A gene truncation strategy generating N- and C-terminal deletion variants of proteins for functional studies: mapping of the Sec1p binding domain in yeast Mso1p by a Mu *in vitro* transposition-based approach

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ABSTRACT

Bacteriophage Mu *in vitro* transposition constitutes a versatile tool in molecular biology, with applications ranging from engineering of single genes or proteins to modification of genome segments or entire genomes. A new strategy was devised on the basis of Mu transposition that via a few manipulation steps simultaneously generates a nested set of gene constructions encoding deletion variants of proteins. C-terminal deletions are produced using a mini-Mu transposon that carries translation stop signals close to each transposon end. Similarly, N-terminal deletions are generated using a transposon with appropriate restriction sites, which allows deletion of the 5′-distal part of the gene. As a proof of principle, we produced a set of plasmid constructions encoding both C- and N-terminally truncated variants of yeast Mso1p and mapped its Sec1p-interacting region. The most important amino acids for the interaction in Mso1p are located between residues T46 and N78, with some weaker interactions possibly within the region E79–N105. This general-purpose gene truncation strategy is highly efficient and produces, in a single reaction series, a comprehensive repertoire of gene constructions encoding protein deletion variants, valuable in many types of functional studies. Importantly, the methodology is applicable to any protein-encoding gene cloned in an appropriate vector.

INTRODUCTION

DNA transposition-based methods have been used to modify genes, genomes and proteins in both prokaryotic and eukaryotic organisms. While earlier applications utilized *in vivo* transposition (1–3), a substantially simplified *in vitro* version with purified components is currently available for several transposons (4,5). The main advantage of using transposons in genetic engineering applications is their ability to integrate efficiently and relatively randomly into target DNA. However, the efficiency and degree of integration randomness vary considerably among different transposons (6). By performing DNA transposition with a plasmid target *in vitro*, large pools of insertion mutants can be produced in a single, simple reaction series. Subsequent manipulation steps for a variety of applications involve only standard molecular biology techniques. Overall, a number of related strategies constitute a multipurpose tool set for various types of molecular and genomic studies (4,5).

One of the most versatile DNA transposition methods is an *in vitro* system derived from bacteriophage Mu transposition (7,8). The minimal Mu transposition reaction requires only a simple reaction buffer and three purified macromolecular components: transposon DNA, transposase protein MuA and target DNA, the latter typically being a gene of interest cloned in an appropriate plasmid. The Mu *in vitro* reaction is highly efficient with relatively low target site selectivity (8–10). These properties make the Mu *in vitro* reaction ideal for generating comprehensive libraries of mutant DNA molecules for a variety of molecular biology applications (11–17). Owing to the malleability of Mu transposons, they can be modified to contain any DNA between specific transposon ends. Typical

constituents include selectable markers, reporter genes, promoters, and in many cases a gene of interest (18).

Sets of protein deletion variants are commonly used to map regions of functionality, particularly those involved in interactions between two distinct proteins (19). Deletion plasmid constructions encoding the protein of interest can be produced by exonuclease digestion or restriction digestions; however, exonuclease reactions are difficult to control and restriction enzymes are limited by the number of restriction sites in the target DNA (20,21). Some strategies are based on PCR, which is susceptible to errors during gene amplification and requires rational design for each deletion clone. In practice, constructions need to be manufactured individually. Accordingly, PCR-based techniques are laborious if the goal is to map the region of interaction at reasonable resolution, particularly in studies of large proteins. Thus, a technique that simultaneously and easily produces a library of deletion constructions would be advantageous for the analysis of protein structure–function relationships. In principle, emerging transposon-based strategies should fulfill the demands, and progress towards this end has been made recently by the use of Tn5 intramolecular transposition *in vitro* (22).

We tested whether the Mu *in vitro* transposition system could be adapted to generate deletion constructions of protein deletion variants. To test the performance of the strategy, we prepared a library of deletion constructions using the yeast *Saccharomyces cerevisiae MSO1* gene as the target. The encoded Mso1p is implicated in the transport and fusion of vesicles destined for the plasma membrane (exocytosis) and has previously been shown to physically interact with yeast Sec1p (23). Sec1p is required for exocytosis (24,25); however, its function in this process is still poorly understood, as is the molecular basis of its interaction with Mso1p. To investigate the interaction between Mso1p and Sec1p, several N- and C-terminally truncated Mso1p deletion variants were analyzed for their ability to interact with Sec1p. The results indicate that the interacting region in Mso1p is located in the N-terminus, primarily between residues T46 and N78 with possible interactions in the region E79–N105. This study demonstrates that, in a single reaction series, the strategy efficiently produces a library of deletion variants that can be further utilized for protein–protein interaction studies.

MATERIALS AND METHODS

Microbes, reagents and DNA techniques

Escherichia coli strains DH5α (Invitrogen) and BL21(DE3) (26) were cultured in Luria broth (27) supplemented with ampicillin (Ap, 100 μg/ml) and chloramphenicol (Cm, 10 μg/ml), when required. Yeast strain H2658 *(MATa ura3-52 leu2-3, 112 Δmso1::hphMX4*; M. Knop, K.J. Miller, M. Mazza, D. Feng, M. Weber, S. Keränen and J. Jäntti, submitted) transformed with plasmid B578 [YEpSEC1aU, (28)] lacks the *MSO1* gene but overexpresses *SEC1*. Yeast cultures were grown in synthetic complete medium lacking uracil (29). MuA transposase was purified in collaboration with Finnzymes (Espoo, Finland) as described (11,30). Restriction enzymes (New England Biolabs) and T4 DNA ligase (Promega) were used as recommended by the suppliers. Complete Mini EDTA-free protease inhibitor cocktail tablets

and AEBSF (4-(2-aminoethyl)benzene sulfonyl fluoride) were from Roche. Imidazole was from Sigma and ProBond protein purification resin (Ni-NTA agarose) from Invitrogen. GelCode Blue stain was from Pierce, Bradford protein assay reagents from Bio-Rad, and ECL reagents from Amersham. Plasmid DNA was prepared using plasmid DNA isolation kits from Qiagen. Standard DNA techniques were performed as described (27). DNA sequencing was performed with the BigDye terminator cycle sequencing kit (Applied Biosystems) and an ABI 377 XL sequencer (Applied Biosystems).

Plasmids and transposons

Plasmid pHis$_6$-MSO1 (23) contains the 633 bp yeast *MSO1* gene cloned in the SmaI site of pGAT-4 (31). This vector is especially suitable for the gene truncation strategy: it carries, 5′ from the multiple cloning site, a NotI site required to produce C-terminal fragments and a poly-His-encoding region for easy purification of the protein fragments. The two transposons used in this study (Figure 1) are derivatives of the cat-Mu transposon (11) and were constructed using standard PCR and cloning techniques (27). The cat-Mu(NotI) transposon has been described previously (16). The cat-Mu(Stop) transposon is similar to cat-Mu(NotI), but instead of NotI restriction sites it contains three translation stop signals close to each transposon end. Transposons were released from their respective carrier plasmids by BglII digestion and purified by anion exchange chromatography as described (11).

In vitro transposition reaction

The standard *in vitro* transposition reaction (25 μl) contained 180 ng transposon DNA as a donor, 500 ng plasmid pHis$_6$-MSO1 as a target, 0.22 μg MuA transposase, 25 mM Tris–HCl, pH 8.0, 100 μg/ml BSA, 15% (w/v) glycerol,

Mu wild type R-end

```
GATCTGAAG CGGCGCACGAAAAACGCGAAAG ...
     ACTTC GCCGCGTGCTTTTTGCGCTTTC ...
   ↑
```

Modified Mu R-end in cat-Mu(Stop)

```
        Stop  Stop  Stop
GATCTGATT GATTGAACGAAAAACGCGAAAG ...
    ACTAA CTAACTTGCTTTTTGCGCTTTC ...
  ↑       * *  * * * *   *
```

Modified Mu R-end in cat-Mu(NotI)

```
         NotI
GATCTGCGG CCGCGCACGAAAAACGCGAAAG ...
    ACGCC GGCGCGTGCTTTTTGCGCTTTC ...
  ↑       * *      *
```

Figure 1. Comparison between wild-type and modified Mu R-end sequences of the two transposons used in this study. The depicted precut configuration with a 4 nt 5′-overhang results from BglII digestion, which is used for transposon isolation. The arrows indicate the endmost 3′ nucleotides, and altered base pairs are labeled with asterisks. The rectangles indicate the endmost MuA transposase binding sites.

0.05% (w/v) Triton X-100, 126 mM NaCl and 10 mM MgCl$_2$. The reaction was carried out at 30°C for 4 h.

Generation of plasmid pools encoding Mso1p deletion variants

N-terminal fragments. Ten *in vitro* transposition reactions were performed otherwise as the standard reaction but containing 720 ng transposon cat-Mu(Stop) donor and 0.88 μg MuA transposase. The reactions were pooled, extracted with phenol and subsequently with chloroform, ethanol precipitated, and resuspended in 30 μl H$_2$O. Several aliquots (1 μl) were electroporated (Bio-Rad Gene Pulser II with 25 μF capacitance, 1.8 kV voltage and 200 Ω resistance, in 0.1 cm cuvettes) into DH5α competent cells (25 μl) prepared as described (32). Transposon-containing plasmid clones were selected on LB-Ap-Cm plates. Approximately 1×10^5 colonies were pooled and grown in LB-Ap-Cm medium at 37°C for 3 h. Plasmid DNA from the pool was isolated, digested with XhoI and HindIII, and subjected to preparative electrophoresis on a 0.8% SeaKemGTG (Cambrex) agarose gel in TAE buffer (27). The 1.9 kb DNA fragment pool, corresponding to transposon insertions into the *MSO1* DNA fragment, was isolated with the QIAquick Gel Extraction kit (Qiagen) and ligated into the vector segment of pHis$_6$-MSO1 digested with XhoI and HindIII. The ligation mixture was electroporated into DH5α cells, transposon-containing clones were selected on LB-Ap-Cm plates, and plasmid DNA was prepared from $\sim 1 \times 10^4$ colonies.

C-terminal fragments. Six standard *in vitro* transposition reactions, with cat-Mu(NotI) as a donor, were pooled, extracted with phenol and subsequently with chloroform, ethanol precipitated, and resuspended in 20 μl water. Electroporation was carried out as above, and plasmid DNA was prepared from a pool of $\sim 6 \times 10^5$ colonies. *MSO1* fragments that carried transposon insertions were cloned into a clean vector backbone as described above, and $\sim 5 \times 10^4$ colonies were pooled. Most of the transposon DNA and the 5′-distal part of the *MSO1* gene were then excised from the plasmid pool by a cleavage with NotI, followed by isolation of the plasmid backbone by preparative electrophoresis as above and recircularization by ligation at low DNA concentration (~ 1 ng/μl). Ligated plasmids were electroporated into DH5α cells as above and selected on LB-Ap plates. The final C-terminal deletion mutant plasmid library contained $\sim 3 \times 10^4$ colonies.

Verification of deletion clones and protein expression

Individual deletion plasmid clones were subjected to restriction analysis (Figure 3A and B) with BamHI (N-terminal fragments) or NotI and HindIII (C-terminal fragments), and the deletion joint sequences were determined by sequencing (Figure 3C). For protein expression, individual plasmid clones (Figure 4A) were introduced into the *E.coli* strain BL21(DE3). Cells were grown in LB-Ap medium at 37°C to an OD$_{600}$ of 0.5, and Mso1p expression was induced for 3 h by the addition of 1 mM IPTG (Fermentas).

Purification of the Mso1p deletion variants

A cell pellet from a 50 ml culture was resuspended in 3 ml urea buffer (100 mM NaH$_2$PO$_4$, 10 mM Tris–HCl and 8 M urea, pH 6.3) containing Complete Mini EDTA-free protease inhibitor cocktail tablets (2 tablets per 10 ml buffer) and lysed on ice by sonication with an MSE Soniprep 150 ultrasonic homogenizer (eight times for 20 s with 30 s intervals). Cell debris was removed by centrifugation at 10 000 *g* for 10 min. The supernatant was mixed with 200 μl of ProBond resin, and the Mso1p was allowed to bind at 4°C for 1 h. The resin was collected by centrifugation at 600 *g* for 3 min at 4°C, washed twice with 10 ml urea buffer, twice with 10 ml imidazole buffer [50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole and 0.05% (v/v) Tween-20, pH 8.0], and once with 10 ml buffer A [100 mM NaH$_2$PO$_4$, 500 mM NaCl and 10% (v/v) glycerol, pH 6.0]. All washes were performed by gently rotating the tubes at 4°C for 10 min. Each resin-bound Mso1p variant was stored in buffer A at 4°C as a 50% slurry. Following boiling in sample buffer (27), the protein content of these preparations was analyzed on 15% SDS–PAGE gels stained with GelCode Blue (Figure 4B).

Binding assay

The interaction of Sec1p with Mso1p variants was analyzed using a Sec1p pull-down assay. Initially, a lysate was prepared from the yeast strain H2658+B578 as follows. Cells were grown in a 600 ml culture to OD$_{600}$ of 1.5, harvested by centrifugation and resuspended in 6 ml MOPS lysis buffer (40 mM MOPS, pH 6.8, 100 mM NaCl, 4 mM AEBSF and 10 mM NaN$_3$) containing protease inhibitors (1 cocktail tablet per 25 ml buffer). Cells were broken with a Bead Beater (Biospec Products) and zirconia/silica beads using six consecutive 45 s treatments at 4°C with 1 min intervals on ice. Triton X-100 (10%, v/v) was added to a final concentration of 1%, and the suspension was incubated on ice for 30 min. The cell debris was removed by centrifugation, and the total protein concentration was determined using the Bradford assay. Resin-bound Mso1p variants (described above; equivalent to ~ 30 μg of Mso1p) were incubated for 1 h at 4°C with the yeast lysate (6 mg total protein) supplemented with 80 mM imidazole. The resin was washed three times for 20 min with MOPS lysis buffer supplemented with 1% Triton X-100 and 80 mM imidazole, and finally rinsed with the same buffer lacking imidazole. The proteins were separated using 10% SDS–PAGE and transferred onto nitrocellulose filters that were then incubated with a polyclonal antibody (1:3000 dilution) against Sec1p (23). Immunoreactive bands indicating Mso1p–Sec1p interaction were detected with the ECL system using Kodak MR-1 film.

RESULTS

Experimental outline

We developed a Mu *in vitro* transposition-based strategy that generates a series of protein deletion variants. To illustrate the method, we used the yeast *MSO1* gene as a target for deletion mutagenesis and subsequently defined the region in Mso1p that interacts with Sec1p. Two deletion mutant libraries were produced, one encoding N-terminal fragments and the other encoding C-terminal fragments of Mso1p. This was accomplished by using custom-designed artificial transposons cat-Mu(Stop) and cat-Mu(NotI), respectively. These two transposons are derivatives of the cat-Mu minitransposon (8,11) and contain defined modifications within the Mu R-end
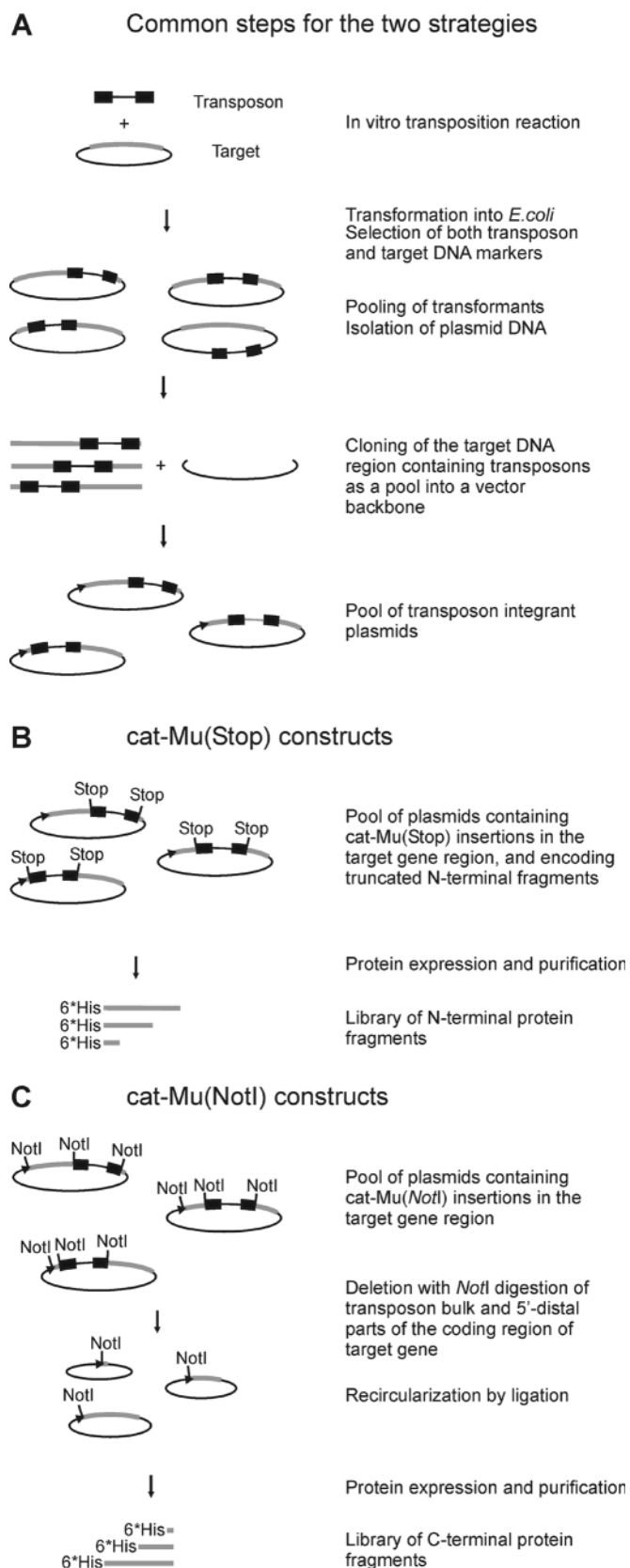
Figure 2. (**A**) Common initial steps of constructing a transposon insertion library for a gene of interest [(39), this study]. (**B** and **C**) Additional steps of constructing the deletion mutant libraries of N- and C-terminal protein fragments with cat-Mu(Stop) and cat-Mu(NotI) transposons, respectively.

sequences (Figure 1). Cat-Mu(Stop) carries three translation stop codons near each transposon terminus, and cat-Mu(NotI) contains a NotI site close to each transposon end. As a selectable marker, these transposons employ the commonly used chloramphenicol acetyl transferase (*cat*) gene.

The reaction series for the generation of the two libraries followed similar outlines (Figure 2). Initially, the plasmid containing the *MSO1* gene was used as a target in an *in vitro* transposition reaction with the modified mini-Mu transposon as a donor. The resulting plasmids were pooled, and the transposon-containing *MSO1* from each pool was then subcloned into a 'clean' plasmid backbone to limit the transposon insertion sites to those within the *MSO1* region (Figure 2A). At this stage, the constructions for N-terminal fragments were ready for screening and protein expression (Figure 2B). Before screening, the constructions encoding C-terminal fragments were further modified by NotI digestion and plasmid recircularization by ligation, thus eliminating the transposon core together with the 5′-distal coding region of *MSO1* (Figure 2C).

Generation of plasmid pools encoding truncated Mso1p variants

Our gene truncation strategy using *MSO1* as a target yielded $\sim 1 \times 10^4$ and $\sim 3 \times 10^4$ clones for the libraries encoding N-terminal and C-terminal protein variants, respectively. Fifteen randomly selected clones from each library were subjected to restriction analysis (Figure 3A and B), in which a variation in length of a particular restriction fragment indicates a successfully generated deletion series. Indeed, such variability was observed, suggesting that each clone represented a unique deletion variant. A number of deletion clones (potentially encoding 23 N-terminal fragments and 45 C-terminal fragments) were then fine-mapped by DNA sequencing to identify the exact location of their respective deletion joints. Among the clones mapped, we detected 20 unique deletion joint positions for N-terminal fragments and 35 for C-terminal fragments. Their distribution along the *MSO1* gene is shown in Figure 3C. Among the 68 clones analyzed, we identified 46 insertion sites once, 8 sites twice and 2 sites three times, evidently reflecting the relatively weak target site selectivity of Mu transposition. All of the sequenced clones contained faultless deletion joint sequences, indicating a high degree of accuracy during the manipulation steps. All the clones encoding N-terminal Mso1p variants were useful for protein expression, as the engineered stop codons terminated Mso1p translation in all three reading frames. Theoretically, however, for *MSO1* 5′-distal deletions, a proper reading frame is expected to be retained in one out of three clones, implying that only one-third of the clones encode a useful C-terminal protein variant (see Discussion). In our collection of the 5′-distal deletion clones, 12 of 45 (27%) retained a proper reading frame for the expression of Mso1p variants, in reasonable agreement with the 33% theoretical yield.

Mso1p–Sec1p binding assay

For the Sec1p binding assay, we purified a representative set of Mso1p deletion variants (Table 1 and Figure 4A) using metal affinity chromatography, as all of the protein fragments contained a His tag at the N-terminus. Using the same standard
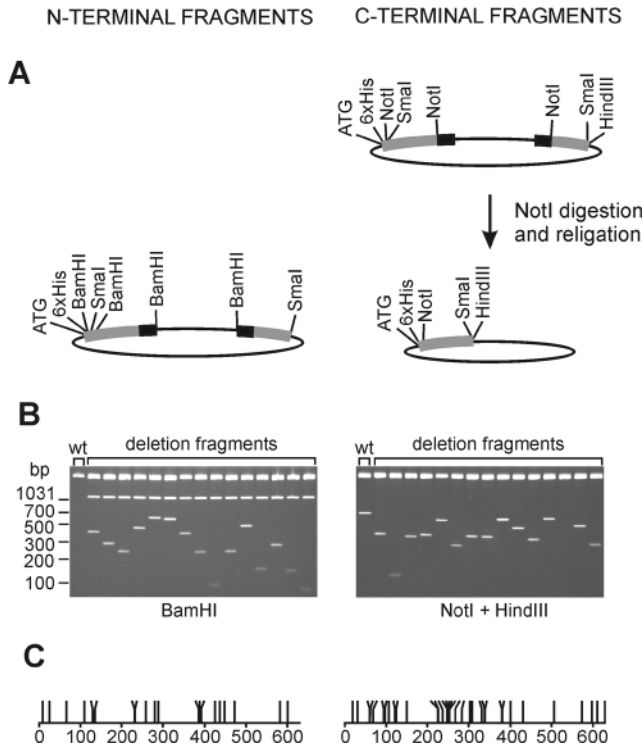
Figure 3. Analysis of individual deletion constructions at the DNA level. Individual plasmid clones were isolated for the analysis. Details of generating the N- and C-terminal fragments are shown on the left and right, respectively. (**A**) Deletion constructions. The target plasmid contains the *MSO1* gene inserted into the SmaI site of pGAT-4. The transposon is shown in black (transposon ends as thick lines) within the protein coding region (thick grey line). For C-terminal fragments, the construction is shown before and after the excision of the transposon and the 5′-coding region with NotI digestion and religation. (**B**) A random set of plasmids analyzed to demonstrate the variability of the insertion sites. Fifteen constructions for N-terminal fragments were digested with BamHI. A diagnostic fragment (0–650 bp) is produced depending on the transposon insertion site. Also, 15 plasmid clones encoding C-terminal fragments were digested with NotI and HindIII; a diagnostic fragment is 50–700 bp in length. Samples were run in a 2% MetaPhor agarose gel at 70 V for 110 min. (**C**) The distribution of transposon insertion sites in randomly selected samples as determined by DNA sequencing. The *MSO1* wt sequence is depicted as a line from 0 to 633 bp, and the insertion sites are indicated (23 N- and 45 C-terminal constructions).

protocol (see Materials and Methods), we purified the wild-type protein as well as seven N-terminal and five C-terminal Mso1p fragments. We obtained a good quality preparation of each protein variant, as verified by SDS–PAGE (Figure 4B).

Finally, to locate the Sec1p-interacting region in Mso1p, we analyzed the ability of the Mso1p deletion fragments to bind Sec1p. The His-tagged Mso1p fragments were bound to Ni-NTA agarose resin via their His tags, and the resin was incubated with soluble fraction of yeast lysate containing over-expressed Sec1p. The resin was collected, washed and the presence or absence of Sec1p bound to Mso1p fragments was analyzed by immunoblotting using an antiserum to Sec1p (Figure 4C). Most of the N-terminal Mso1p fragments, including the fragment containing amino acids 1–78, bound Sec1p efficiently; and only the shortest fragment (1–46) failed to bind. Two of the longest C-terminal fragments (9–210 and 35–210) also bound Sec1 efficiently. The fragment 78–210 bound less efficiently than the wild-type protein, and binding

Table 1. Details of the selected deletion mutants verified by DNA sequencing

|	Amino acids	Size (kDa)	Sequence	Extra amino acids	Sec1p-binding
N-terminal fragments	1–194	26	—**ASSGVID***	2	+
	1–158	22	—**RPTRWID***	3	+
	1–146	20	—**HTSSSSD***	1	+
	1–131	18	—**PEYTPAS***	0	+
	1–96	15	—**KQNSLID***	3	+
	1–78	13	—**KEDLPND***	1	+
	1–46	9	—**GDTVNTD***	1	−
C-terminal fragments	9–210	25	***QEGSGR**—	0	+
	35–210	22	***IKAEKD**—	0	+
	78–210	17	***NERKIL**—	0	±
	105–210	14	***NRRKAS**—	0	−
	136–210	11	***DIYNNH**—	0	−

In the set of N-terminal fragments, there can be up to three extra amino acids at the C-termini, depending on which stop codon in the transposon end matches the reading frame of the encoded protein. The bolded sequences correspond to Mso1p, and extra C-terminal amino acids are shown in regular font. The predicted size of each fragment includes the N-terminal His$_6$ tag. Asterisks denote the protein termini. The result of Sec1p binding assay is denoted as + (binding), −(no binding), ± (intermediate binding).

was abolished upon further deletion from the N-terminus (i.e. fragments 105–210 and 136–210). These results indicate that the most critical region for Sec1p binding lies between residues 46 and 78 in Mso1p and that there probably are some weaker interactions within the region E79–N105; however, the latter interactions are not necessary for efficient Sec1p binding given that fragment 1–78 bound Sec1p equivalently to wild-type Mso1p.

DISCUSSION

A new strategy to produce pools of plasmids encoding N- and C-terminal deletion variants of proteins is presented in this paper. The methodology is based on the *in vitro* transposition of phage Mu. We produced deletion variants of the yeast *MSO1* gene and mapped the region in Mso1p that interacts with yeast Sec1p.

The Mu *in vitro* transposition system is highly efficient, easily producing sizeable insertion mutant libraries of target plasmids (8). Due to easy selection of integrants by the use of appropriate antibiotics, 100% of the selected plasmid clones contain a transposon insertion. Owing to the symmetric nature of the transposon ends, all these plasmids are appropriate for further manipulation steps irrespective of the relative orientation of the inserted transposon. Importantly, in addition to a 5 bp target site duplication, mini-Mu insertions do not induce other rearrangements, such as deletions or nonpredictable base pair additions, at the site of integration [(8,16), this study]. Mini-Mu transposons utilized in *in vitro* reactions can carry essentially any DNA between the short Mu-specific ends (8). This DNA typically contains selectable genes or other convenient genetic markers. The transposon ends, including the sequences required for initial transposase binding and subsequent transpososome assembly, also tolerate some sequence variation (16). In this study, we utilized a transposon that carries three translation stop signals close to each terminus; and similarly, a second transposon variant that contains an engineered NotI site at each end. Despite these modifications,
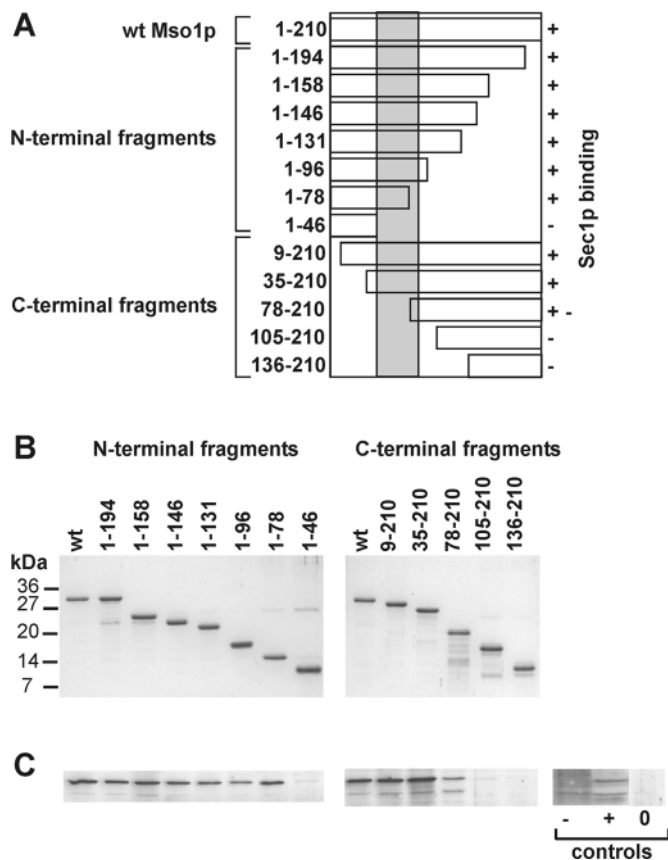
Figure 4. Analysis of Mso1p–Sec1p interaction. (**A**) Graph illustrating the Mso1p part of the different deletion variants and their Sec1p-binding characteristics. Number ranges indicate the Mso1p residues contained in the fragments. The grey rectangle depicts the binding region. (**B**) Analysis of purified Mso1p deletion variants by SDS–PAGE. (**C**) Mso1p–Sec1p binding analysis. Mso1p fragments that were bound to agarose beads were incubated with yeast lysate containing overexpressed Sec1p. The proteins were resolved by 10% SDS–PAGE, transferred to nitrocellulose filters, and the presence of Sec1p was detected with a polyclonal antiserum and ECL reagents. The fragment order is the same as in (B). Controls: (−) yeast lysate not overexpressing Sec1p; (+) yeast lysate overexpressing Sec1p; (0) a sample with no Mso1p added as a negative control.

the transposons retained their proficiency of generating integrant plasmid clones, although with lower efficiency as compared with the corresponding wild-type transposon. In a direct comparison experiment (standard reaction conditions, 4 h incubation time), cat-Mu(Stop) and cat-Mu(NotI) yielded integrant clones with roughly 10 and 70% efficiency of the wild-type transposon (cat-Mu), respectively (data not shown). Even with these efficiencies, the number of integrant clones obtained in standard reaction conditions should easily be far greater than the potential number of unique deletion variants for a standard size protein. For more demanding projects, the efficiency can further be increased by addition of more transposon DNA and MuA transposase in the reaction. For example, with cat-Mu(Stop) we used four times (4×) higher concentrations of these reaction components. The number of integrant clones obtained in these conditions approaches the level of that obtained with the wild-type cat-Mu transposon in standard (1×) reaction conditions (data not shown).

We constructed a new transposon, cat-Mu(Stop), which carries translation stop signals in all three reading frames,

thereby terminating translation near the transposon insertion site (Figure 1). This transposon can generate a library of gene constructions encoding C-terminally truncated protein variants, given that the gene of interest is cloned in an appropriate plasmid. We designed the stop codons to reside as close as possible to the transposon ends, and most of the modified nucleotides do not overlap with the critical transposase binding site (Figure 1). All of the C-terminally truncated deletion variants will be translated from the common initiation codon, and the transposon insertion site will dictate which stop codon is used for each individual clone. Some clones will directly terminate translation without the addition of extra amino acids. However, the rest of the clones will encode one to three additional amino acids at the C-terminus of each fragment. The exact composition of the encoded amino acids is dependent on the original sequence. Such short additions will be tolerated for most applications and would not be expected to significantly affect the biological function of the fragment, particularly in comparison with the effect of the deletion itself.

We used the cat-Mu(NotI) transposon (16) to produce constructions encoding N-terminally truncated protein variants. This strategy requires that the vector contains a suitably located unique NotI restriction site 5′ from the cloned target gene. By NotI digestion of plasmids, each carrying a transposon insertion in the target gene, the 5′-distal part of the gene and the transposon core is eliminated. Since the original translation start signal is retained in each clone, the resulting protein variants are truncated at their N-termini. An apparent drawback of the strategy is that only one-third of the clones will encode a C-terminal protein fragment in a proper reading frame with the start codon, depending on the number of base pairs deleted. However, due to high efficiency of the strategy, this does not limit the number of different deletion variants generated; but for further analyses, suitable clones should be selected by an appropriate initial screening.

The described deletion mutagenesis strategy does not require specially engineered vectors, although certain aspects need to be considered carefully. The vector must be of sufficient size for the adequate electrophoretic separation of the vector backbone from the pool of fragments representing transposon insertions into the target gene (Figure 2). For the generation of C-terminal fragments, the vector must contain a unique NotI restriction site between the translation start signal and the beginning of the actual coding sequence of the target gene. Complementarily, the target gene must not contain NotI sites. This typically is of little concern, as NotI is a rare-cutting enzyme with 8 nt recognition sequence. For isolation of Mso1p variants, we used a vector that encodes an additional His$_6$ tag at the N-terminus of each expressed protein. This feature is not mandatory for the strategy but constitutes a means for convenient protein purification.

Mu inserts into a broad spectrum of target sites in DNA (9,10,16). With the described strategy, it is possible to produce extensive libraries of deletion constructions containing the majority of all potential variants. We aimed at producing a saturating library of a minimum of 10^4 clones that would represent a broad diversity of insertions; theoretically, every possible insertion site becomes targeted several times in a library of this magnitude. However, the spectrum of different mutant clones is to a certain degree influenced by the target gene sequence (10). Individual clones can be screened by

restriction analysis or sequencing to find a suitable set of different deletion plasmids to map protein interaction regions at high precision.

We chose a small set of Mso1p deletion constructions for protein purification and Sec1p binding assays. The results indicate that within Mso1p, the most important amino acids for the Sec1p interaction are located between residues 46 and 78. The region between 79 and 105 probably is involved in the interaction as well, but this region is not essential for binding. Previous Mso1p–Sec1p interaction studies showed that the C-terminal part of Mso1p is not necessary for the Sec1p interaction; a deletion protein containing the 164 N-terminal amino acids of Mso1p bound Sec1p in a yeast two-hybrid assay (23). The current findings expand on the knowledge of Mso1p–Sec1p interaction, and they are in full agreement with yeast two-hybrid results and other *in vivo* analyses of functionally important domains of Mso1p (M. Knop, K.J. Miller, M. Mazza, D. Feng, M. Weber, S. Keränen and J. Jäntti, submitted).

Previous methods for deletion mutagenesis of proteins include partial exonuclease digestions or restriction enzyme cleavages of DNA constructions (20,33). However, exonuclease digestions can be difficult to control, and restriction enzyme cleavages are limited to the existing sites within the gene. Accordingly, these strategies are not convenient for fine mapping of specific regions in proteins. Intramolecular transposition-based procedures for producing deletion variants of plasmids have been described for transposon Tn9 and members of the Tn3 and Tn5 families (22,34–36). However, most of these methods suffer from low frequency of transposition and low overall efficiency of producing deletions. Until now, the Tn*5 in vitro* system probably represents the most efficient transposition-based method for the purpose of generating protein deletion variants (22). While useful, the strategy has certain drawbacks, however. It requires a special vector for intramolecular transposition, and <70% of the produced clones are true deletion constructions. In addition, the published protocol includes a description for the generation of C-terminally truncated protein variants only, although the possibility of using a similar strategy for N-terminally truncated variants is being discussed (22). For comparison, the Mu transposition-based strategy described in this paper can utilize a wider selection of vectors and all the plasmid clones obtained are genuine deletion constructions. In addition, both N- and C-terminally truncated protein variants can be produced with relative ease by the use of similar procedures.

Nested sets of deletions are useful for studies of relationships between sequence, structure and function. Most often they are used to define regions involved in protein–protein interactions between protein monomers in homomultimers or between separate proteins. They are also used to specify regions that are required for different functions. One useful application for deletion libraries is protein fragmentation, i.e. dissection of proteins into distinct subdomains to identify independently folding units that interact non-covalently to form a functional protein (37). Furthermore, deletion libraries can be used to minimize proteins into fragments when developing drugs for the purpose of disrupting interactions at protein–protein interfaces (38). Finally, by screening deletion libraries, it is also possible to identify proteins with altered properties.

The strategy described in this study is rapid and efficient, and it constitutes an economical way of producing a large number of protein deletion derivatives. The inherent accuracy of Mu *in vitro* transposition, in combination with effective selection schemes during DNA manipulation steps, guarantees high quality protein-expressing gene truncation libraries.

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