

# Translocation in Yeast and Mammalian Cells: Not All Signal Sequences are Functionally Equivalent

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**Abstract.** In *Saccharomyces cerevisiae*, nascent carboxypeptidase Y (CPY) is directed into the endoplasmic reticulum by an NH<sub>2</sub>-terminal signal peptide that is removed before the glycosylated protein is transported to the vacuole. In this paper, we show that this signal peptide does not function in mammalian cells: CPY expressed in COS-1 cells is not glycosylated, does not associate with membranes, and retains its sig-

nal peptide. In a mammalian cell-free protein-synthesizing system, CPY is not translocated into microsomes. However, if the CPY signal is either mutated to increase its hydrophobicity or replaced with that of influenza virus hemagglutinin, the resulting precursors are efficiently translocated both *in vivo* and *in vitro*. The implications of these results for models of signal sequence function are discussed.

THE signal hypothesis (Blobel and Dobberstein, 1975) provides a satisfying explanation of the targeting and translocation of newly synthesized proteins across the membrane of the endoplasmic reticulum (ER)<sup>1</sup> of eukaryotic cells. Essential to the hypothesis are the interactions between a hydrophobic segment of the secretory protein (the signal or leader) and the translocation machinery of the cell. In some circumstances, these interactions begin as the hydrophobic signal of a nascent polypeptide emerges from the ribosome and binds to signal recognition particle (SRP; Walter et al., 1984). Synthesis of the remainder of the protein may then be halted or slowed until the bound SRP attaches to its receptor on the membrane of the ER, establishing a functional ribosome-membrane junction through which the nascent polypeptide is translocated (Walter and Blobel, 1981; Walter et al., 1984; Meyer, 1985). In other cases, interaction between the signal sequence and the translocation machinery may not occur until synthesis of the polypeptide is completed, or nearly so (Ainger and Meyer, 1986; Perara et al., 1986; Mueckler and Lodish, 1986). Although the factors that determine whether translocation is co- or posttranslational are not well understood, it is clear that neither process occurs in the absence of a functional signal: removal or mutation of the sequence coding for the signal results in proteins that cannot be translocated (Gething and Sambrook, 1982; Carlson and Botstein, 1982; Chao et al., 1987). Conversely, addition of a signal peptide causes proteins that normally would be sequestered on the cytoplasmic side of the ER to be trans-

located both *in vivo* and in cell-free systems (Sharma et al., 1985; Lingappa et al., 1984).

Signal peptides have been demonstrated on secretory and membrane proteins of all organisms studied to date (for review, see Rapoport, 1986). Although there is no obvious conservation of primary amino acid sequence or length, even between signals on proteins from the same organism, statistical analyses have suggested that signals from both prokaryotes and eukaryotes are organized along similar lines (von Heijne, 1981; Perlman and Halvorson, 1983). A typical signal sequence appears to consist of three regions: a positively charged amino terminal (n) region, a central hydrophobic (h) region, and a more polar carboxy terminal (c) region, which defines the cleavage site (von Heijne, 1985). Of particular interest is the h region, since a number of studies have suggested that the overall hydrophobicity of a signal sequence is important to its function (Rapoport, 1986). In most cases, a mutation which abolishes the function of a signal peptide replaces a hydrophobic residue in the h region with a charged residue (Rapoport, 1986).

Because of the structural similarities between signal sequences from organisms widely separated on the evolutionary scale, it was perhaps not surprising to find that signals from one organism can function in another. For example, the bacterial  $\beta$ -lactamase signal sequence is functional in vertebrate systems *in vivo* and *in vitro* (Mueller et al., 1982; Wiedmann et al., 1984); the rat preproinsulin signal works in bacteria (Talmadge et al., 1980); and the signals of human interferon (Hitzeman et al., 1983) and influenza virus hemagglutinin (Jabbar et al., 1985) function in yeast. In addition, yeast invertase is translocated in mammalian systems both *in vivo* (Bergh et al., 1987), and *in vitro* (Perlman and Halvorson, 1981), and the precursors to several other yeast proteins such as  $\alpha$ -factor (Julius et al., 1984) and killer toxin (Bostian

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1. *Abbreviations used in this paper:* CPY, carboxypeptidase Y; ER, endoplasmic reticulum; HA, hemagglutinin; tun, tunicamycin.

et al., 1983) have been shown to translocate in mammalian *in vitro* systems.

In this paper we show that the transfer of signal function between different organisms is not universal: a signal sequence that works efficiently in yeast is incapable of directing translocation in mammalian systems. Carboxypeptidase Y (CPY) from *Saccharomyces cerevisiae* contains a cleavable amino terminal signal sequence that directs it into the lumen of the yeast ER (Blachly-Dyson and Stevens, 1987; Johnson et al., 1987). However, CPY cannot be translocated across mammalian membranes either *in vivo* or *in vitro*, unless its own signal sequence is replaced by a signal from a mammalian secretory protein. We also show that mutating the CPY signal to increase its hydrophobicity—by replacing either one of its two glycine residues with a leucine—allows it to direct CPY into the mammalian ER.

## Materials and Methods

### Plasmids

The plasmids pKC3 and pXKH (Hanahan, D., unpublished work) were derived from pko (Van Doren et al., 1984). In pXKH, the pBR322 sequences of pko are replaced by those of pX3 (Maniatis et al., 1982). In pKC3, the pBR322-derived sequences of pko from the *Ava* I site to the *Eco* RI site (which contain the origin and beta-lactamase gene) have been replaced by the corresponding *Pvu* II to *Eco* RI fragment of pUC 8; a polylinker has been inserted into the unique *Hind* III site; and the *Pvu* II site upstream of the SV40 origin has been replaced by a *Cla* I site. The expression vector, pSVT7 was obtained by inserting a 20-bp DNA fragment containing a T7 RNA polymerase-specific promoter (Pharmacia Fine Chemicals, Piscataway, NJ) into the unique *Stu* I site of pKC3. This *Stu* I site lies within sequences derived from the early region of SV40 (at nucleotide 5190 in the SV40 sequence) and is ~30 bp downstream from the point of initiation of the early transcript (Tooze, 1981).

The construction of the plasmids, pSVTCPY and pSVTHA, is outlined in Fig. 1 A. The plasmid (pT5Y1) containing the yeast *PRC1* gene was obtained from Dr. T. Stevens. The sequence of this gene has been determined by Valls et al. (1987). The cDNA for the hemagglutinin (HA) of the A/Japan/305/57 strain of influenza virus was obtained on a *Cla* I–*Bam* HI fragment from an SV40 late-replacement vector similar to those described by Gething and Sambrook (1981). The *Cla* I site lies within 12 bp of the HA initiation codon. A *Hind* III site lies between the *Cla* I site and the initiation codon.

Fig. 1 B shows the steps taken in the construction of pSVHA/NEO and pSVmHA/NEO. A 1,400-bp *Hind* III fragment containing the neomycin/kanamycin (NEO) resistance gene from the transposon Tn5 (Beck et al., 1982) was cloned into the *Hind* III site of M13mpl9 so that the end of the gene encoding the NH<sub>2</sub>-terminus of NEO was adjacent to the other sites in the polylinker. An 800-bp *Kpn* I–*Xba* I fragment containing the HA signal sequence (from pMTHA; Sambrook et al., 1985) was subsequently cloned into the mpl9 NEO derivative cleaved with *Kpn* I and *Xba* I (these enzymes cut in the polylinker and do not cleave NEO).

Oligonucleotide-directed mutagenesis (Zoller and Smith, 1984) was then used to delete the sequences between the end of the HA signal and the second codon of the NEO gene. The deduced amino acid sequence of the resulting HA/NEO fusion is shown in Fig. 1 B. The signal sequence of the HA/NEO chimera was then altered by site-directed mutagenesis to give the mHA/NEO derivative. In this construct (Fig. 1 B), the leucine residues at positions six and eight in the HA signal are replaced by arginines. Finally, *Hind* III-generated fragments containing the HA/NEO and mHA/NEO fusions were isolated from the mpl9 derivatives and cloned into the *Hind* III site of pXKH (Fig. 1 B). The signal sequence:NEO fusions in the resulting plasmids, pSVHA/NEO and pSVmHA/NEO were verified by DNA sequencing.

The plasmid pSVTCPY/HA was constructed as follows: plasmid pSVTCPY was digested with *Eco* RI and *Mst* II, and the resulting termini were filled in using the Klenow fragment of *E. coli* DNA polymerase. The 550-bp fragment containing the CPY signal sequence was isolated and inserted into a filled *Hind* III site just upstream of the full-length HA cDNA (which had previously been cloned between the *Hind* III and *Bam* HI sites of

M13mpl8). Using oligonucleotide-directed mutagenesis, the sequences between the end of the CPY signal and the end of the HA signal were deleted. The deduced amino acid sequence surrounding the junction of CPY and HA in the resulting CPY/HA fusion is shown in Fig. 4. The CPY/HA fusion was excised from the mpl8 derivative on a *Sac* I–*Pvu* II fragment, which was used to replace the *Sac* I–*Pvu* II fragment (containing most of HA) from pSVTHA. The presence of the CPY signal sequence on the resulting plasmid, pSVTCPY/HA, was verified by restriction mapping and DNA sequencing.

The plasmid pSVTHA/CPY was constructed by first inserting a 480 bp *Eco* RI (filled) to *Bam* HI fragment, derived from pSVTCPY, into the *Sal* I (filled) and *Bam* HI sites of the HA mpl8 vector described above. The resulting construct carried the HA signal sequence upstream of the CPY signal and flanking regions, but did not contain the entire CPY coding region. Using oligonucleotide-directed mutagenesis the sequences between the end of the HA signal and the end of the CPY signal were deleted. The deduced amino acid sequence around the junction of HA and CPY in this construct is also shown in Fig. 4. The HA/CPY fusion was excised from the mpl8 derivative using *Hind* III (upstream of HA) and *Sma* I (downstream of CPY sequences). The *Hind* III terminus was filled and the fragment was cloned into pSVT7 that had been digested with *Sma* I. The remainder of the CPY coding sequence—on an *Xba* I fragment—was inserted after digesting the resulting plasmid with *Xba* I. The presence of the HA signal fused to proCPY on the final plasmid, pSVTHA/CPY, was confirmed by DNA sequencing.

The CPY signal sequence was altered by oligonucleotide-directed mutagenesis. Plasmid pSVTCPY was digested with *Nco* I (filled) and *Eco* RI. *Nco* I cuts at nucleotide 1394 in the CPY gene (Valls et al., 1987). The fragment containing the CPY sequence was inserted into M13mpl9 cut with *Eco* RI and *Sma* I. The resulting mpl9 derivative was mutagenized using oligonucleotides designed to yield the signal sequence mutants shown in Fig. 6, which were verified by DNA sequencing. Each mutant was excised from its mpl9 derivative on an *Eco* RI–*Bgl* II fragment (the *Bgl* II site is upstream of the *Nco* I site in the CPY sequences) which was then used to replace the wild type *Eco* RI–*Bgl* II fragment in pSVTCPY. The signal sequence mutations in the resulting plasmids, pSVTCPYml, pSVTCPYm2, and pSVTCPYm3 were confirmed by DNA sequencing.

### Transfection of Cells and Analysis of Proteins

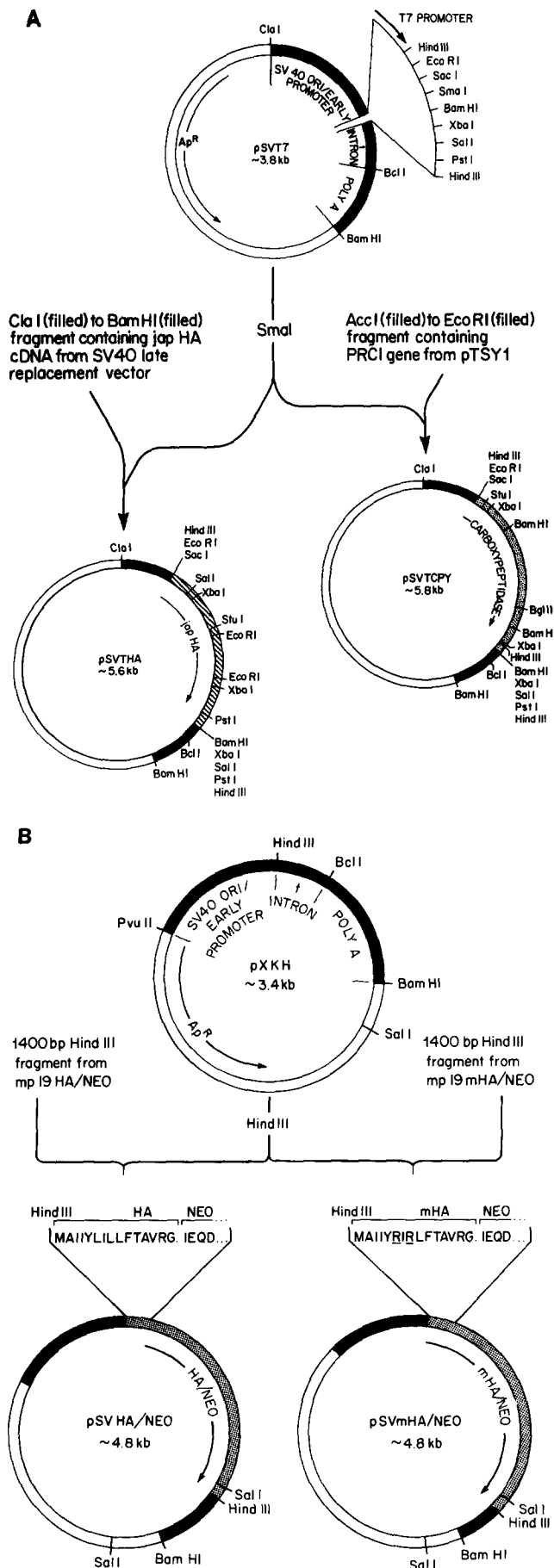
Approximately 10<sup>6</sup> COS-1 cells (Gluzman, 1981) per 100-mm dish were transfected with 2–5 µg of DNA as described by Shubeita et al. (1987). 30 h after the addition of DNA, the cells from each 100-mm dish were split into two 60-mm dishes and tunicamycin (20 µg/ml) was added to one. After a further 18 h, the medium was removed and the cells were starved for 30 min in serum- and methionine-free medium, including tunicamycin in the appropriate dishes. This medium was then removed and 300 µl of the same medium containing 50 µCi of [<sup>35</sup>S]methionine (Translabel, 10 mCi/ml, 1000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) was added and the cells were incubated for a further 30–60 min (with or without tunicamycin). Cell extracts were prepared and immunoprecipitations were performed as described by Gething et al. (1986), except that the protein A–Sepharose was added at the same time as the antiserum. Unless otherwise noted, immunoprecipitated proteins were analyzed on 8% SDS–polyacrylamide gels and fluorographed using Amplify (Amersham Corp., Arlington Heights, IL).

### Antisera

To immunoprecipitate CPY from ~5 × 10<sup>5</sup> transfected cells, we used 1 µl of a polyclonal rabbit antiserum obtained from Dr. T. Stevens. To immunoprecipitate HA, we used a mixture of the anti-HA (0.1 µl) and IH2 (1 µl) antisera described by Gething et al. (1986). HA produced *in vitro* was immunoprecipitated using IH2 only. The NEO proteins were precipitated using 1 µl of a polyclonal rabbit serum provided by Dr. J. Davies.

### Fractionation of COS-1 Cells

Transfected COS-1 cells were fractionated into membrane or cytoplasmic portions in the following fashion. Cell monolayers on 90-mm dishes were washed twice in ice-cold PBS (GIBCO, Grand Island, NY). The cells were then scraped from the dishes and resuspended in cold PBS containing the protease inhibitors (Sigma Chemical Co., St. Louis, MO) aprotinin (10 U/ml), leupeptin, antipain, pepstatin (each at 1 µg/ml), and phenylmethylsulfonyl fluoride (PMSF; 0.2 mM). These inhibitors were included in all the solutions used in succeeding steps. After collection by centrifugation at



2,000/rpm for 5 min at 4°C, the cells were washed once in 10 mM Tris-HCl (pH 8.0), 1 mM MgCl<sub>2</sub>, resuspended in the same solution and left for 15 min on ice before homogenization by five strokes of a Dounce homogenizer (VWR Scientific, Dallas, TX). An equal volume of 0.5 M sucrose, 40 mM Tris-HCl (pH 8.0), 2 mM MgCl<sub>2</sub> (STM) was added and the homogenate centrifuged at 2,000–3,000 rpm for 10 min at 4°C to remove cell debris and nuclei. The resulting supernatant was centrifuged at 100,000 g for 60 min. The supernatant (cytoplasmic fraction) was carefully removed and proteins of interest were immunoprecipitated as described above in the presence of 1% Nonidet P-40 (NP-40). The pellet (membrane fraction) was resuspended to the same volume as the supernatant in half-strength STM and immunoprecipitation was then carried out in the presence of 1% NP-40.

### Protein Sequencing

20 100-mm dishes of transfected COS-1 cells producing CPY were starved in medium lacking leucine, then labeled for 2 h with 200 μCi [<sup>3</sup>H]leucine (1 mCi/ml, 60 Ci/mmole; New England Nuclear, Boston, MA). CPY was extracted and immunoprecipitated as described above. Amino-terminal sequencing of labeled CPY (~4 × 10<sup>5</sup> dpm) was performed by Dr. Clive Slaughter using a gas-phase protein sequencer.

### In Vitro Transcription and Translation

RNA was synthesized in vitro from the pSVT7-based plasmids after each had been linearized using an appropriate restriction endonuclease which recognized a unique site downstream from the gene of interest. Bacteriophage T7 DNA-dependent RNA polymerase (Bethesda Research Laboratories, Bethesda, MD), which specifically recognized the T7 promoter on these plasmids, was used under the conditions recommended by the manufacturer. Typically, 10 μg of RNA was produced from 1–2 μg of template. 200–400 ng of RNA was used to program a rabbit reticulocyte lysate translation system (nuclease-treated; Promega Biotec, Madison, WI). A 25 μl reaction included 2 μl RNA, 1 μl of an amino acid mixture lacking methionine (Bethesda Research Laboratories), 2.5 μl Translabel (ICN Biomedicals, Inc.) and 18 μl of the lysate. Reactions were incubated for 60 min at 30°C, then 500 μl of NET-gel buffer as described in Gething et al. (1986) containing 1% NP-40 and including the appropriate antiserum was added. The immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography.

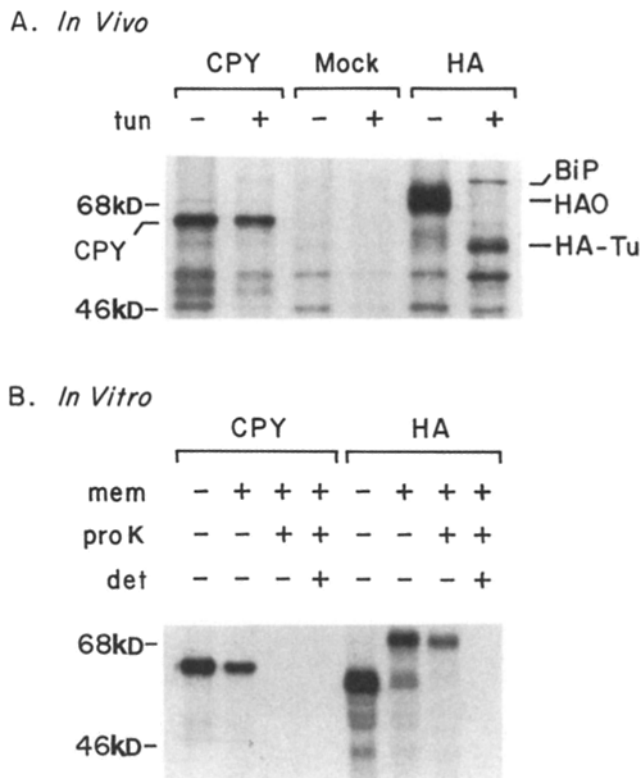
Canine pancreas microsomes (2 U equivalents) were included in most translation reactions. The microsomes were provided by Dr. P. Walter and were treated with nuclease as described by Walter and Blobel (1983). The sensitivity of the newly-synthesized proteins to protease was determined by adding proteinase K (120 μg/ml in the presence or absence of 1% Triton X-100) to the translation reactions after the 60 min incubation period at 30°C. After 45–60 min on ice, PMSF (1.2 mg/ml; Sigma Chemical Co.) was added, the samples were left for a further 10 min on ice then immunoprecipitated as described above. Protease sensitivity in the presence of 1% Triton X-100 (Sigma Chemical Co.) was determined by adding the detergent at the same time as the proteinase K.

### Results

#### Plasmids Used to Study the Expression and Targeting of CPY in Mammalian Cells

The structural gene encoding yeast CPY (Valls et al., 1987) was cloned into an expression vector (pSVT7), which contains a bacteriophage T7 promoter (Fig. 1 A) as well as regulatory elements that direct expression in COS-1 cells (Gluzman, 1981). In the resulting plasmid (pSVTCPY), the CPY coding region was flanked on the upstream side by the SV40 early promoter/origin and the bacteriophage T7

*Figure 1.* Construction of plasmids for expression of CPY, HA, HA/NEO, and mHA/NEO. Details of the construction of pSVTCPY and pSVTHA (A), as well as pSVHA/NEO and pSVmHA/NEO (B), are given in Materials and Methods. The maps are not drawn to scale. The SV40 sequences in the vectors pSVT7 and pXKH account for ~15% of each plasmid's DNA.



**Figure 2.** Expression of CPY and HA in vivo and in vitro. (A) COS-1 cells transfected with pSVTCPY, pSVTHA, or pSVT7 (*Mock*) were radiolabeled in the absence (–) or presence (+) of tunicamycin (*tun*), as described in Materials and Methods. CPY antiserum was used to immunoprecipitate the *Mock*-transfected cell extracts. *HAO* is the glycosylated form of *HA*; *HA-tun* is the unglycosylated form. *BiP* is the 77-kD heavy chain-binding protein. The extra bands (*M*, ~48 kD) in the *CPY* lanes are breakdown products of *CPY* which were generated during the extraction and immunoprecipitation procedures. (B) *CPY* and *HA* were synthesized in vitro and translocated using a rabbit reticulocyte lysate supplemented with canine pancreas microsomes, as described in Materials and Methods. RNAs synthesized in vitro from *pSVTHA* (linearized with Bam HI) and *pSVTCPY* (linearized with Sal I) were translated in the absence (–) or presence (+) of microsomes (*mem*). The resulting proteins were assayed for sensitivity to proteinase K (*pro K*) in the presence or absence of 1% Triton X-100 (*det*).

promoter, and downstream by the SV40 small *t* intron and polyadenylation signal. The T7 promoter was used in conjunction with the bacteriophage DNA-dependent RNA polymerase (Tabor and Richardson, 1985) to generate CPY mRNA in vitro for translation in a rabbit reticulocyte lysate cell-free system. The plasmid pSVTHA is similar in structure to pSVTCPY except that it contains a cDNA encoding the HA from the A/Japan/305/57 strain of influenza virus (Fig. 1 A). When this cDNA is expressed in mammalian cells, HA is directed into the ER by a 15-amino acid amino terminal-signal sequence, which is later cleaved from the nascent polypeptide (McCauley et al., 1979). HA receives mannose-rich oligosaccharide side chains in the ER, travels through the Golgi apparatus where it is terminally glycosylated and then moves to the cell surface (for review, see Roth et al., 1988).

Plasmids pSVHA/NEO and pSVmHA/NEO (Fig. 1 B) are

similar in design to the pSVT7-based vectors, except that they lack the bacteriophage T7 promoter. In mammalian cells these plasmids express chimeric proteins consisting of the HA signal sequence (or a mutated HA signal sequence, mHA) fused to the amino terminus of a mutant form of the bacterial enzyme, aminoglycoside 3' phosphotransferase (NEO). This altered enzyme, which was created by site-directed mutagenesis, carries a consensus sequence for *N*-glycosylation (<sup>199</sup>Asn-Gly-Thr). The increase in the size of the protein that occurs when a mannose-rich oligosaccharide is attached to the Asn residue provides a convenient assay for translocation (Bird, P., M. J. Gething, and J. Sambrook, manuscript in preparation).

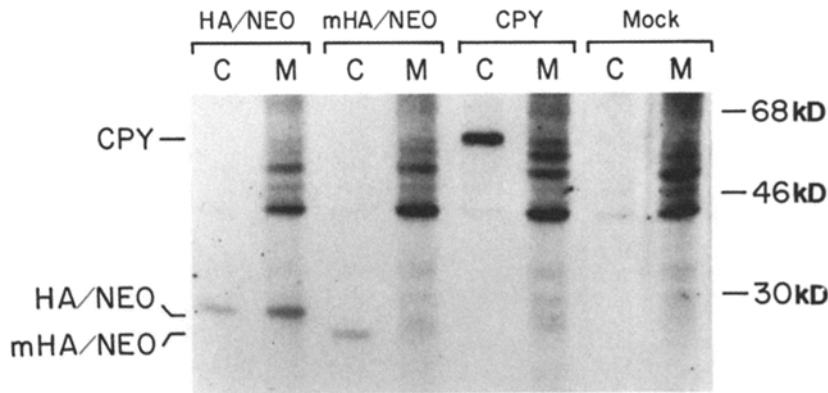
### CPY is not Glycosylated in Mammalian Cells

COS-1 cells transfected with pSVTCPY, pSVTHA, or pSVT7 were radiolabeled with [<sup>35</sup>S]methionine in the presence or the absence of tunicamycin—a drug which inhibits *N*-linked glycosylation (Tkacz and Lampen, 1975). Proteins encoded by the plasmids were then immunoprecipitated from cell extracts and analyzed by SDS-PAGE (Fig. 2 A).

In *Saccharomyces cerevisiae*, the addition of four oligosaccharide side-chains to CPY causes a marked increase in the molecular mass of the translocated protein (Hasilik and Tanner, 1978; Stevens et al., 1982). However, in COS-1 cells transfected with pSVTCPY, CPY is synthesized as a protein of ~62 kD, which shows no reduction in its apparent molecular mass in the presence of tunicamycin (Fig. 2 A), and which co-migrates with full-length preproCPY synthesized in a reticulocyte cell-free system in the absence of membranes (results not shown). This result suggests that CPY is not glycosylated in COS-1 cells and raises the possibility that it is not translocated. Consistent with this hypothesis is the observation that the molecular mass of CPY synthesized in COS-1 cells is greater than that reported for unglycosylated proCPY isolated from yeast (Stevens et al., 1982). In yeast, the signal peptide (20-amino acids long) is cleaved from preproCPY to yield proCPY in the ER (Blachly-Dyson and Stevens, 1987). However, an untranslocated form of preproCPY synthesized in COS-1 cells would retain the signal peptide and would therefore migrate more slowly through polyacrylamide gels.

To verify that translocation and glycosylation occurred normally in transfected cells, the biosynthesis of HA was analyzed in COS-1 cells transfected with pSVTHA. HA synthesized in a 60-min pulse in the absence of tun was detected as a broad band (*HAO*, ~70 kD), which has previously been shown to consist of mixture of core-glycosylated and terminally-glycosylated molecules (Gething and Sambrook, 1981; Doyle et al., 1985). HA synthesized in the presence of tun appears as a 61-kD unglycosylated protein (*HA-tun*). The 77-kD protein that co-precipitates with *HA-tun* (Fig. 2 A) is the immunoglobulin heavy chain-binding protein (*BiP*; Haas and Wabl, 1983), which has been shown to bind to unglycosylated or mal-folded forms of HA in the ER (Gething et al., 1986). These results indicate that the transfected COS-1 cells are fully competent to translocate and glycosylate newly-synthesized proteins.

To test further the idea that CPY is not glycosylated because it is not translocated, and to rule out the possibility that



**Figure 3.** CPY is localized in the cytosol of transfected COS-1 cells. COS-1 cells were transfected with pSVHA/NEO, pSVmHA/NEO, pSVTCPY, or pSVT7 (*Mock*), radiolabeled and fractionated into cytosolic (C) and membrane (M) preparations (see Materials and Methods). After immunoprecipitation with anti-NEO serum (lanes 1-4) or anti-CPY serum (lanes 5-8), the samples were subjected to electrophoresis through a 10% SDS-polyacrylamide gel. The mock fractions were immunoprecipitated using CPY antiserum. HA/NEO is glycosylated and is therefore larger than the cytosolic mHA/NEO.

a splicing artifact may have produced a form of CPY lacking a signal sequence, we studied the behavior of CPY in an *in vitro* translation and translocation system. CPY and HA mRNAs were synthesized *in vitro* from linearized pSVTCPY and pSVTHA DNAs, using bacteriophage T7 DNA-dependent RNA polymerase. The RNAs were then used to program a rabbit reticulocyte lysate translation system in the presence or absence of canine pancreas microsomes. Translocation into microsomes leads to the acquisition of resistance to exogenously-added protease; glycosylation causes an increase in the molecular mass of the translocated proteins (Walter and Blobel, 1983). As shown in Fig. 2 B, the vast majority of HA molecules synthesized in the presence of microsomes migrate as the higher molecular mass, glycosylated form indicating that they have been translocated across the microsomal membrane. Translocation of HA was qualitatively confirmed by its resistance to digestion with proteinase K. Disruption of the microsomes with Triton X-100 renders the translocated HA completely sensitive to protease. By contrast, CPY was synthesized *in vitro* as a protease-sensitive protein of 62 kD whose size was not affected by the presence of microsomes (Fig. 2 B). These studies eliminate the possibility of splicing artifacts and strongly suggest that CPY is translocated very inefficiently, if at all, in mammalian systems.

#### ***CPY does not Become Associated with Membranes in Mammalian Cells and its Signal Sequence is not Cleaved***

Because the first site for addition of oligosaccharide to CPY is located 124 amino acids from its amino terminus (Valls et al., 1987), we considered the possibility that the failure to detect glycosylated CPY *in vivo* or *in vitro* might be due to incomplete translocation caused by a cryptic "stop-transfer" (Blobel, 1980) sequence located within these first 124 residues. This would leave the molecule stranded in the membrane, with its amino terminal portion in the lumen of the ER and the remainder in the cytoplasm. In this case, CPY might be expected to sediment with ER-derived vesicles if the cells were gently broken and fractionated. To test this possibility, COS-1 cells were transfected with pSVTCPY, pSVHA/NEO, pSVmHA/NEO, or pSVT7, radiolabeled with [<sup>35</sup>S]methionine, then gently broken by Dounce homogenization. After removal of nuclei and large cellular debris, the homogenates were separated by ultracentrifugation into mem-

brane (ER, Golgi apparatus, and plasma membrane-derived vesicles) and cytosolic fractions (Wills et al., 1984). The proteins of interest were then immunoprecipitated from each fraction and analyzed by SDS-PAGE (Fig. 3). Although a significant amount of background was obtained in immunoprecipitates from the membrane fractions, comparison with the results obtained for the mock-transfected (pSVT7) samples allowed estimation of the distribution of the proteins in the membrane or cytosolic fractions.

We have previously shown that the HA/NEO chimera is translocated and glycosylated both *in vivo* and *in vitro*, whereas the mHA/NEO which carries a mutated signal sequence is not (Bird, P., M. J. Gething, and J. Sambrook, manuscript in preparation). We therefore expected the HA/NEO protein to co-fractionate with membranes and the mHA/NEO protein to be in the cytosolic fraction. Fig. 3 shows that the majority of the HA/NEO chimera (~30 kD) was found in the membrane fraction. Some of the chimeric protein was also detected in the cytosolic fraction, indicating that leakage of translocated proteins occurs during fractionation of membranes. By contrast, the majority of the smaller, non-glycosylated 28-kD mHA/NEO protein was found in the cytosolic fraction. The majority of CPY was detected in the cytosolic fraction, suggesting that the newly-synthesized protein is incapable of associating efficiently with microsomal membranes.

To determine whether the cytoplasmic form of CPY has an intact signal sequence, CPY was immunoprecipitated from COS-1 cells that had been transfected with pSVTCPY and radiolabeled with tritiated leucine. The positions of labeled leucine residues near the amino terminus of the molecule were determined by protein sequencing. Radioactivity was liberated in the 7th, 8th, 11th, and 13th of the first 15 cycles of Edman degradation (data not shown). The positions of the radiolabeled leucine residues therefore correspond exactly to the positions of the first four leucines in the CPY signal sequence. Analysis of the entire preproCPY sequence (Valls et al., 1987) shows that this distribution of leucine residues occurs only in the signal sequence of preproCPY and would not have been observed if the signal had been cleaved, if proCPY had been proteolytically processed to its mature form, or if CPY had been proteolytically cleaved at any other position in the molecule.

The simplest explanation for these results is that the CPY signal does not function in mammalian cells. However, since signal cleavage is not necessarily a consequence of transloca-

CPY      M K A F T S L L C G L G L S T T L A K A I S L Q R P L . .  
                   5           10           15           20 ↓  
 CPY/HA M K A F T S L L C G L G L S T T L A K A D Q I C I G Y . .  
  
 HA                    5           10           15 ↓  
                   M A I I Y L I L L F T A V R G D Q I C I G Y . .  
 HA/CPY            M A I I Y L I L L F T A V R G I S L Q R P L . .

**Figure 4.** Signal sequences of CPY, HA, HA/CPY, and CPY/HA. The amino acid sequences deduced from the relevant nucleotide sequences are shown. The arrows indicate the sites of signal peptide processing for CPY (Blachly-Dyson and Stevens, 1987) and HA (McCauley et al., 1979). The cleavage sites for HA/CPY and CPY/HA have not been determined.

tion (Rapoport, 1986), an alternative, but less likely, explanation is that the function of the CPY signal is abrogated by a downstream amino acid sequence.

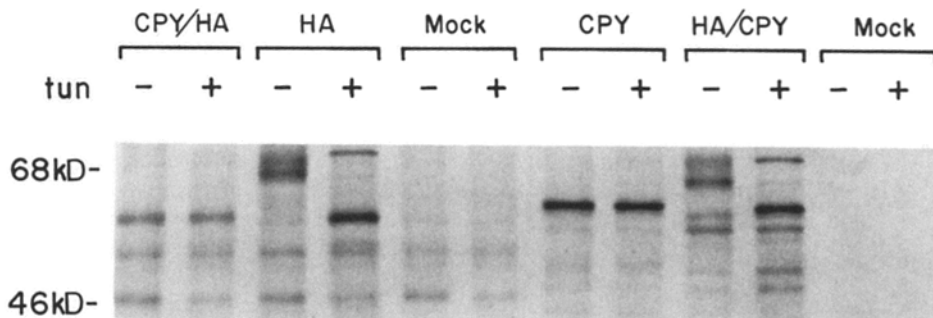
**The HA Signal Sequence can Direct CPY into the ER of COS-1 Cells**

To test whether the failure of CPY to be translocated was due to a defect in its signal sequence or to the influence of some downstream stretch of amino acids, two plasmids were constructed using standard in vitro mutagenic techniques (see Materials and Methods). In pSVTHA/CPY, the CPY

signal sequence was replaced by the HA signal sequence and the resulting composite gene was cloned into pSVT7. In pSVTCPY/HA, the HA signal was replaced by the CPY signal and the resulting chimera was cloned into pSVT7.

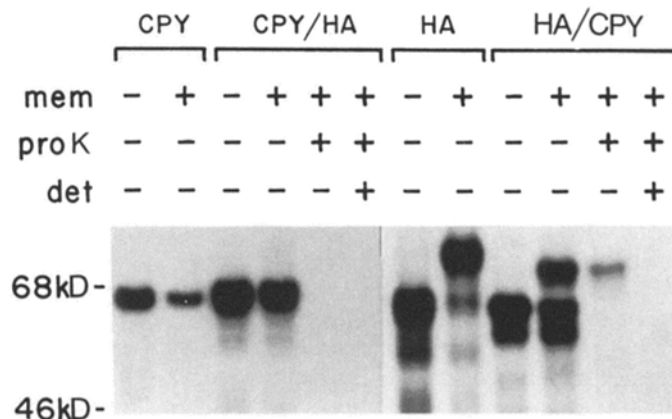
Fig. 4 shows the deduced amino acid sequences of the signal sequences of the fusion proteins, CPY/HA and HA/CPY. The CPY/HA gene codes for a chimeric protein consisting of the first 20 amino acids of CPY (i.e., all the amino acids that lie upstream of the signal cleavage site, [Blachly-Dyson and Stevens, 1987]) fused to sequences of HA that begin immediately downstream of the HA signal cleavage site

**A. In Vivo**



**Figure 5.** Expression of HA/CPY and CPY/HA in vivo and in vitro. (A) COS-1 cells were transfected with pSVTCPY/HA, pSVTHA/CPY, pSVTCPY, pSVTHA or pSVT7 (Mock). Cells were radiolabeled in the absence (-) or presence (+) of tunicamycin (tun), and samples were prepared and analyzed as described in Materials and Methods. The mock samples adjacent to the HA samples on the gel were immunoprecipitated with HA antisera. The mock samples adjacent to the CPY samples were immunoprecipitated with CPY antiserum. (B) RNAs were synthesized in vitro using linearized plasmids as templates: pSVTHA/CPY was linearized with Sal I, pSVTCPY/HA with Bam HI. As described in Materials and Methods, the RNAs were translated in a rabbit reticulocyte lysate translation system in the presence (+) or absence (-) of microsomes (mem), and the resulting proteins were assayed for sensitivity to proteinase K (pro K) in the presence or absence of 1% Triton X-100 (det).

**B. In Vitro**



(McCauley et al., 1979). Conversely, the chimeric protein encoded by the HA/CPY gene consists of the 15-amino acid signal sequence of HA joined to proCPY at the first residue after the CPY cleavage site.

The expression of these chimeric proteins was analyzed in transfected COS-1 cells (see Fig. 5 A). Genes that contain a CPY signal (CPY/HA and CPY) are expressed as unglycosylated proteins whose molecular masses are not altered by the presence of tun. By contrast, genes that contain an HA signal (HA and HA/CPY) are expressed as glycosylated proteins of higher molecular mass. The presence of terminally-glycosylated forms of both proteins (Fig. 5 A) indicates that they had been transported from the ER to the Golgi apparatus during the labeling period. Indeed, some HA/CPY was detected in the medium of transfected COS-1 cells (data not shown), a result that further supports the conclusion that CPY is successfully translocated under the influence of the HA signal. HA and HA/CPY synthesized in the presence of tun migrate slightly faster than CPY/HA and CPY, respectively. This difference is presumably due to cleavage of the HA signal from the translocated proteins. The 77 kD protein that co-precipitates with HA and HA/CPY isolated from tun-treated cells (Fig. 5 A) is the immunoglobulin heavy chain-binding protein (BiP) referred to earlier. These findings show that the CPY signal itself rather than a downstream amino acid sequence is responsible for the failure of CPY to be translocated in mammalian cells.

These conclusions were confirmed by studies in which mRNA transcribed *in vitro* from the chimeric genes were translated in a cell-free protein-synthesizing system in the presence and absence of canine pancreas membranes. As shown in Fig. 5 B, ~90% of wild-type HA molecules and ~30% of chimeric HA/CPY were translocated into microsomes (indicated by a membrane-dependent increase in size and by acquisition of resistance to protease). In Fig. 5 B, no translocation of CPY or the CPY/HA chimera is detectable. However, over-exposure (not shown) of the autoradiogram revealed that in this system a very small proportion (<1%) of the CPY/HA chimera was translocated. Because we could not detect translocation of CPY/HA *in vivo*, we do not know whether this result reflects a difference in sensitivity between the two systems, or a lack of stringency in the *in vitro* system. Whatever the explanation, it is clear that the CPY signal functions very inefficiently in mammalian *in vitro* systems and at an undetectably low level *in vivo*.

### Increasing the Hydrophobicity of the CPY Signal Sequence Allows it to Function in Mammalian Cells

As discussed below, the CPY signal contains a core or h region that is less hydrophobic than most others. To determine

whether the CPY signal would function in mammalian cells if the hydrophobicity of its h region were increased, we used standard *in vitro* mutagenic techniques to alter the glycine codons at position 10 or 12 in the CPY signal to leucine codons. In addition, we constructed a mutant in which both glycine codons were changed to leucine codons (Fig. 6). These CPY mutants were cloned into expression vectors and the resulting plasmids (pSVTCPYml, pSVTCPYm2 and pSVTCPYm3) were used both to transfect COS-1 cells and to generate RNA for use in the mammalian *in vitro* translation and translocation system.

Fig. 7 A shows that all the mutants were translocated *in vivo* as indicated by the appearance of glycosylated CPY. Terminally-glycosylated forms of CPY were observed in cells expressing the CPY signal mutants or the HA/CPY chimera. In each case radiolabeled CPY was detected in the culture medium (data not shown). As before (Fig. 5 A), translocated, unglycosylated CPY was associated with BiP.

The CPY signal mutants were also translocated in the mammalian cell-free system, as indicated by their microsome-dependent increase in size (Fig. 7 B). We did not observe any significant difference in the efficiency of translocation between the various mutants and HA/CPY. Once again protease protection experiments provide qualitative confirmation of this result.

### Discussion

Our results show that signal sequences that function efficiently in one organism do not necessarily work in another. Specifically, the inability of the yeast CPY signal to work in mammalian cells is attributable to the structure of its central hydrophobic core. Based on the study of 118 vertebrate, 2 yeast, and 32 prokaryotic signals, von Heijne (1985) has suggested that the minimal requirement for the central core (or h region) is seven hydrophobic residues interrupted by no more than one serine, threonine, glycine or proline residue. Fig. 6 illustrates that the putative h region of the CPY signal does not meet these requirements. In fact, the seven-residue CPY h region consists of only five hydrophobic amino acids that are interrupted by two glycine residues. While the yeast translocation machinery clearly tolerates such a patchy distribution of hydrophobic residues, mammalian systems apparently require a more coherent arrangement. From the data presented in this paper, it seems that a core composed of six hydrophobic residues with no more than one interruption may be sufficient for signal function in mammalian systems. However, further work is necessary to define such matters as (I) the overall length of the h region (II) the positions at which interruptions can be tolerated (III) the amino acids that are allowed at the sites of interruption and (IV) the degree of hydrophobicity required of each non-polar residue.

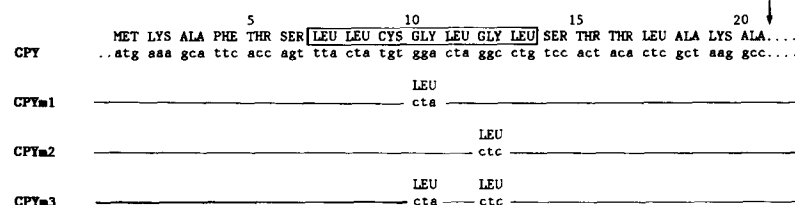
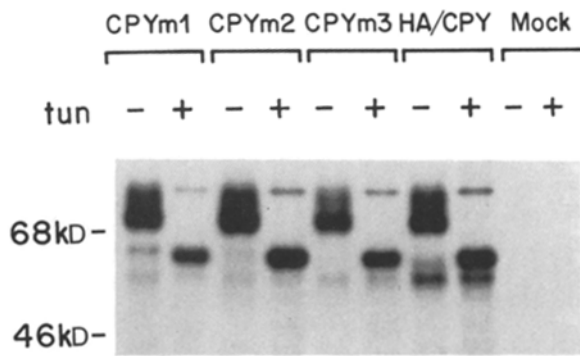


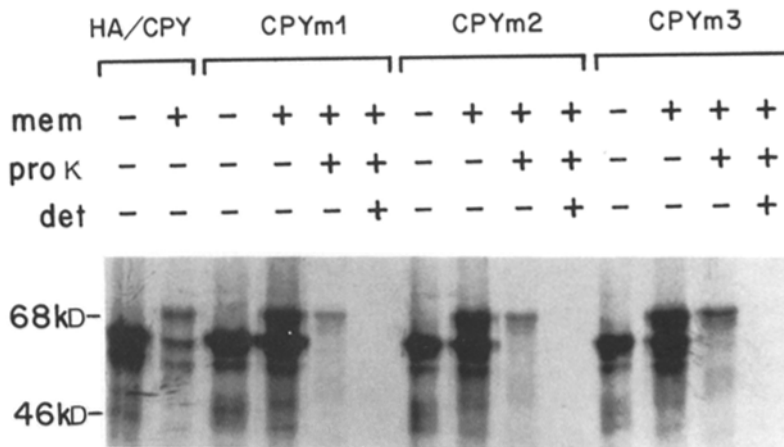
Figure 6. Mutations constructed in the CPY signal sequence. Oligonucleotide-directed mutagenesis was used to alter the CPY signal sequence to yield the mutants CPYm1, CPYm2, and CPYm3 (see Materials and Methods). The putative h region of the CPY signal (von Heijne, 1985) is boxed. The nucleotide sequences of each of the mutants is identical to that of the wild type except at the positions indicated.



### A. *In Vivo*



### B. *In Vitro*



**Figure 7.** Translocation of the CPY mutants in mammalian systems. (A) COS-1 cells were transfected with pSVTCPYm1, pSVTCPYm2, pSVTCPYm3, pSVTHA/CPY or pSVT7 (*Mock*) and radiolabeled in the absence (-) or presence (+) of tunicamycin (*tun*). The mock samples were immunoprecipitated using CPY antiserum. (B) Plasmids were linearized with Sal I and used to synthesize RNA *in vitro*. The resulting mRNAs were used to program a rabbit reticulocyte lysate in the presence (+) or absence (-) of microsomes (*mem*), and the resulting proteins were assayed for sensitivity to proteinase K (*pro K*) in the presence or absence of 1% Triton X-100 (*det*). See Materials and Methods for details.

Although the numbers are not large, most of the yeast secretory proteins studied to date have signal sequences which resemble mammalian signals in structure. For example, the 19 residue invertase signal sequence—which functions in mammalian cells (Bergh et al., 1987)—has an h region consisting of 10 hydrophobic residues interrupted by one glycine (Carlson et al., 1983). Such yeast signals can interact productively with mammalian SRP, as shown by studies on their ability to direct translocation in mammalian cell-free systems (Schekman, 1985). Our finding that the CPY signal does not function in mammalian cells raises an interesting question about the nature of signal sequences in yeast. Why should yeast have two classes of signal sequences, as defined by the ability to function in mammalian systems?

Studies using yeast cell-free systems have shown that proteins such as  $\alpha$ -factor (Hansen et al., 1986; Rothblatt and Meyer, 1986) and CPY (P. Walter, personal communication) may be posttranslationally translocated. That is, translocation of these proteins may occur after most or all of the polypeptide has been synthesized. Indeed, it has been demonstrated that a fraction of CPY molecules can be translocated even when their signal sequence has been deleted (Blachly-Dyson and Stevens, 1987). It is possible that such proteins may be inherently competent for translocation either because

they possess another internal signal sequence or because they do not assume a conformation in the cytoplasm which prevents translocation. By contrast, a protein like invertase, which cannot be posttranslationally translocated (Rothblatt and Meyer, 1986), might rapidly assume a translocation-incompetent conformation in the cytoplasm. In this case, tight coupling between translation and translocation would be necessary to prevent synthesis and folding in the cytoplasm.

Recently, Rapoport et al. (1987) have proposed a model in which they suggest that the interaction between SRP and a signal peptide can be thought of as an equilibrium between unbound SRP and signal on one hand and the SRP-signal complex on the other. This model predicts that SRP should have different binding affinities for different signals. Although a counterpart to mammalian SRP has not yet been demonstrated in yeast, we think that the apparent existence of more than one type of signal in yeast may be consistent with this model. We postulate that putative yeast SRP binds more efficiently to signal sequences that are also recognized by mammalian SRP, and less efficiently to signals, such as that found on CPY, that do not work in mammalian systems. A consequence of tighter binding to SRP might be a stricter coupling between translation and translocation.

Rather than falling into two classes exemplified by inver-



tase and CPY, it is possible that yeast signals form a continuum with respect to their affinity for SRP. Signals lying at one end of this continuum would contain h regions that conform to von Heijne's (1985) rules and would interact strongly with SRP. At the other end of the continuum would lie signals which contain less conventional h regions and that interact weakly, if at all, with SRP. In addition to the natural signal of CPY, others of this type might include the surrogate signals that have recently been shown to mediate the translocation of a derivative of invertase lacking its natural signal sequence (Kaiser et al., 1987). However, it remains to be determined whether these surrogate sequences would still function as signals when fused to another protein which is not normally translocated, or whether they merely facilitate translocation in conjunction with other sequences in the body of invertase.

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## References

- Ainger, K. J., and D. I. Meyer. 1986. Translocation of nascent secretory proteins across membranes can occur late in translation. *EMBO (Eur. Mol. Biol. Org.) J.* 5:951-955.
- Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller. 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene.* 19:327-336.
- Bergh, M. L. E., C. L. Cepko, D. Wolf, and P. H. Robbins. 1987. Expression of the *Saccharomyces cerevisiae* glycoprotein invertase in mouse fibroblasts: glycosylation, secretion, and enzymatic activity. *Proc. Natl. Acad. Sci. USA.* 84:3570-3574.
- Blachly-Dyson, E., and T. H. Stevens. 1987. Yeast carboxypeptidase Y can be translocated and glycosylated without its amino-terminal signal sequence. *J. Cell. Biol.* 104:1183-1191.
- Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA.* 77:1496-1500.
- Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell. Biol.* 67:835-851.
- Bostian, K. A., S. Jayachandran, and D. J. Tipper. 1983. A glycosylated protoxin in killer yeast: models for its structure and maturation. *Cell.* 32:169-180.
- Carlson, M., and D. Botstein. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. *Cell.* 28:145-154.
- Carlson, M., R. Taussig, S. Kustu, and D. Botstein. 1983. The secreted form of invertase in *Saccharomyces cerevisiae* is synthesized from mRNA encoding a signal sequence. *Mol. Cell. Biol.* 3:439-447.
- Chao, C. C.-K., P. Bird, M.-J. Gething, and J. Sambrook. 1987. Post-translational translocation of influenza virus hemagglutinin across microsomal membranes. *Mol. Cell. Biol.* 7:3842-3845.
- Doyle, C., M. G. Roth, J. Sambrook, and M.-J. Gething. 1985. Mutations in the cytoplasmic domain of the influenza virus hemagglutinin affect different stages of intracellular transport. *J. Cell. Biol.* 100:704-714.
- Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell.* 46:939-950.
- Gething, M.-J., and J. Sambrook. 1981. Cell-surface expression of influenza hemagglutinin from a cloned DNA copy of the RNA gene. *Nature (Lond.)* 293:620-625.
- Gething, M.-J., and J. Sambrook. 1982. Construction of influenza hemagglutinin genes that code for intracellular and secreted forms of the protein. *Nature (Lond.)* 300:598-603.
- Gluzman, Y. 1981. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell.* 23:175-182.
- Haas, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature (Lond.)* 306:387-389.
- Hansen, W., P. D. Garcia, and P. Walter. 1986. In vitro protein translocation across the yeast endoplasmic reticulum: ATP-dependent post-translational translocation of the prepro-a-factor. *Cell.* 45:397-406.
- Hasilik, A., and W. Tanner. 1978. Carbohydrate moiety of carboxypeptidase Y and perturbation of its biosynthesis. *Eur. J. Biochem.* 91:567-575.
- Hitzeman, R. A., D. W. Leung, L. J. Perry, W. J. Kohr, H. L. Levine, and D. V. Goeddel. 1983. Secretion of human interferon by yeast. *Science (Wash. DC)* 219:620-625.
- Jabbar, M. A., and D. P. Nayak. 1987. Signal processing, glycosylation, and secretion of mutant hemagglutinins of a human influenza virus by *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7:1476-1485.
- Johnson, L. M., V. A. Bankiatis, and S. D. Emr. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. *Cell.* 48:875-885.
- Julius, D., R. Schekman, and J. Thorner. 1984. Glycosylation and processing of prepro-a-factor through the yeast secretory pathway. *Cell.* 36:309-318.
- Kaiser, C. A., D. Preuss, P. Grisafi, and D. Botstein. 1987. Many random sequences functionally replace the secretion signal sequence of yeast invertase. *Science (Wash. DC)* 235:312-317.
- Lingappa, V. R., J. Chaidez, C. S. Yost, and J. Hedgpeth. 1984. Determinants for protein localization: beta-lactamase signal directs globin across membranes. *Proc. Natl. Acad. Sci. USA.* 81:456-460.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 545 pp.
- McCauley, J., J. Bye, K. Elder, M.-J. Gething, J. J. Skehel, A. Smith, and M. D. Waterfield. 1979. Influenza virus signal sequences. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 108:422-426.
- Meyer, D. I. 1985. Signal recognition particle (SRP) does not mediate a translational arrest of nascent secretory proteins in mammalian cell-free systems. *EMBO (Eur. Mol. Biol. Org.) J.* 4:2031-2033.
- Mueckler, M., and H. F. Lodish. 1986. The human glucose transporter can insert posttranslationally into microsomes. *Cell.* 44:629-637.
- Mueller, M., I. Ibrahim, C. N. Chang, P. Walter, and G. Blobel. 1982. A bacterial secretory protein requires Signal Recognition Particle for translocation across mammalian endoplasmic reticulum. *J. Biol. Chem.* 257:11860-11863.
- Perara, E., R. E. Rothman, and V. R. Lingappa. 1986. Uncoupling translocation from translocation: implications for transport of proteins across membranes. *Science (Wash. DC)* 232:348-352.
- Perlman, D., and H. O. Halvorson. 1981. Distinct repressible mRNAs for cytoplasmic and secreted yeast invertase are encoded by a single gene. *Cell.* 25:525-536.
- Perlman, D., and H. O. Halvorson. 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J. Mol. Biol.* 167:391-409.
- Rapoport, T. A. 1986. Protein translocation across and integration into membranes. *CRC Crit. Rev. Biochem.* 20:73-137.
- Rapoport, T. A., R. Heinrich, P. Walter, and T. Schulmeister. 1987. Mathematical modelling of the effects of the signal recognition particle on translation and translocation of proteins across the endoplasmic reticulum membrane. *J. Mol. Biol.* 195:621-636.
- Roth, M. G., M.-J. Gething, and J. Sambrook. 1988. Intracellular transport and membrane insertion of the influenza viral glycoproteins. In *The Influenza Viruses*, R. M. Krug, editor. Plenum Publishing Corp., New York. In press.
- Rothblatt, J. A., and D. I. Meyer. 1986. Secretion in yeast: translocation and glycosylation of prepro-a-factor in vitro can occur via an ATP-dependent post-translational mechanism. *EMBO (Eur. Mol. Biol. Org.) J.* 5:1031-1036.
- Sambrook, J., L. Rogers, J. White, and M.-J. Gething. 1985. Lines of BPV-transformed murine cells that constitutively express influenza virus hemagglutinin. *EMBO (Eur. Mol. Biol. Org.) J.* 4:91-103.
- Schekman, R. 1985. Protein localization and membrane traffic in yeast. *Annu. Rev. Cell Biol.* 1:115-143.
- Sharma, S., L. Rodgers, J. Brandsma, M.-J. Gething, and J. Sambrook. 1985. SV40 T antigen and the exocytotic pathway. *EMBO (Eur. Mol. Biol. Org.) J.* 4:1479-1489.
- Shubeita, H. E., J. F. Sambrook, and A. M. McCormick. 1987. Molecular cloning and analysis of functional cDNA and genomic clones encoding bovine cellular retinoic acid binding protein. *Proc. Natl. Acad. Sci. USA.* 84:5645-5649.
- Stevens, T., B. Esmon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* 30:439-448.
- Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA.* 82:1074-1078.

- Talmadge, K., S. Stahl, and W. Gilbert. 1980. Eukaryotic signal sequence transports insulin antigen in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. 77:3369-3373.
- Tkacz, J. S., and J. O. Lampen. 1975. Tunicamycin inhibition of polyisoprenol N-acetylglucosaminyl pyrophosphate formation in calf liver microsomes. *Biochem. Biophys. Res. Commun.* 65:248-257.
- Tooze, J. 1981. In DNA Tumour Viruses. Molecular Biology of Tumour Viruses. 2nd Edition. J. Tooze, editor. Cold Spring Harbor Laboratory, New York.
- Valls, L. A., C. P. Hunter, J. H. Rothman, and T. H. Stevens. 1987. Protein sorting in yeast: the localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. *Cell*. 48:887-897.
- Van Doren, K., D. Hanahan, and Y. Gluzman. 1984. Infection of eukaryotic cells by helper-independent recombinant adenoviruses: early region 1 is not obligatory for integration of viral DNA. *J. Virol.* 50:606-614.
- von Heijne, G. 1981. On the hydrophobic nature of signal sequences. *Eur. J. Biochem.* 116:419-422.
- von Heijne, G. 1985. Signal sequences. The limits of variation. *J. Mol. Biol.* 184:99-105.
- Walter, P., and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. III. Signal Recognition Protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. *J. Cell. Biol.* 91:557-561.
- Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* 96:84-93.
- Walter, P., R. Gilmore, and G. Blobel. 1984. Protein translocation across the endoplasmic reticulum. *Cell*. 38:5-8.
- Wiedmann, M., A. Huth, and T. A. Rapoport. 1984. *Xenopus* oocytes can secrete bacterial beta-lactamase. *Nature (Lond.)*. 309:637-639.
- Wills, J. W., R. J. Srinivas, and E. Hunter. 1984. Mutations in the Rous sarcoma virus *env* gene that affect the transport and subcellular location of the glycoprotein products. *J. Cell. Biol.* 99:2011-2023.
- Zoller, M. J., and M. Smith. 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *DNA*. 3:479-488.