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Signal-sequence Trap in Mammalian and Yeast Cells: A Comparison

G. Galliciotti, H. Schneider, L. Wyder, A. Vitaliti, M. Wittmer, M. Ajmo, R. Klemenz
Department of Pathology, Division of Cancer Research, University Hospital, Schmelzbergstrasse 12, 8091 Zurich, Switzerland

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Abstract. Membrane associated and secreted proteins are translated as precursors containing a signal peptide that allows protein-insertion into the membrane of the endoplasmic reticulum and is co-translationally removed in the lumen. The ability of the signal peptide to direct a polypeptide into the secretory pathway is exploited in methods developed to select cDNAs encoding such proteins. Different strategies are known in which cDNA libraries can be screened for signal peptides by the ability of the latter to rescue the translocation of signal sequence-less proteins. In one method, a cDNA library is tested for interleukin 2 receptor α chain translocation to the membrane in COS cells, in another one for invertase secretion from yeast. In this work, we compared the two systems by testing six mouse signal peptides in COS and yeast cells. All of them were functional in the mammalian system, whereas only three of them in yeast. Two other sequences needed the 5′ cDNA sequence flanking the ATG codon to be removed in order to enable protein translocation. Although the structure of signal sequences and the functioning of the secretory machinery are well conserved from prokaryotes to eukaryotes, it seems evident that not all signal peptides can be interchanged between different proteins and organisms. In particular, signal peptides that are functional in the mammalian system do not necessarily lead to protein translocation in yeast.

Key words: Signal sequence — Signal sequence trap — Membrane protein — Protein translocation — Yeast

Present address for L. Wyder: Richard Dimbleby Department of Cancer Research/ICRF Laboratory, St. Thomas' Hospital, Lambeth Palace Road, SE1 7EH London, United Kingdom.

Present address for A. Vitaliti: Novartis Pharma AG, WKL 125 10 45, Klybeckstrasse 141, 4057 Basel, Switzerland.

Correspondence to: R. Klemenz; email: roman.klemenz@pty.usz.ch

Introduction

Both prokaryotes and eukaryotes have evolved numerous mechanisms for the proper segregation of proteins to and within various compartments of the cell. A great number of proteins are directed either co- or post-translationally across the membrane of the endoplasmic reticulum (ER). These include secretory proteins, proteins anchored in the plasma membrane, or located in lysosomes, endosomes and all organelles of the secretory pathway (Rapoport, 1992). Such proteins need a specific targeting signal for their transport to the ER: the signal sequence, a transient extension of the amino terminus of the protein, which is removed once its targeting function has been carried out (von Heijne, 1990).

Signal sequences from both prokaryotes and eukaryotes seem to be built along the same general lines. They are 15–30 amino acids in length and consist of three regions: a positively charged N-terminal region, a central hydrophobic region, critical for the signal sequence function, and a more polar C-terminal region that contains the signal peptide cleavage site (von Heijne, 1985). However, there is no apparent conservation of the signal peptide amino acid sequence.

Membrane-associated and secreted proteins are of particular importance because among them are most of the mediators of cell-to-cell communication. In order to isolate such proteins, one can either choose a biochemical approach to directly isolate them or one can first isolate mRNAs, then search among these molecules for the clones that encode the desired proteins. The latter strategy is the preferred choice when the starting material is available in only very limited amounts. It is usually performed by subjecting a cDNA library to a signal sequence-trap screening procedure. Different methods have been described which consist of cloning a cDNA library upstream of a DNA fragment encoding a known membrane-anchored or secreted protein lacking its own signal sequence and initiation methionine codon. In-

frame-ligation of a cDNA fragment encoding a signal peptide should result in surface expression or secretion of the tester protein.

In one method (Tashiro et al., 1993), a cDNA library is cloned in front of a signal peptide-less IL-2 receptor α chain (Tac) sequence in an eukaryotic expression vector. After transfection of the plasmids into COS cells, the expression of the Tac-fusion protein on the cell surface is easily detectable by flow cytometry analysis using an anti-Tac antibody. Most of the positive clones contain cDNA sequences encoding a signal peptide preceded by an ATG codon. However, due to the detection limit, only pools of no more than 24 plasmids can be tested in one transfection reaction, which makes this method very laborious.

An attractive alternative method has been published (Klein et al., 1996). cDNA fragments are cloned upstream of a signal peptide-less invertase sequence in a yeast expression vector. Invertase is needed by yeast to grow on sucrose. A whole cDNA library can be cloned into this plasmid and transfected into an invertase-less yeast strain. In-frame-ligation of a cDNA fragment encoding a signal peptide allows expression and secretion of invertase and thus the selective growth of yeast on sucrose. This procedure is much less laborious than the previously described one, because it involves only a single transfection reaction and allows the screening of thousands of colonies by positive selection on sucrose plates. However, as already postulated by others (Kojima & Kitamura, 1999), we found that several mammalian signal sequences that were identified with the COS expression system were not functional in yeast. Some clones, which were functional in the mammalian but not in the yeast system, could direct invertase secretion only after the removal of the 5′ cDNA sequences flanking the translation start site. These data demonstrate that the yeast signal sequence-trap procedure allows the identification of only a subset of mammalian signal sequences that can be isolated with the COS system.

Materials and Methods

Cell Lines, Tumor Models and Cell Culture

The Lewis Lung Carcinoma (LLC) cell line was kindly obtained from R. Schwendener (Zürich), the ras-transformed rat fibroblast cell line FE-8 from R. Schäfer (Berlin). They were grown in DMEM (Gibco-BRL, Paisley, UK) containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. 1×10^6 FE-8 cells were injected s.c. into each flank of Balb/c nude mice (BRL, Fuellinsdorf, Switzerland). The animals were sacrificed when tumors reached a size of approximately 800 to 900 mm^3. 1.2×10^5 LLC cells were injected i.v. in C57BL/6 mice (BRL, Fuellinsdorf, Switzerland). The mice were sacrificed after 3 weeks.

Endothelial Cell Isolation

Primary endothelial cells were isolated from tumors and normal organs (lung, liver, kidney, and skin) according to Wyder et al. (2000).

RNA Preparation and cDNA Synthesis

Total RNA was isolated from primary endothelial cells with the RNeasy kit (QIAGEN, Hilden, Germany). cDNA synthesis was performed according to the protocol of the SMART PCR cDNA synthesis kit from Clontech (Palo Alto, CA; User manual PT304-1), using 1 μg of total RNA and a CapSwitch II oligonucleotide containing a DpnII site (underlined) (5′-AAGCAGTGGTAACAACGCAGAGATCGCGGG-3′).

Suppression Subtractive Hybridization (SSH) and Signal Sequence Trap (SST) in COS Cells

Subtractive hybridization was carried out using a modified version of the Clontech PCR-Select cDNA Subtraction kit protocol (Clontech; User manual PT1117-1) as described by Wyder et al. (2000). cDNA derived from endothelial cells of FE-8 and LLC tumor-penetrating blood vessels were used as tester, and either a mixture of cDNAs derived from endothelial cells of blood vessels in lung, liver, kidney, and skin (FE-8-subtraction) or only lung (LLC-subtraction) as driver. The subtracted cDNA sequences were cloned as DpnII fragments into the mammalian expression vector pTac and tested for the presence of signal peptide sequences using the signal sequence trap method in COS cells according to Wyder et al. (2000). Four of the isolated fragments, corresponding to the signal sequences of MCP-5, MCP-1, H-Cadherin and C10, were then analyzed together with two control signal peptides (T1 and Flk-1) for invertase secretion using the yeast SST method.

SST in Yeast Cells

In order to clone the cDNA fragments encoding the six signal peptides analyzed in this study into the NotI site of the yeast expression vector, they were first inserted into the BamHI site of modified pBluescript SK (pBS) vectors. pBS (Stratagene, La Jolla, CA) was modified by replacing the KpnI and SacI polylinker fragment with adaptor A, B or C generated by annealing 2.5 nmol of two different oligonucleotides in a 1:1 ratio in 10× PCR buffer (Gibco BRL, Paisley, UK) with 50 mM $MgCl_2$, heated at 95°C for 10 minutes and slowly allowed to cool down to 16°C. The oligonucleotide sequences were the following: 5′-CGCGGCCGC*GGATCC*ttgGCGGCCGCGAGCT-3′ and 5′-CGCGGCCGCcaa*GGATCC*GCGGCCGCGGTAC-3′ for adaptor A, 5′-CGCGGCCGC*GGATCC*cGCGGCCGCGAGCT-3′ and 5′-CGCGGCCGCg*GGATCC*GCGGCCGCGGTAC-3′ for adaptor B, 5′-CGCGGCCGC*GGATCC*agGCGGCCGCGAGCT-3′ and 5′-CGCGGCCGCct*GGATCC*GCGGCCGCGGTAC-3′ for adaptor C. All three adaptors contain two NotI restriction sites (underlined) separated by a BamHI site (italics) and they are identical except for the nucleotides marked by lower case letters which allow the cloning in all three reading frames. Moreover, all three adaptors contain 3′ overhangs that allowed cloning into the KpnI and SacI sites of pBS.

The cDNA inserts cloned into the modified pBS were excised with NotI and cloned into the yeast expression vector pRK18. This plasmid was obtained by excision from the cloning vector (λRK18) kindly provided by R.D. Klein (Genentech, San Francisco) by co-infecting BB4 bacteria with λRK18 and a M13 helper phage. Plasmid DNA was recovered from ampicillin-resistant colonies. Plasmid DNA containing the six signal peptides was prepared from *E. coli* with the QIAprep Spin Miniprep Kit (QIAGEN). 0.5 μg of each plasmid were transformed into competent cells of the yeast strain YT455 (*suc2*Δ*9, ade 2-101, ura 3-52*) using the lithium-acetate protocol, and Ura^+ transformants were selected by growth on solid medium containing glucose

for 2 to 3 days. They were replica-plated on agar plates containing sucrose and grown for 7 to 14 days at 30°C. Single yeast colonies were used to inoculate 4 ml of selective medium (minimal medium with 2% sucrose). Plasmid DNA was prepared from this culture as follows. The yeast was resuspended in 500 μl 1M sorbitol/50 mM EDTA and the cell wall was digested for one hour at 37°C with 100 μg lyticase (Sigma). The spheroplasts were resuspended in 500 μl TE and the plasma membrane was disrupted by the addition of 25 μl 20% SDS and incubation at 65°C for 30 minutes. 200 μl 5M potassium acetate were added and the mixture again incubated for one hour on ice. After isopropanol precipitation, traces of RNA were digested with 15 μg RNase A. The DNA was again precipitated and resuspended in 20 μl water. Half of the amount of DNA was used to amplify the insert by two rounds of PCR, using the primers 5′-CTATAATCCTTCCTCC-3′ and 5′-GTCTTGTGATTTGGC-3′ for the first PCR, 5′-CTAACGAAGCTTATC-3′ and 5′-ACCAAAGGTCTATCGC-3′ for the second nested PCR. All primers hybridize to sequences in pRK18 flanking the inserts. The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) and sequenced to verify the integrity of the sequence.

DpnII Clones

MCP-5 and MCP-1 sequences were isolated as DpnII fragments from a subtracted cDNA library derived from endothelial cells of an FE-8 tumor using the mammalian signal sequence trap system.

H-Cadherin and C10 sequences were similarly isolated as DpnII fragments from a subtracted cDNA library derived from endothelial cells of a LLC tumor.

The cDNA encoding the signal peptide of the T1 gene was amplified by PCR using pT1.11 (Klemenz, Hoffmann & Werenskiold, 1989) as a template and the primers 5′-CATTGCCATAGAGCGGCCGCAGCCATC-3′ and 5′-GATAAGTCGAGCGGCCGCGCTTTGGGGG-3′. The signal peptide sequence of the Flk-1 gene was amplified by PCR using the endothelial cell cDNA library from an LLC tumor as a template and the primers 5′-GTCTTGCGCTGCGGCCGCCATACCGCC-3′ and 5′-GATACTGTCACCGCGGCCGCATTCAG-3′. All primers contain a NotI site (underlined). After digestion with NotI the PCR products were cloned into the pRK18 yeast plasmid. In order to clone the T1 and the Flk-1 signal sequence into the BamHI site of pTac, the same fragments inserted into the pRK18 vector were PCR-amplified with the primers 5′-CATTGCCATAGAGGATCCTCAGCCATC-3′ and 5′-GATAAGTCGAGGATCCTCTTTGGGGG-3′, 5′-GTCTTGCGCTGCGGGATCCATACCGCC-3′ and 5′-GATACTGTCACCACCGCCGGATCCAG-3′, respectively. All primers contain a BamHI site (underlined).

The clones without 5′ and/or 3′ flanking regions were generated by PCR using the DpnII fragments in pRK18 vector as a template. In order to delete the 5′ flanking region, PCR-fragments were generated using the following upper primers 5′-CGGCGGCCGCGGATCC*GCCACC*atgattgacagacagagaatgg-3′ for T1, 5′-CGGCGGCCGCGGATCC*GCCACC*atggagagcaaggcgctgctag-3′ for Flk-1, 5′-CGGCGGCCGCGGATCC*GCCACC*atgcagccgagaactccgctc-3′ for H-Cadherin and 5′-CGGCGGCCGCGGATCC*GCCACC*atgagaaactccaagactgcc-3′ for C10. They hybridize to the first 21 bp of the signal sequence (marked in lower case letters) and contain a NotI site and a BamHI site (underlined) as well as a Kozak sequence (italics). The lower primer hybridized to a vector sequence (5′-ACCAAAGGTCTATCGC-3′). Similarly, in order to delete the 3′ flanking region, the following lower primers were used: 5′-TTGCGGCCGCCGGATCCACTGCCctccgtaactgtcaaataca-3′ for T1, 5′-TTGCGGCCGCCGGATCCAGAGGCggctcgggtctccacgcaga-3′ for Flk-1, 5′-TTGCGGCCGCCGGATCCATCATCtgcagacgtgaccaggagca-3′ for H-Cadherin, and 5′-TTGCGGCCGCCGGATCCGAGGCCagcctgggacccaaggacag-3′ for C10. They hybridize to the last 20 bp of the signal sequence (marked in lower case letters) and contain a NotI site and a BamHI site (underlined). The upper primer hybridized to a vector sequence (5′-CTATAATCCTTCCTCC-3′). In order to delete both flanking regions, the upper and lower sequence-specific primers listed above were used for the amplification of each of the four templates.

All PCR products were cloned in pGEM-T (Promega, Madison, USA) and the inserts were sequenced. The NotI fragments were excised and cloned into pRK18.

Abbreviations

ER, endoplasmic reticulum; FE-8, ras-transformed rat fibroblast cell line; IL-2, interleukine 2; LLC, Lewis Lung Carcinoma; MCP, monocyte chemotactic protein; s.c., subcutaneous; SRP, signal recognition particle; SSH, suppression subtractive hybridization; SST, signal sequence trap; Tac, interleukine 2 receptor α chain.

Results and Discussion

In a seach for membrane-anchored proteins, which are overexpressed on the surface of endothelial cells lining tumor penetrating blood vessels, we have generated subtracted cDNA libraries by the SSH method. cDNAs derived from endothelial cells of FE-8 and Lewis Lung Carcinoma tumors were used as tester and cDNA derived from a mix of lung, liver, kidney and skin endothelial cells or only lung endothelial cells as driver, respectively. These libraries were cloned into the pTac vector encoding IL-2 receptor α chain (Tac) lacking its own signal sequence and start codon. Pools of 24 plasmids were transfected into COS cells. Cells expressing Tac at their surface were detected by flow-cytometry analysis using an anti-Tac antibody. Four pools of plasmids were identified that directed surface expression of Tac. Subsequently, individual clones were tested and four different plasmids were found that allow for the synthesis and membrane anchorage of Tac (Fig. 1). Sequence analysis of these plasmids revealed that the four inserts encode signal peptide sequences of known proteins, one membrane bound (H-cadherin) and three secreted (C10, MCP-1 and MCP-5) (Fig. 2). As positive controls the signal peptide sequences of the two receptors Flk-1 and T1 were cloned into pTac and the resulting plasmids subjected to the same analysis. The proportion of Tac-positive cells varied between 7 and 29% and reflects the efficiency of COS cell transfection.

In order to compare the two signal sequence-trap methods described above, the inserts of these six plasmids were subsequently cloned in-frame into the yeast vector pRK18 containing the signal sequence-less invertase gene. The plasmids were transfected into invertase-deficient yeast cells and the transfectants were first grown on glucose containing agar plates. Afterwards, they were transferred onto plates which contained sucrose instead of glucose as the major carbon source. Due

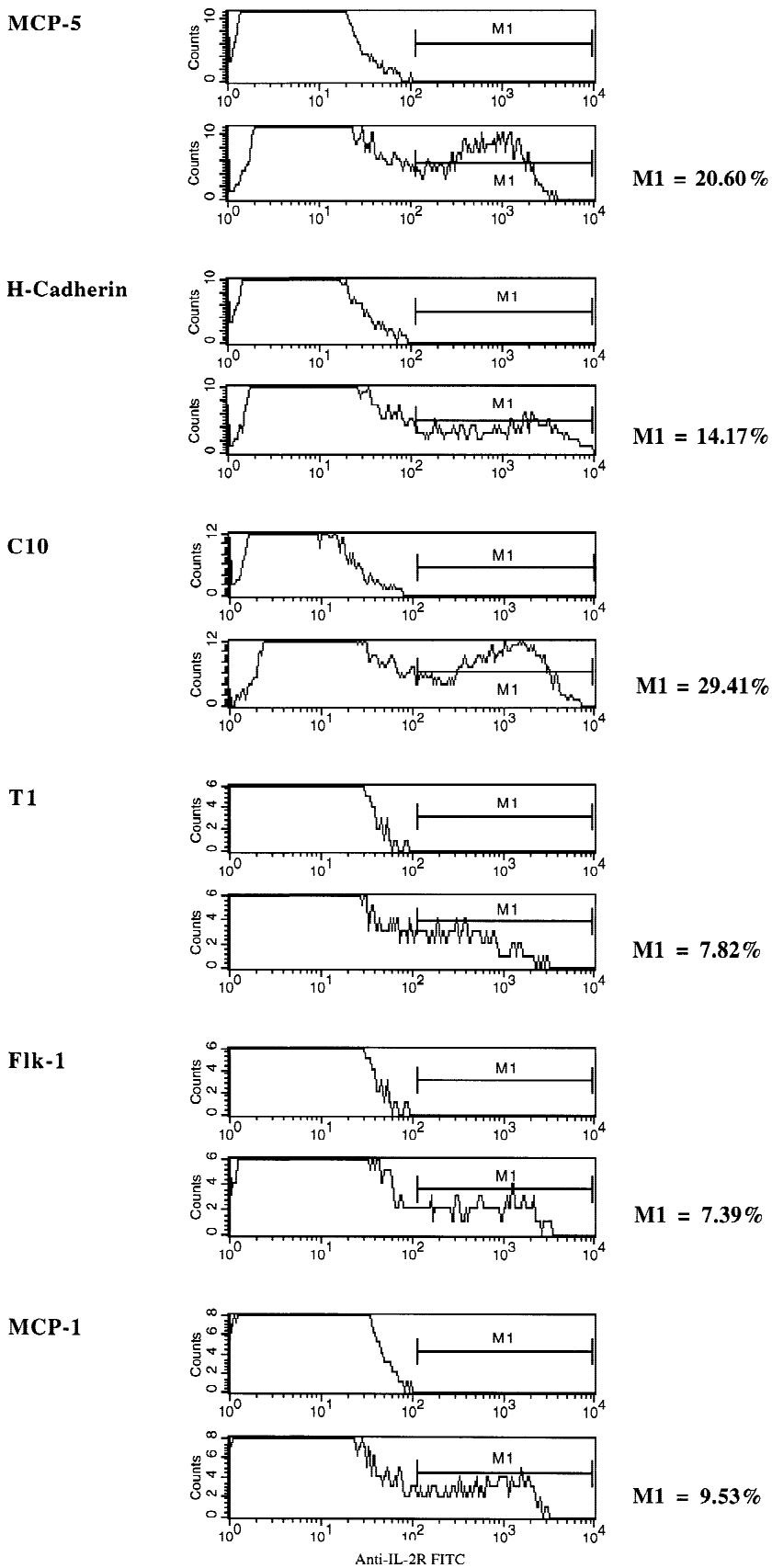

Fig. 1. Flow-cytometric analyses of COS cells that were transfected with the six plasmids encoding fusions of signal peptides with Tac. Cells were transfected with vector only (upper panel) or with the six constructs (lower panel) and stained with an FITC-modified anti-Tac antibody. The major peak representing nontransfected cells was cut off to better visualize the Tac-positive cells. M1 indicates the gate that was set to distinguish between positive and negative cells.

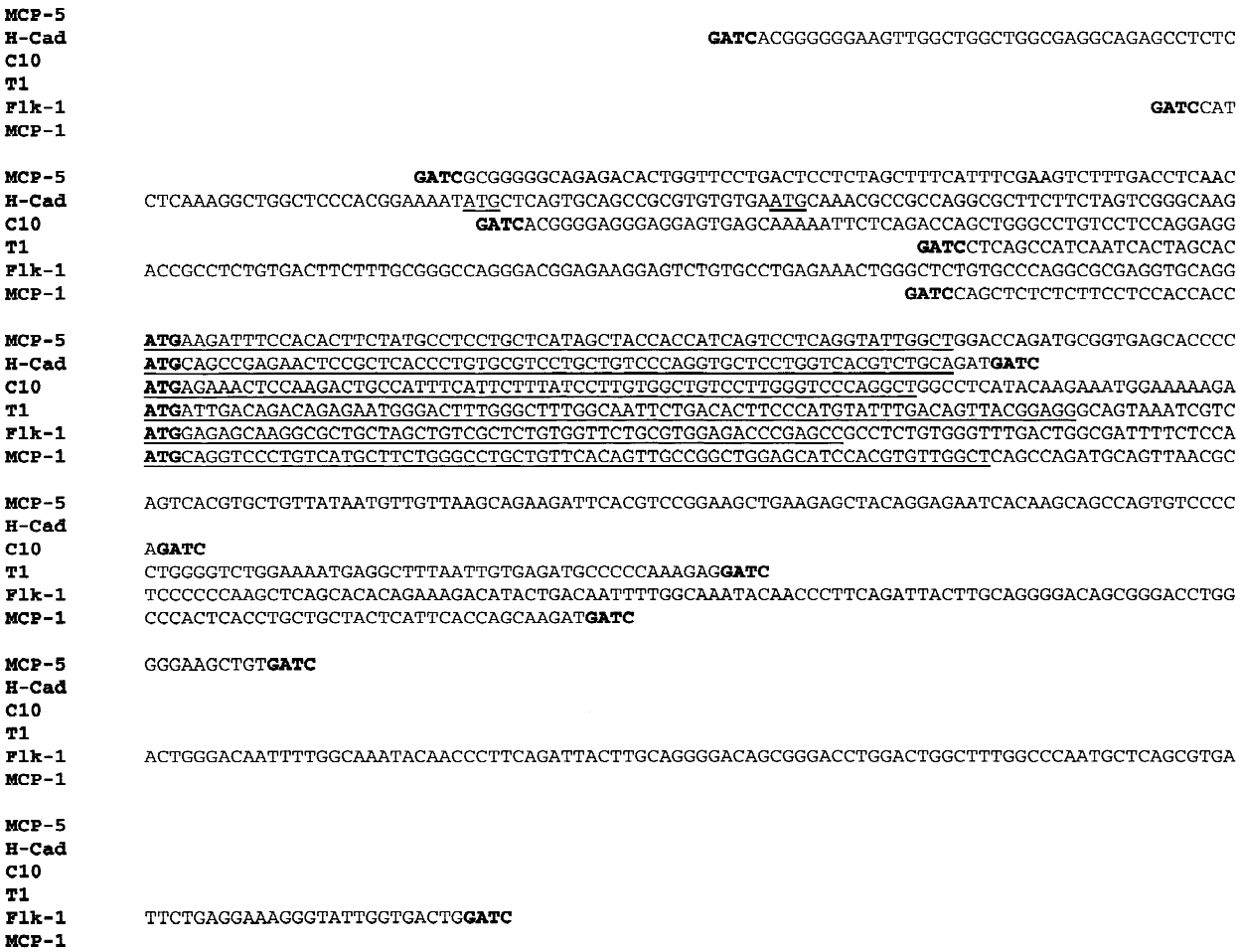

Fig. 2. Sequences of the six clones encoding the signal peptides tested with the two signal sequence-trap methods. The sequences are aligned at the ATG initiation codon. Upstream ATG triplets in H-Cadherin and signal peptide sequences are underlined, DpnII sites and ATG initiation codons are indicated in bold.

to a deletion in the endogenous invertase gene, only those cells which secrete invertase encoded by the pRK18 vector can grow on sucrose plates. All transfectants grew well on glucose-containing plates. However, only those containing the signal sequences of T1, MCP-1, and MCP-5 grew on sucrose plates (Table 1). To exclude the possibility that the inability to grow on sucrose was due to some changes of the DNA sequence, plasmids from several colonies of each transfectant growing on glucose plates were isolated and subjected to sequence analysis. No sequence rearrangements or other mutations were detected.

Table 1. Functionality of murine signal peptides in the mammalian and yeast signal sequence trap assay

Signal peptide	IL-2R Expression in COS	SUC2 Expression in yeast
MCP-5	YES	YES
H-Cadherin	YES	NO
C10	YES	NO
T1	YES	YES
Flk-1	YES	NO
MCP-1	YES	YES

One possible explanation for the observation that only three of six signal peptides from mouse proteins function in yeast could be that the secretory machineries of different organisms prefer different signal sequences, i.e., a signal sequence of higher eukaryotes might not necessarily work in *S. cerevisiae*, and vice versa. In fact, although it was demonstrated that several signal sequences from one organism could function in another (Muller et al., 1982; Sleep, Belfield & Goodey, 1990; Talmadge, Stahl & Gilbert, 1980; Yost, Hedgpeth & Lingappa, 1983) it has also been shown that this is not universally true. For example, the yeast carboxypeptidase Y is inserted into the ER-membrane in *S. cerevisiae* but not in COS-1 cells, unless the signal sequence is

Table 2. Influence of DNA sequences flanking the signal peptides on invertase secretion in yeast

Signal peptide	—SS—	—SS	SS—	SS
H-Cadherin	NO	NO	YES	NO
C10	NO	NO	YES	YES
T1	YES	YES	YES	YES
Flk-1	NO	NO	NO	NO

The 3′ and/or 5′ sequences flanking the signal peptides were removed from those cDNA fragments that did not allow invertase secretion as well as from the T1 sequence, used as a positive control.

SS signal sequence

— normal sequence upstream/downstream of the SS

either mutated to increase its hydrophobicity or replaced with that of influenza virus hemagglutinin (Bird, Gething & Sambrook, 1987). Likewise, a hybrid protein consisting of the bovine prolactin signal sequence fused to the mature sequence of yeast invertase is only very inefficiently secreted in *S. cerevisiae* unless an amino acid in the signal peptide is replaced by a residue that favors an α-helical conformation. In addition, truncation of the 5′ sequence flanking the ATG initiation codon in the same hybrid protein resulted in increased invertase secretion (Ngsee & Smith, 1990). This observation led us to test whether the sequences flanking the signal peptides might negatively influence the secretion in yeast. Expression plasmids were constructed in which all mouse sequences either upstream or downstream of the signal peptide, or both, were deleted. The consensus GCCACC Kozak sequence was introduced upstream of the ATG in those cases where the 5′ flanking region was deleted to ensure efficient translation initiation. Two of the sequences (H-Cadherin and C10), which did not allow invertase secretion in the previous experiment, could enable growth on sucrose after deletion of the 5′ region upstream of the signal sequence. However, the Flk-1 signal sequence lacking the 5′ region was still inactive. Truncation of the 3′ flanking region had no positive effect for all the clones tested. Surprisingly, the H-Cadherin signal peptide lost its capability to mediate invertase secretion if both 5′ and 3′ regions were eliminated (Table 2).

The reason why the 5′ sequence flanking the initiation codon exerts a negative effect on invertase secretion for the H-Cadherin and C10 signal peptide is not clear. Since the deleted sequences are not part of the coding regions, the proteins encoded by the deletion constructs do not differ from the original ones. Therefore, we assume that the 5′ untranslated regions of the murine H-Cadherin and C10 mRNA negatively influence translation in yeast. However, if so, this negative effect must be very strong since it was reported that as little as 0.6% of the wild-type invertase activity is sufficient to allow growth on sucrose medium, and 4.7% of the wild-type activity results in growth rates indistinguishable from those supported by a normal invertase protein (Klein et al., 1996).

We assume that the 5′ untranslated regions of H-Cadherin and C10 contain sequence elements that might inhibit translation in a cell type-specific way, i.e., in yeast but not in COS cells. Such an element could be a hairpin structure that impedes the binding or the scanning of the preinitiation complex to the mRNA. The 5′ untranslated region of ornithine decarboxylase (Manzella & Blackshear, 1990) and of transforming growth factor β1 (Kim et al., 1992) mRNA form secondary structures that inhibit translation in some cells but not in others. Another element could be one of the two ATG triplets found in the H-Cadherin sequence upstream of the open reading frame (Fig. 2, underlined). These ATG triplets might be recognized as translation initiation codons in yeast but not in COS cells giving rise to aberrant translation products and thus preventing initiation at the ATG of the H-Cadherin open reading frame. The recognition of the initiator methionine was indeed shown to be cell-type specific. Although some features of the vertebrate consensus sequence for translation initiation are shared by yeast, similarities are not evident over the whole context (Kozak, 1989). As shown by others, translation of S-adenosylmethionine decarboxylase mRNA is inhibited by an upstream ATG codon in lymphoid but not in non-lymphoid cells (Hill & Morris, 1992). Likewise, the BTEB transcription-factor mRNA is translated only in nerve cells in the brain, in spermatocytes in the testis, and in a neuroblastoma cell line, although other organs and cell lines examined were found to express the mRNA (Imataka et al., 1994).

The 3′ flanking region immediately following the signal peptide influenced invertase secretion in one case, H-Cadherin, where its absence, together with the deletion of the 5′ flanking sequence, abolished protein translocation. We postulate that the 3′ flanking region of the H-Cadherin clone but not of T1 and C10 is necessary to maintain the nascent protein in a conformation that allows its translocation to the membrane of the ER. The 3′ region following the signal peptide was indeed found to

be particularly important for protein secretion. It acts like a spacer, an unstructured region that guarantees accessibility of signal sequences to the export machinery. It was demonstrated that by cloning a signal sequence in front of different open reading frames, the corresponding amino-acid sequences from the mature portion of the protein greatly influence the efficiency of the export (Ferenci & Silhavy, 1987). Other found that a signal peptide could promote the export of a protein only if an arginine residue immediately following the signal peptide was removed (von Heijne, 1990). The importance of the amino acids adjacent to the signal peptide probably relies on the fact that a precursor protein is translocated to the ER only if partially unfolded (von Heijne, 1990). The first critical step in translocation, the binding of the SRP particle to the signal sequence, can occur only during a short time window. If translation continues, the signal sequence is eventually sequestered as the protein folds (Bird, Gething & Sambrook, 1990). The duration of ribosome pausing and the strength of the interaction between the nascent protein chain and cytoplasmic chaperones determine how long a polypeptide remains in a translocation-competent state (Arnold et al., 1998). Thus, it is possible that some mammalian signal sequences are not functional in the yeast signal sequence-trap method because in combination with the C-terminal sequence of invertase they fold quickly, preventing a prolonged interaction with the secretory machinery. In contrast, the same sequences could direct the translocation of the IL-2 receptor because in the mammalian system the mature portion of the protein might fold slower or not at all, thus allowing the signal sequences more time to bind to the SRP particle. This prolonged interaction could be particularly important for weak signal peptides.

This theory suggests that signal sequences have been optimized not only for a particular cell-type but also for a particular protein. Thus, some signal peptides might be functional if fused to certain proteins but not to others, irrespective of the organism that is used to monitor their functionality.

In conclusion, this work demonstrates that, although many features of the secretory apparatus are shared by all species (Emr et al., 1984; Hitzeman et al., 1983), and the specificity involved in signal sequence recognition by the SRP particle seems to be very low (Kaiser et al., 1987), signal sequences are not just short, hydrophobic, N-terminal peptides that can be interchanged between different proteins or different organisms. A more complex mechanism seems to regulate the secretory process.

For practical purposes, the results presented in this report indicate that due to various reasons, only a fraction of the signal peptides isolated by the mammalian signal sequence-trap method are functional in yeast. This is inconsistent with the hypothesis of Klein et al. (1996) that even an inefficient mammalian signal peptide should be detectable with the yeast system.

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