavior is associated with the *O*-glycosylated viral glycoprotein E1, which, after its synthesis, is confined to membranes of the endoplasmic reticulum and, perhaps, the Golgi region (13, 14) and only ever reaches the cell surface as part of the mature virion. The fact that the restricted localization of E1 is an intrinsic property of the protein itself was further demonstrated both by microinjection of specific mRNA[2] as well as by expression of the E1 cDNA[2,3] in cultured cells. In both cases, the protein was detected solely in intracellular membranes of ER and possibly Golgi. Thus, the E1 molecule represents a model intracellular membrane protein.

Previously, we described the cotranslational assembly of E1 into dog pancreatic microsomal vesicles *in vitro* (15) and showed both that it lacks a cleavable signal sequence and that it spans the membrane several times, a feature confirmed by determining the primary structure of the molecule (16). Interestingly, the N-terminal amino acid sequence does not resemble the signal sequences of either eukaryotic or prokaryotic secretory or membrane proteins (17). There are, however, three stretches of about 20 uninterrupted hydrophobic or uncharged amino acids at positions 27–46, 57–77, and 82–103 respectively (16).[4] It was therefore of interest to see if E1 needs SRP for its insertion into membranes and whether or not such putative "internal" signals participate in this event. Additionally, choosing E1 has the advantage in that purified preparations of E1 mRNA can be obtained, eliminating the need to immunoprecipitate either complete or incomplete products of cell-free translations.

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[1] The abbreviations used are: SRP, signal recognition particle; MHV, mouse hepatitis virus; RM_K, salt-extracted, nuclease-treated rough microsomes; SDS, sodium dodecyl sulfate.

[2] J. Armstrong and B. Timm, unpublished observations.

[3] H. Niemann and M. Wirth, personal communication.

[4] P. J. M. Rottier, G. W. Welling, S. Welling-Wester, H. G. M. Niesters, and B. A. M. van den Zeijst, manuscript submitted.

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In this study, we demonstrate that SRP is required for the correct insertion of E1 into pancreatic microsomes in a wheat germ cell-free system. SRP brings about a stable arrest of translation which can subsequently be released by the addition of salt-extracted rough microsomes. Significant is the fact that the SRP arrest can still be induced relatively late during the synthesis of E1, suggesting an internal signal sequence. These arrests did not generate a specific blocked peptide of uniform length.

EXPERIMENTAL PROCEDURES

Preparation of mRNA—Poly(A)$^+$ RNA was prepared from MHV-A59-infected Sac$^-$ cells as described (18). mRNA enriched for E1 (RNA6) was purified by agarose gel electrophoresis as described (18) and then bound to oligo(dT)-cellulose, eluted, and precipitated with ethanol.

In Vivo Labeling of E1—Monolayers of Sac$^-$ cells were infected with MHV-A59 at multiplicities of 60 and pulse-labeled at 6.5 h post-infection with 120 μCi of [^{35}S]methionine/ml (19). Cells were then chilled, washed with Dulbecco's phosphate-buffered saline, and allowed to swell for 10 min in 10 mM Tris-Cl (pH 8.0), 2 mM $MgCl_2$, 40 μg/ml phenylmethylsulfonyl fluoride. Cell breakage was facilitated by pipetting up and down. Homogenates were centrifuged for 5 min at 1300 × *g* to remove nuclei and debris, and the supernatants were treated as described below.

Proteolysis—Samples of 1300 × *g* supernatants or the products of *in vitro* translations were treated for 15 min at 37 °C with proteinase K (Merck, Darmstadt, West Germany) at a final concentration of 1 mg/ml. The reaction was stopped by addition of phenylmethylsulfonyl fluoride to a concentration of 40 μg/ml. Samples were placed on ice for 10 min, and proteins were precipitated in 10% trichloroacetic acid prior to SDS-polyacrylamide gel electrophoresis.

In Vitro Translation—The preparation of wheat germ extract, dog pancreas microsomes, SRP, and salt-extracted, nuclease-treated rough microsomes (RM_K) was described previously (4). Details of each translation are presented in the figure legends. SRP units are those defined by Walter and Blobel (20). Typically, translation in a wheat germ extract was performed with 0.2 μg of mRNA/25-μl translation. Synchronization was achieved by adding 7-methylguanosine 5′-monophosphate (P-L Biochemicals) (21) to a final concentration of 4 mM after 2 min of translation. Aliquots were removed at various times and further treated as stated in the figure legends. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, fluorography, and densitometric scanning as previously described (22).

RESULTS

SRP Requirement for Assembly of E1—In order to establish the requirement for SRP in the membrane integration of E1, poly(A)$^+$ mRNA from infected cells was translated in a wheat germ system. The products of this translation are shown in

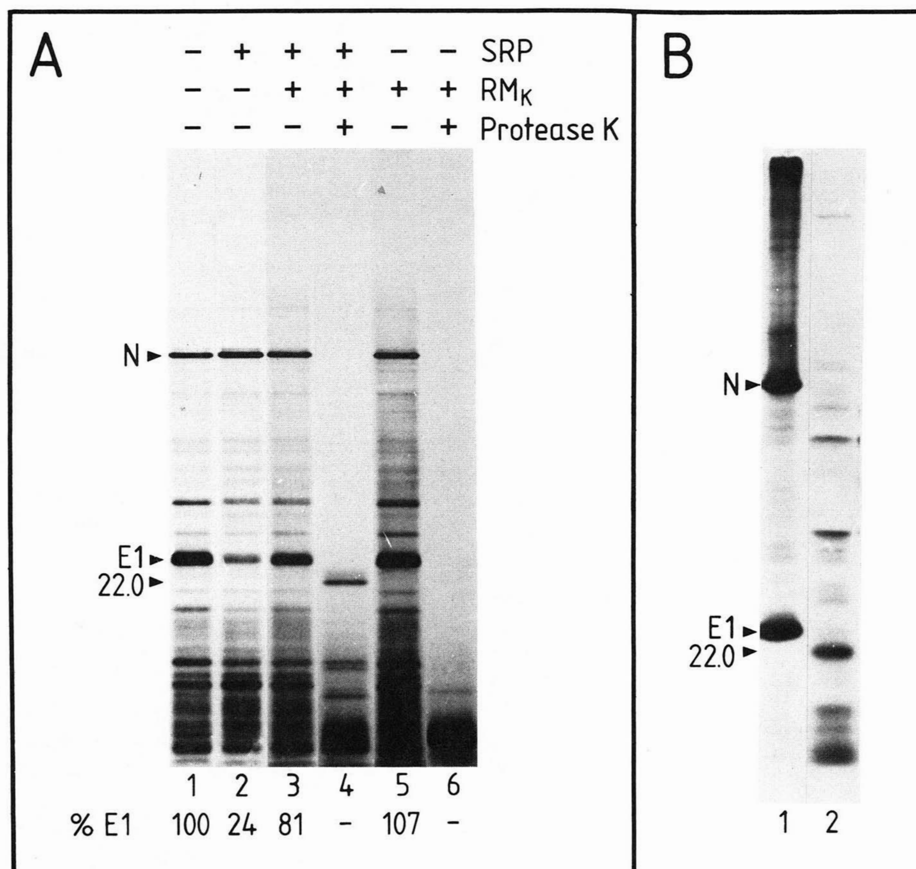

FIG. 1. **SRP-dependent insertion of E1 into dog pancreas rough microsomes.** *A*, *in vitro* translations of MHV mRNA. Poly(A)$^+$ RNA was translated in a wheat germ cell-free system at 25 °C for 1 h. Shown above are fluorograms of the translation products separated on 10–15% SDS-polyacrylamide gels. *Lane 1*, control, without SRP or RM_K; *lane 2*, translation in the presence of 40 units/ml SRP; *lane 3*, as in *lane 2* with the inclusion of RM_K to 40 A_{280} units/ml; *lane 4*, products of the translation shown in *lane 3* treated with proteinase K as described under "Experimental Procedures"; *lane 5*, translation in the presence of RM_K without SRP; *lane 6*, translation as in *lane 5* treated post-translationally with proteinase K. *B*, proteolytic digestion of E1 protein labeled *in vivo*. Membranes were prepared from pulse-labeled Sac$^-$ cells and proteolyzed as described under "Experimental Procedures." *Lane 1*, immunoprecipitation of MHV proteins using a mouse anti-MHV serum; *lane 2*, proteins derived from proteinase K-treated membranes of Sac$^-$ cells. *N* indicates the nucleocapsid protein of MHV and *22.0* the major fragment of E1 protected by the membrane from exogenous proteolysis (M_r = 22,000).

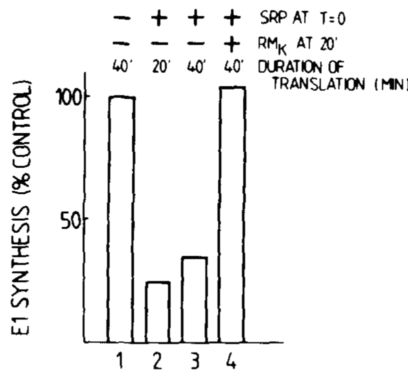

FIG. 2. **Stability and reversibility of the SRP-mediated arrest of a synchronized translation.** E1 mRNA was translated in wheat germ lysate. Translation was carried out at 27 °C. *Lane 1*, amount of E1 synthesized at 40 min; *lane 2*, amount of E1 synthesized by 20 min in the presence of 40 units/ml SRP; *lane 3*, amount of E1 synthesized at 40 min in the presence of 40 units/ml SRP; *lane 4*, amount of E1 synthesized in 40 min when RM_K were added to a final concentration of 40 A_{280} units/ml at 20 min to a duplicate of the sample shown in *lane 2*. Translation products were separated on 10–15% SDS-polyacrylamide gels and detected by fluorography. Amounts of E1 were determined by densitometric analysis of fluorograms and normalized to the control translation performed in the absence of SRP (*lane 1*).

Fig. 1*A* (*lane 1*) and are composed primarily of E1 and nucleocapsid protein (*N*). Addition of SRP caused a sharp reduction in the amount of E1 synthesized (*lane 2*). Based on densitometic quantitation normalized to the amount of *N* (not affected by SRP), the yield of E1 was reduced to 24% of the control (*lane 1*) by the addition of SRP at 40 units/ml. This inhibition was relieved by the inclusion of salt-extracted microsomal membranes (RM_K) (*lane 3*) to a level corresponding to 81% of control. The products of translation with SRP and RM_K were treated with proteinase K, which caused the disappearance of nucleocapsid protein, and a reduction in molecular mass of E1 (Fig. 1*A*, *lane 4*) to a 22,000-dalton form indistinguishable from that found by protease treatment of microsomes from virally infected cells (Fig. 1*B*). RM_K in the absence of SRP caused neither a block of E1 synthesis (Fig. 1*A*, *lane 5*) nor a conversion to protease resistance (Fig. 1*A*, *lane 6*). Thus, SRP causes a specific block of E1 synthesis and is required for correct assembly of the protein into the microsomal membrane.

Stability of the SRP-dependent Translation Block—A recent study indicated that the inability of SRP to induce a translation arrest in the case of Ca^{2+}-ATPase might be due to the lack of a cleavable signal sequence. In such cases, SRP would affect the rate of synthesis instead of producing a stable block (8). To characterize further the specific arrest of E1 translation by SRP, the stability of the translation block was measured. E1 RNA was translated *in vitro* in a synchronized system (as described under "Experimental Procedures") to prevent reinitiation of synthesis. In this study, we initially allowed translation to proceed in the presence of SRP for 20 min. At this point, only about one quarter of the total possible E1 which can be synthesized in the absence of SRP has been completed (compare Fig. 2, *lane 2* to *lane 1*). When this translation was allowed to continue for a total of 40 min, only a small additional quantity of E1 appeared (*lane 3*). This inhibition was completely rescued by the addition of RM_K after 20 min (*lane 4*). Thus, rather than causing a general inhibition of E1 synthesis, SRP brings about a stable, and completely reversible, arrest of translation.

Time of Addition of SRP—An examination of the primary structure of E1 (16) indicates that its N terminus does not possess the hydrophobic properties characteristic of signal sequences (17). Instead, three hydrophobic stretches can be found in the region between amino acids located 27–46, 57–77, and 82–103 from the N terminus. The use of timed SRP addition should enable one to determine if the hydrophobic sequences distal to the N terminus can also function as insertion signals. In principle, the further away from the N terminus the signal resides, the longer the period during which SRP should be able to be added to the translation and still bring about an arrest.

Such an experiment is detailed in Fig. 3. Completed E1 (228 amino acids) first appears between 18 and 22 min of translation (Fig. 3, *A* and *B*). SRP was added to parallel synchronized translations at various times, and these were allowed to continue for a total of 30 min. It can be seen in Fig. 3, *C* and *D*, that addition of SRP as late as 17 min after the start of translation still results in a half-maximal inhibition of E1 synthesis (*arrow* T_1).

Since the rate of elongation appears to be linear (see Fig. 3*A*), one can calculate that at 16–18 min approximately two thirds of the molecule, or roughly 150 amino acids of the total 228, have been polymerized. The effectiveness of such a late addition of SRP suggests that even the most C-terminal stretch of hydrophobic amino acids (82–103) could serve as a signal sequence.

It is clear from Fig. 3*C* that bands were not observed that could represent a blocked peptide. This was corroborated by identical studies carried out using gel systems capable of even better resolution of such smaller peptides (data not shown).

DISCUSSION

We have characterized the involvement of SRP in the membrane insertion of E1, a model intracellular membrane protein. In the case of most plasma membrane and secretory proteins, SRP functions by bringing about an arrest of translation which can be relieved by the presence of rough microsomes (3). Our results indicate that insertion of E1 is carried out in the same manner. We have shown that SRP causes a specific (Fig. 1*A*) and quite stable (Fig. 2) arrest of synthesis of E1 *in vitro*. Addition of salt-washed microsomal membranes causes a release of the translation block (Fig. 1*A*, *lane 3*) which is apparently quantitative (Fig. 2). The resulting protein is assembled into the membrane, in a form which is indistinguishable, as judged by protease resistance, from the protein produced *in vivo* (Fig. 1*B*, *lane 2*; Fig. 1*A*, *lane 4*).

As we used mRNA which encoded virtually only E1, it was thought that the signal could be defined by identifying the arrested peptide produced by the addition of SRP. We have consistently failed, however, to observe such a species. In these translations, arrested peptides of a defined length, if present, would have been visible. A very recent report has shown a similar insertion scheme for cytochrome P-450 (10). In this case, no blocked peptide could be found by immunoprecipitation of the cytochrome synthesized *in vitro*. This finding is strengthened by our results in which immunoprecipitation is eliminated through the use of a specific E1 mRNA. One must conclude, therefore, that the translation arrest, although stable, is relatively imprecise, such that specific bands cannot be seen on a gel. Last, the results of this study as well as those of others (10) demonstrate that no correlation exists between the lack of a cleavable signal sequence and the failure to observe translation arrest by SRP *in vitro* (9).

Although we prove that E1 is treated like most membrane and secretory proteins, studies using timed SRP addition to

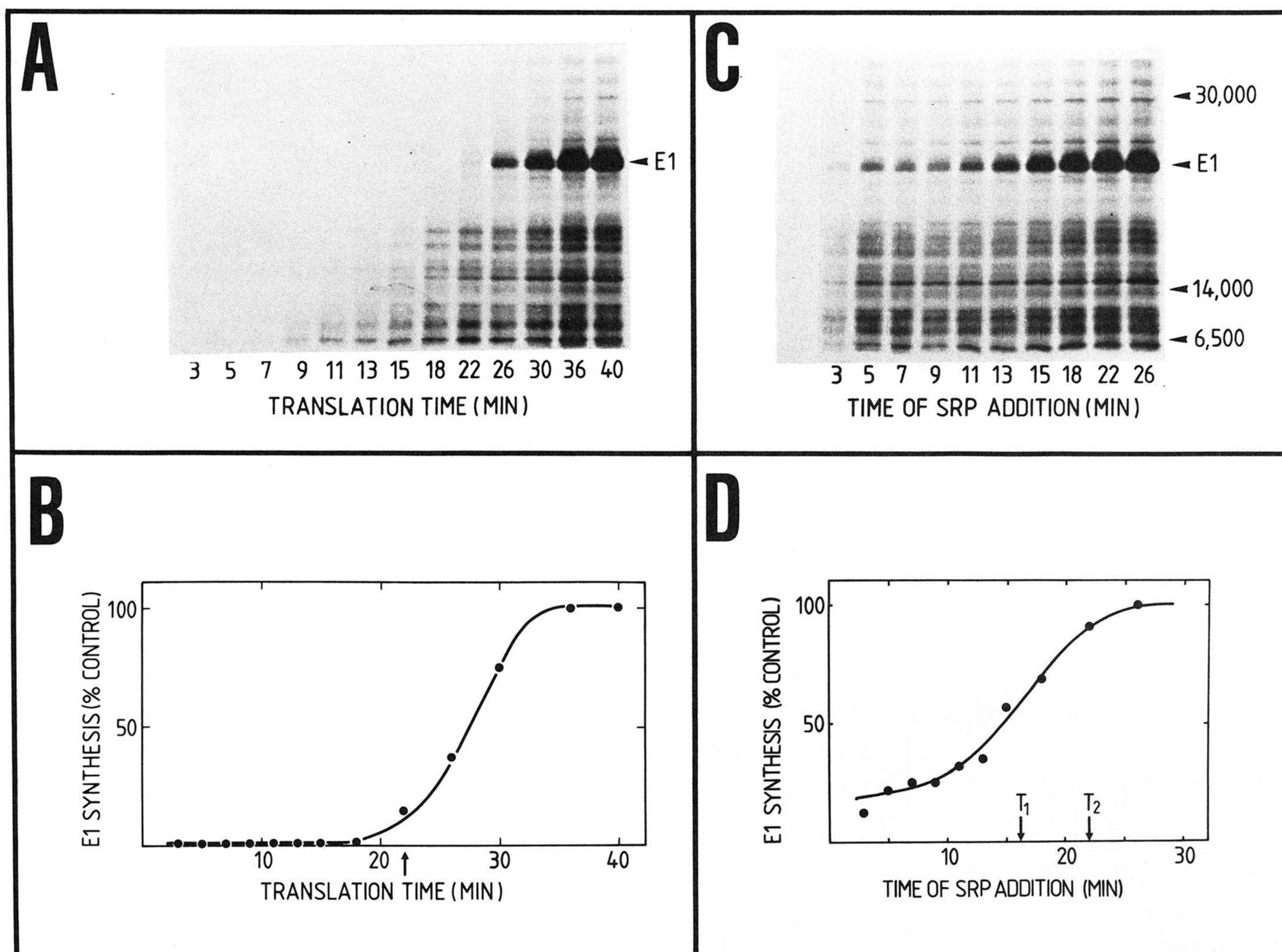

FIG. 3. **Time dependence of SRP-induced arrests in synchronized translations.** E1 mRNA (RNA6) was translated in a wheat germ cell-free system at 25 °C for varying periods of time. Translation products were separated on 10–15% gradient gels and quantitated by densitometric analysis of fluorograms. *A* and *B*, fluorogram and quantitation of E1 mRNA translated in the absence of SRP for the times indicated. The *arrow* indicates the time at which completed E1 first appeared on fluorograms. *C* and *D*, fluorogram and quantitation of the appearance of E1 in translations to which SRP was added at various time points. In control translations, buffered salt solutions not containing SRP were added. In this way, it was demonstrated that the 1 M KAc present in SRP preparations did not influence the total E1 synthesized (data not shown). Indicated are the amounts of E1 detected at 40 min of translation when SRP was added at a given time point during translation. The *arrow* at T_1 corresponds to the time point at which added SRP still exerts a half-maximal blocking effect. T_2 corresponds to the time at which completed E1 first appears and reflects the data presented in *A* and *B*.

synchronous translations suggest possibilities that warrant further attention. Apparently SRP can exert its effect on E1 synthesis even when it is added quite late in translation (Fig. 3, *C* and *D*); approximately 150 amino acids of a total of 228 can be polymerized before translations can no longer be arrested. Based on known measurements of arrested N-terminal peptides (3, 4), one assumes that a block occurs when the signal sequence emerges from the large ribosomal subunit. This would entail having about 40 amino acids in addition to the signal sequence in order to span the ribosome. Thus, in the case of E1, a block could occur earliest at a chain length of 85–90 amino acids (approximately 10,000 daltons) if the signal sequence was represented by the first hydrophobic domain (amino acids 27–46). Similarly, the size of the arrested nascent chain would correspond to 115–120 amino acids (12,500 daltons) and 140–150 amino acids (16,000 daltons) in the case of the second and third domains, respectively. On the basis of our finding that SRP still brings about an arrest when two thirds of the protein (approximately 17,000 daltons) is completed, we interpret these results to mean that even the most C-terminal hydrophobic domain can interact with SRP resulting in this arrest. We cannot completely rule out the possibility that the most N-terminal hydrophobic domain is the sole signal and is still being recognized by SRP at a point as late in translation when two thirds of E1 is completed. In light of the fact that the signal sequence of most secretory proteins is inaccessible to cleavage by signal peptidase late in their translation (23), it seems unlikely that such signals would still be accessible to the much larger SRP.

These findings substantiate the notion that a considerable fraction of a protein can be synthesized and still become inserted into the membrane. The insertion of E1 into microsomes late in its synthesis has been documented previously using similar synchronized translation in the reticulocyte

lysate system (15). The kinetics of insertion in this system paralleled those of the SRP arrests seen here, implying that the same signals are functioning in both cases.

Of the three possible signals of E1, all are internal, *i.e.* not N- or C-terminal. Several proteins having uncleaved N-terminal signals have been shown to become inserted into the membrane in an SRP-dependent fashion (8–10). E1 represents the first case of a protein with an implicit internal signal which has been shown to require SRP. This result points out that SRP is also capable of recognizing this type of sequence and that such sequences can be functional in more C-terminal locations.

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