

## Signal Sequence Cleavage of Peptidyl-tRNA Prior to Release from the Ribosome and Translocon\*

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**Many secretory polypeptides undergo cleavage of their signal sequence. In this study, we observed and quantitated the presence of a tRNA-bound, ribosome-associated polypeptide subpopulation present *in vitro*. This subpopulation was accessible to signal peptidase on ribosome-associated polypeptides longer than 114 amino acids. This demonstrates that it is possible for a peptidyl-tRNA species, in the midst of translation, to be processed by the endoplasmic reticulum signal peptidase implying that the peptidase is closely associated with the mammalian translocon.**

“Signal sequence” refers to a stretch of amino acids necessary and sufficient to target a polypeptide to a cellular location (1, 2) such as the peroxisomal (3, 4), chloroplast (5, 6), or mitochondrial (7, 8) membrane or lumen, eukaryotic or prokaryotic plasma membranes (9), the Gram-negative bacterial periplasm (10), or extracellular milieu (11–13). Many secretory proteins possess an amino-terminal signal sequence (13). This sequence is a substrate for a family of conserved proteases called type I signal peptidases (SPase I)<sup>1</sup> (2), which cleave the signal sequence before the protein is secreted from the cell (14). Functional assays show that bacterial proteins can be cleaved by eukaryotic SPase I (15) and eukaryotic proteins can be cleaved by bacterial SPase I (16).

Nascent polypeptides translocate across the membrane through a proteinaceous channel to reach the lumen of the ER (17, 18) or the periplasm/extracellular environment (19, 20). Nascent proteins can be modified within the ER by removal of the signal sequence, addition of *N*-linked sugars (21), or formation of disulfide bonds (22). However, the temporal order of these events relative to each other and relative to translocation through the channel has not been resolved. In this paper, we observe that polypeptide intermediates still attached to their biosynthetic ribosomes can be cleaved by the ER signal peptidase.

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<sup>1</sup> The abbreviations used are: SPase I, type I signal peptidase; ER, endoplasmic reticulum; RM, rough microsomes; Puro, puromycin.

dase. We conclude that the peptidase can access a nascent chain that has not completed translocation or translation.

### EXPERIMENTAL PROCEDURES

**Materials**—Restriction enzymes were from New England Biolabs. PerkinElmer Life Sciences supplied [<sup>35</sup>S]methionine. Except when indicated, all other chemicals were supplied by either Fisher or Sigma.

**Plasmids**—pGEM-BP1, containing both the *Staphylococcus aureus*  $\beta$ -lactamase gene and *Bos taurus* preprolactin cDNA, was provided by Dr. C. Nicchitta (23). The secretory protein *Rattus norvegicus* immunoglobulin  $\kappa$  light chain VJC cDNA was provided by Dr. M. Nussenzweig and subcloned into a pGEM-3Z plasmid (Promega) using EcoRI and AcclI to create plasmid pGEM- $\kappa$ LC.

**PCR, Transcription, and Translation**—DNA was amplified by PCR using Pfu DNA polymerase (Stratagene); PCR products were purified (Qiagen). Transcription was carried out using instructions and reagents provided in the Megascript T7 kit from Ambion. *In vitro* [<sup>35</sup>S]methionine translations were performed using a rabbit reticulocyte lysate system (Promega) treated with 2 mM puromycin, purified, separated with Tris-glycine SDS-PAGE, and visualized using STORM autoradiography (Amersham Biosciences) in a manner comparable with the manufacturer's instructions and previous descriptions (24, 25).

**Protein Quantification**—Using ImageQuant 5.2 (Amersham Biosciences), we drew rectangles around the four potential bands in each lane of an SDS gel. Background subtraction and normalization were done as described previously (26). Percent tRNA-bound polypeptide = the intensity of the top two bands of any given lane divided by the total intensity of all four rectangles in that same lane; percent total tRNA-bound polypeptide without signal sequence = the intensity of the lower of the top two rectangles divided by the total intensity of all four rectangles in a lane; percent tRNA-bound polypeptide without (or with) signal sequence released by puromycin addition = the percent total tRNA-bound polypeptide with or without signal sequence was determined with (A) and without (B) puromycin. The ratio (E) of the percentage released by puromycin to the percentage present in the absence of puromycin was calculated:  $(B - A)/B = E$ .

**Examples of Construct Nomenclature**— $\beta$ lac FL-1 Trp =  $\beta$ -lactamase full-length minus stop codon(s), tryptophan as final amino acid; pPI 0.75 Pro = preprolactin, 75% of full-length, proline as final amino acid;  $\kappa$ LC FL =  $\kappa$  light chain full-length with stop codon(s).

### RESULTS

**Background and Characterization of the Experimental System**—During translation of mRNA truncated without stop codon(s), the ribosome stalls at the terminal codon and the cognate tRNA molecule remains bound to the polypeptide chain and to the ribosome (23, 27). This resulting peptidyl-tRNA migrates more slowly on SDS-PAGE and can be quantified when labeled with [<sup>35</sup>S]methionine. Concomitantly, the peptidyltransferase of the ribosome may add puromycin to the carboxyl end of the stalled chain thereby releasing the tRNA (28, 29). Puromycin increases the electrophoretic mobility of ribosome-bound, nascent peptidyl-tRNA but has no effect on peptidyl-tRNA that have dissociated from their ribosomes (24).

**Several Higher Molecular Mass Bands Are Present in  $\beta$ lac FL-1 Trp Translation Product**—The transcription/translation product for  $\beta$ -lactamase without stop codons,  $\beta$ lac FL-1 Trp, was predicted to encode a 31.6-kDa preprotein of 286 amino acids and a 28.9-kDa mature form of 263 amino acids. For both  $\beta$ lac FL-1 Trp and  $\beta$ lac FL, translation products at 31 kDa (major) and 25 kDa (minor) were observed in the absence of RM (RM<sup>-</sup>) (Fig 1A). In the presence of RM (RM<sup>+</sup>), an additional, major band at 28 kDa was visible (Fig. 1A, lanes 2 and 4). The 31-kDa and the 28-kDa bands have been characterized as the full-length polypeptide with and without signal sequence, respectively. The 25-kDa product has not been characterized but

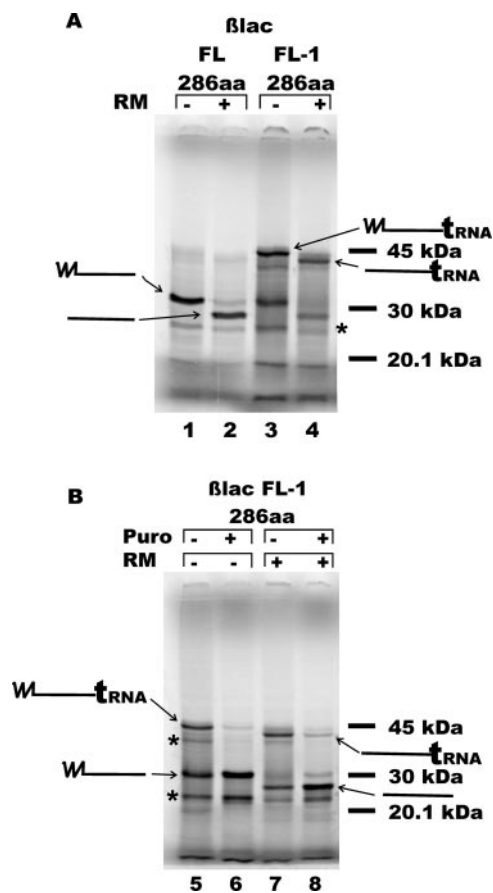


FIG. 1. *In vitro* translation of  $\beta$ lac FL and  $\beta$ lac FL-1. mRNA from these constructs was translated and characterized by SDS-PAGE as described under “Experimental Procedures” in the presence or absence of ER-derived canine pancreatic rough microsomes (RM). A,  $\beta$ lac FL; B,  $\beta$ lac FL-1. In both A and B, asterisk = unidentified translation products; tRNA = aminoacyl-tRNA; long horizontal line = mature polypeptide; capital W with long horizontal line = unprocessed polypeptide; capital W = signal sequence. aa, amino acids

appears elsewhere (30). The intensity of the 28-kDa band as a percentage of total (28 kDa + 31 kDa) band intensity was  $76 \pm 24\%$  ( $n = 13$ ) for  $\beta$ lac FL and  $72 \pm 14\%$  ( $n = 4$ ) for  $\beta$ lac FL-1 Trp.

**Higher Molecular Mass  $\beta$ lac FL-1 Trp Bands Are Released by Puromycin**—Translation products of  $\beta$ lac FL-1 Trp showed several additional major translation products of higher molecular mass (Fig. 1A). In the absence of RM, the 44-kDa band was more intense than the 40-kDa band (paired, two-tailed Student’s *t* test,  $p < 0.01$ ). An additional band of 42 kDa was seen in the presence of RM (Fig. 1A, lane 4). To test whether these higher molecular mass bands were nascent polypeptide chains with carboxyl-terminal amino acids still attached to their cognate tRNA, we added puromycin to translation mixtures (Puro +) (24).

When a RM – translation mix encoding  $\beta$ lac FL-1 Trp was treated with 2 mM puromycin, both the 44- and 40-kDa bands decreased in intensity, while the 31- and 25-kDa bands gained intensity (Fig. 1B, lanes 5 and 6). In RM +/Puro + mixtures, the major band of 42 kDa lost intensity, while the 28-kDa band gained intensity (Fig. 1B, lanes 7 and 8). These data suggest that higher molecular mass bands represent tRNA-bound forms of nascent polypeptide with (44 kDa) and without (42 kDa) a signal sequence. For all experiments with  $\beta$ lac FL-1 ( $n = 4$ ), the percentage of total tRNA-bound protein without a signal sequence (the 42-kDa band) was  $\sim 0 \pm 0\%$  in samples untreated with RM as opposed to  $75 \pm 6\%$  in samples treated

with RM (paired, two tailed Student’s *t* test,  $p < 0.01$ ) (Table I). The percentage intensity of tRNA-bound, processed polypeptide of total tRNA intensity was decreased an average of  $91 \pm 3\%$  by addition of puromycin (Table I).

**Truncated Eukaryotic Constructs Show Results Similar to  $\beta$ -Lactamase**—To observe whether the signal sequence can be cleaved from polypeptide intermediates of eukaryotic origin, we synthesized several bovine preprolactin and rat  $\kappa$  light chain constructs. Construct pPl 0.75 Pro was predicted to encode a 19.1-kDa preprotein of 172 amino acids and a 15.9-kDa mature protein of 142 amino acids. When pPl 0.75 Pro was translated in the absence of RM, two major bands of  $\sim 35$  and 20 kDa were observed (Fig. 2A, lanes 1 and 2). In the presence of RM, two additional bands appeared at 32 and 16 kDa (Fig. 2A, lanes 3 and 4). In the presence of RM,  $27 \pm 6\%$  of the pPl 0.75 Pro ( $n = 4$ ) tRNA-bound polypeptide species were without a signal sequence, while in the absence of RM  $2 \pm 3\%$  of the peptidyl-tRNA lacked a signal sequence (paired, two-tailed Student’s *t* test,  $p < 0.01$ ) (Table I). Puromycin released an average of  $95 \pm 5\%$  of tRNA-bound, processed polypeptide chains. We observed lower but similarly patterned results for construct pPl 0.57 Pro (Table I).

The longer IgG  $\kappa$  light chain construct,  $\kappa$ LC 0.89 Ser, coded for a 22.5-kDa preprotein of 206 amino acids and a 19.4-kDa mature protein of 177 amino acids. For translation products of both  $\kappa$ LC constructs, the results were similar to  $\beta$ -lactamase and preprolactin truncated constructs (Fig. 2B and Table I).

**Signal Sequence Cleavage of  $\beta$ -Lactamase tRNA-bound Constructs Is Length-dependent**—To test whether length of the polypeptide intermediate affected processing of the signal sequence, we created truncated mRNA constructs encoding various lengths of  $\beta$ -lactamase. When primed with mRNA coding for a polypeptide greater than 114 amino acids, translations in the presence of RM contained a significantly greater amount of processed peptidyl-tRNA than translations in the absence of RM. Translation mixtures primed with truncated  $\beta$ -lactamase constructs coding for less than 132 amino acids did not show a similar, significant difference (Fig. 3 and Table I, paired, two-tailed Student’s *t* test).

When mRNA constructs encoding more than 114 amino acids of  $\beta$ -lactamase were translated in the presence of RM, the tRNA-bound translation products were without a signal sequence. These translation products, upon puromycin addition, were reduced in excess of 80–90% of their original intensity. Similarly, all constructs (except  $\beta$ lac 0.27) coding for less than 132 amino acids showed release of tRNA-bound, signal sequence present species in excess of 60% upon puromycin addition (Table I).

## DISCUSSION

Can ER signal peptidase cleave the signal sequence from polypeptide chains still associated with functional ribosomes? To address this question we built on previous experimental paradigms (24, 26, 31, 32) to resolve and quantitate the existence of tRNA-bound, ribosome-associated polypeptide subpopulations present *in vitro*.

In this study, typically 25% or greater of the polypeptide was tRNA-bound, and of that generally more than 60% was associated with functional ribosomes (Table I). Our results show that the percentage of tRNA-bound, ribosome-associated polypeptide is a variable quantity, specific to, but not uniformly predicted by, the identity of the parental mRNA. The results indicate that in many *in vitro* translations, and potentially in many *in vivo* reactions, as much as 85% of the nascent polypeptide is either not tRNA-bound or ribosome-associated. This tRNA-bound, ribosome-associated subpopulation is the only

TABLE I  
Quantitative data from *in vitro* translations of various mRNA constructs

Each mRNA construct listed was translated using a rabbit reticulocyte lysate in the presence or absence of ER-derived canine pancreatic RM. The translation mixtures were split in half; one half incubated with 2 mM puromycin, the other half incubated with buffer. Samples were precipitated with 2.3 mM  $(\text{NH}_4)_2\text{SO}_4$ , separated by SDS-PAGE, and quantitated using autoradiography. Data are listed as follows. Percentage of total polypeptide that is tRNA-bound in both RM + and RM - samples represents average  $\pm$  S.D. from *n* independent experiments; the percentage of total, tRNA-bound polypeptide without a signal sequence compared between RM + and RM - samples represents average  $\pm$  S.D. from *n* independent experiments - statistical significance: *p* values from paired, two-tailed, Student's *t* test; ratio of the difference between the percentages of total, tRNA-bound polypeptide without a signal sequence in RM + samples incubated with and without puromycin to the percentage of total, tRNA-bound polypeptide without a signal sequence in RM + samples incubated without puromycin represents average  $\pm$  S.D. from *n* independent experiments.

<i>n</i>	Construct	Length (amino acid)	% total polypeptide as tRNA bound form ( $\pm$ S.D.)		% total tRNA-bound polypeptide without signal sequence ( $\pm$ S.D.)			% tRNA bound polypeptide ( $\pm$ S.D.)
			RM -	RM +	RM -	RM +	<i>p</i>	
4	$\beta$ lac FL-1 Trp	286	27 $\pm$ 3	58 $\pm$ 6	0 $\pm$ 0	75 $\pm$ 6	<0.01	91 $\pm$ 3 <sup>a</sup>
5	$\beta$ lac 0.87 Pro	249	60 $\pm$ 8	62 $\pm$ 10	3 $\pm$ 2	34 $\pm$ 6	<0.01	93 $\pm$ 9 <sup>a</sup>
4	$\beta$ lac 0.75 Pro	217	44 $\pm$ 4	23 $\pm$ 2	3 $\pm$ 4	82 $\pm$ 4	<0.01	95 $\pm$ 2 <sup>a</sup>
4	$\beta$ lac 0.63 Asp	177	46 $\pm$ 4	20 $\pm$ 2	2 $\pm$ 3	70 $\pm$ 1	<0.01	93 $\pm$ 1 <sup>a</sup>
4	$\beta$ lac 0.50 Pro	143	47 $\pm$ 9	43 $\pm$ 22	2 $\pm$ 2	57 $\pm$ 9	<0.01	95 $\pm$ 4 <sup>a</sup>
4	$\beta$ lac 0.46 Ala	132	25 $\pm$ 3	18 $\pm$ 7	2 $\pm$ 3	58 $\pm$ 12	<0.01	82 $\pm$ 5 <sup>a</sup>
3	$\beta$ lac 0.39 Gly	114	19 $\pm$ 10	12 $\pm$ 7	3 $\pm$ 5	0 $\pm$ 1	0.4	84 $\pm$ 15 <sup>b</sup>
3	$\beta$ lac 0.38 Glu	108	37 $\pm$ 15	21 $\pm$ 12	2 $\pm$ 3	4 $\pm$ 4	0.7	88 $\pm$ 18 <sup>b</sup>
4	$\beta$ lac 0.37 Pro	105	26 $\pm$ 15	17 $\pm$ 11	3 $\pm$ 6	0.1 $\pm$ 0.2	0.4	62 $\pm$ 27 <sup>b</sup>
3	$\beta$ lac 0.27 Asp	83	23 $\pm$ 6	49 $\pm$ 6	2 $\pm$ 2	1 $\pm$ 1	0.5	28 $\pm$ 16 <sup>b</sup>
4	$\kappa$ LC 0.89 Ser	206	36 $\pm$ 5	60 $\pm$ 5	3 $\pm$ 4	61 $\pm$ 9	<0.01	77 $\pm$ 2 <sup>a</sup>
3	$\kappa$ LC 0.75 Val	181	84 $\pm$ 2	79 $\pm$ 6	1 $\pm$ 2	60 $\pm$ 2	<0.01	28 $\pm$ 6 <sup>a</sup>
4	pPI 0.75 Pro	184	71 $\pm$ 18	76 $\pm$ 11	2 $\pm$ 2	25 $\pm$ 4	<0.01	95 $\pm$ 5 <sup>a</sup>
3	pPI 0.57 Pro	139	54 $\pm$ 11	64 $\pm$ 3	1 $\pm$ 1	22 $\pm$ 3	<0.01	73 $\pm$ 8 <sup>a</sup>

<sup>a</sup> Without signal sequence, released by puromycin addition.

<sup>b</sup> With signal sequence, released by puromycin addition.

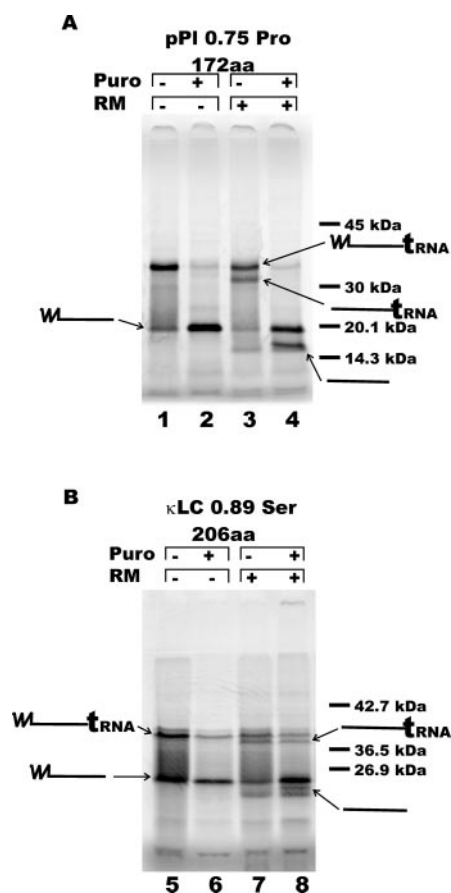


FIG. 2. *In vitro* translation of pPI 0.75 Pro and  $\kappa$ LC 0.89 Ser. mRNA from these constructs was translated and characterized by SDS-PAGE as described under "Experimental Procedures" in the presence or absence of ER-derived canine pancreatic RM. A, pPI 0.75 Pro; B,  $\kappa$ LC 0.89 Ser. In both A and B, tRNA = aminoacyl-tRNA; capital W with long horizontal line = unprocessed polypeptide; lone horizontal line = mature polypeptide; capital W = signal sequence. aa, amino acids.

biologically relevant intermediary in protein translation, a factor that must be taken into consideration in all studies.

To assess the activity of ER signal peptidase, we used this system to observe the differences between polypeptide species in translation mixtures containing or lacking RM. Without RM in the translation mixture, the tRNA-bound population was generally homogenous (e.g. Fig. 1B, lane 5). When the same translation was done in the presence of RM, the tRNA-bound population became heterogeneous, with one band similar to the RM - lane representing unprocessed, tRNA-bound polypeptide and one band of generally 1–3 kDa lower apparent molecular mass representing the tRNA-bound polypeptide without its signal sequence (e.g. Fig. 1B, lanes 5 and 7). Therefore, in the RM + translation mixtures, some tRNA-bound polypeptides were processed and some were unprocessed, and a reproducible percentage of these processed constructs were susceptible to puromycin release (Table I). These results demonstrate that tRNA-bound, ribosome-associated polypeptides can be accessed by ER signal peptidase *in vitro*.

Once it was established that polypeptides still attached to the ribosome could be processed by the ER signal peptidase, we examined how early during the biosynthesis of a polypeptide a signal sequence could be cleaved. We synthesized increasingly shorter  $\beta$ -lactamase mRNA constructs and translated them. Ribosome-associated polypeptides 114 amino acids and shorter could no longer be resolved from their signal sequence cleaved form (Fig. 3, Table I). However, by our puromycin release assay, tRNA-bound polypeptide chains from these shorter constructs were still attached to functional ribosomes (Table I).

Of the 114 amino acids in the longest unprocessed  $\beta$ -lactamase construct, 70 are buried in the translation and translocation "machinery" of the cell (24, 33–35) and ~25 are the signal sequence itself. This allows ~40 amino acids to be exposed to the lumen of the ER. We hypothesize that, in this study, these 20–50 amino acids span the distance from the active site of the signal peptidase to the point where the translocon protects the nascent chain from protease. This would necessitate the subcellular location of the ER signal peptidase

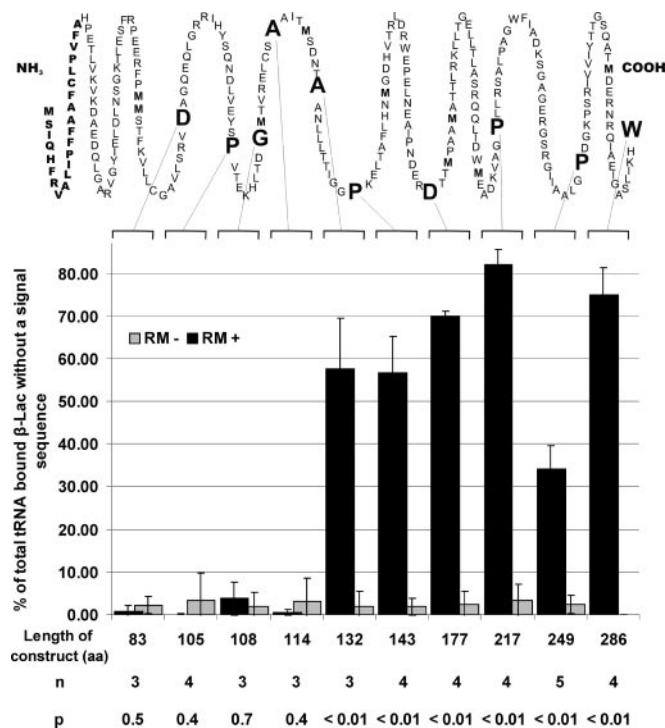


FIG. 3. Signal sequence cleavage of tRNA-bound  $\beta$ -lactamase constructs. *Top*, the letters at the top of the figure represent the amino acid sequence of pre- $\beta$ -lactamase. The amino terminus is labeled "NH<sub>2</sub>" and the carboxyl terminus is labeled "COOH." The  $\beta$ -lactamase signal sequence contains 23 amino acids in boldface type. Boldface letters also designate potentially radiolabeled methionines. Amino acids corresponding to ultimate codons of truncated constructs are printed in large font and connected with the appropriate column of the histogram below. *Bottom*, mRNA encoding  $\beta$ -lactamase truncated at various points was translated. The fraction of ribosome-conjugated nascent peptides whose signal sequences were cleaved was quantified and plotted as a function of predicted length of the polypeptide. Samples represent average  $\pm$  S.D. from  $n$  independent experiments. Puromycin was used as a control to make sure that the tRNA-bound polypeptides were associated with functional ribosomes. Statistical significance:  $p$  values from paired, two-tailed, Student's  $t$  test. *aa*, amino acids.

to be in close proximity to the mammalian translocon. Based on these ideas, we propose that signal sequence cleavage is an event tightly coupled, both temporally and spatially, to the movement of the polypeptide through the translocon.

$\beta$ -Lactamase is a single protein, and this study examines three canonical polypeptides from both prokaryotes and eukaryotes within a mammalian translation/translocation model. Obvious extensions include using alternative models for ER translocation such as permeabilized cell extracts (36) or prepared bacterial membranes (37) along with a wider range of constructs, including viral and yeast, to explore these results further.

We have previously demonstrated that nascent polypeptides, still attached to their biosynthetic ribosomes in the cytosol, can be modified by the oligosaccharyltransferase in the lumen of the endoplasmic reticulum (24). The analysis of a nascent polypeptide containing both a cleaved signal sequence and glycosylation site would allow us to assay the relative timing of these events and further our *in vitro* biochemical understanding of the functional organization of the translocon complex.

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