2 Material and Methods

2.1 Materials

2.1.1 Bacterial Strains

- *E. coli* strain DH5 Stratagene
- *E. coli* strain XL10-Gold Ultra competent cells Stratagene
- E. coli strain DH5-RIL, Ultra competent cells Stratagene

2.1.2 Yeast strains

- Wild type DF5 a or α (haploid)
- Sec63prA
- Srp68prA

2.1.3 Plasmids

- PET28a Novagen, Madison, WI
- PET21a Novagen, Madison, WI
- pGEX4-T2 Amersham Biosciences Corp., NJ
- pGEX6-P1 Amersham Biosciences Corp., NJ
- pBXAHis5 Beckmann et al., 1997

2.1.4 E. coli Media

LB (Luria Bertani) medium:

Bacto-tryptone	10 g/l
Bacto yeast extract	5 g/l
NaCl	5 g/l

Autoclave before use for 20 minutes.

For use as liquid medium 0.4% (w/v) glucose were added. For use as solid medium 2% agar were added before autoclaving. The medium was cooled to approx. 50 °C before pouring it into sterile petri dishes of appropriate size.

SOC medium:

Per liter:

yeast extract	5 g
bacto-tryptone	20 g
NaCl	10 mM
KCl	2.5 mM
MgCl2	10 mM
MgSO4	10 mM
glucose	20 mM
H2O to 1 liter	

The media is autoclaved for 35 minutes, aliquoted into 5 ml batches and stored at -20ºC.

2.1.5 Yeast media

2.1.5.1 Rich media

YPD media (liquid):

Per liter:

yeast extract	10 g
bacto-peptone	20 g
dextrose	20 g

The yeast extract and the bacto-peptone were dissolved in 700 ml of water and autoclaved for 35 minutes (YP). The dextrose was dissolved in 300 ml of water and autoclaved for 35 minutes. The YP and the dextrose solution were mixed directly before use.

YPD media (solid)

For all plates, agar was added at a concentration of 2% (20 g/liter). A pellet of sodium hydroxide (~0.1 g) was added per liter to raise the pH enough to prevent agar breakdown during autoclaving.

2.1.5.2 Minimal Media (Drop Out Plates)

Per 500 ml:

3.35g	yeast nitrogen base without amino acids
0.40 g	of CSM-HIS drop-out powder
450 ml	of water
9.50 g	agar
150 µl	10N NaOH
10g	dextrose

The yeast nitrogen base (without amino acids) and the drop-out powder (amount depends on amino acid) were mixed with the water. After the addition of the NaOH and the agar, the media was autoclaved for 35 minutes. The dextrose was dissolved in 50 ml of water and autoclaved for 35 minutes. The dextrose was added to the media which was poured into plates of appropriate size after letting it cool to 50 ºC.

2.1.6 Solutions

2.1.6.1 Solutions for Gel Electrophoresis

4x SDS-PAGE stacking buffer

Tris-HCl 0.5 M
pH adjusted to 6.8.

4x SDS-PAGE separating buffer

Tris-HCl 1.5 M
pH adjusted to 8.8.

5x SDS-PAGE loading buffer

4x SDS-PAGE stacking buffer	1 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
β-mercaptoethanol	0.4 ml
1% (w/v) Bromophenol Blue	1.2 ml
dH_2O	ad 8 ml

SDS-PAGE electrode buffer

Tris base	3.0 g
Glycine	14.4 g
SDS	1.0 g
dH_2O	ad 1 l

Coomassie staining solution

Coomassie Brilliant Blue R-250	0.05 g
Methanol	500 ml
HAc	100 ml
dH_2O	add 1 l

Destaining solution

Methanol	100 ml
HAc	50 ml
dH_2O	ad 1 l

50x TAE buffer

Tris base	146 g
HAc	28.55 ml
0.5 M EDTA	50 ml
dH_2O	ad 500 ml

2.1.6.2 Buffers for the Manipulation of Yeast

10xLithium acetate

1M	lithium acetate
	filter sterile

10xTE buffer

100 mM	Tris·Cl, pH 7.5
10 mM	EDTA, pH 7.5
	filter sterile

PEG-4000

50% (w/v)	PEG 4000
	filter sterile

Reduction solution

100 mM	TRIS, pH=8.0
10 mM	DTT

2.1.7 Chemicals

Standard chemicals were purchased from Sigma, St. Louis, MO.

2.1.8 Enzymes, Kits, and Miscellaneous Materials

300 µm Mesh Copper Grids	EMS, Fort Washington, PA
Benzalkonium chloride	Sigma-Aldrich, St. Louis, MO
Cellulose acetate butyrate	Sigma-Aldrich, St. Louis, MO
Carbon Rods (Grade 1)	Ted Pella Inc., Redding, CA
Carbon tetrachloride	Sigma-Aldrich, St. Louis, MO
Cryo-Transfer Holder	Gatan, Warrendale, PA
Dynabeads M-280, Strepavidin	Dynal ASA, Oslo, Norway
Genomic Yeast DNA	Promega, Madison, WI
GFX DNA and Gel Band Purification Kit	Amersham Bioscience Inc., NJ
GFX Micro Plasmid Prep Kit	Amersham Bioscience Inc., NJ
Glutathione Sepharose	Amersham Bioscience Inc., NJ
Herring Sperm DNA	Promega, Madison, WI
mantGDP	Jena Bioscience GmbH, Jena, Germany
mMESSAGE mMACHINE™	Ambion Inc., Austin, TX
Nitro-cellulose membrane	Schleicher & Schuell, Keene, NH
PAGE Gold Precast Gels, (4-20%)	BioWhittaker, Inc., Walkersville, MD
Pfu or Taq DNA polymerase	Stratagene, La Jolla, CA
Q-Sepharose	Amersham Bioscience Inc., NJ
Restriction enzymes	New England Biolabs, Beverly, MA
RNasin	Promega, Madison, WI
Ruby Red Mica	EMS, Fort Washington, PA
Simply Blue Safe Stain	Invitrogen, Carlsbad, CA
SO-163 Electron Image Film	Kodak, Rochester, NY
SP-Sepharose	Amersham Bioscience Inc., NJ
Superdex-75 column	Amersham Bioscience Inc., NJ
SuperSignal® Chemiluminescent Detection Kit	Pierce, Rockland, IL
TALON Metal Affinity Resin	Clontech, Palo Alto, CA

2.1.9 Detergents

DeoxyBigCHAP	Calbiochem, San Diego, CA
Digitonin	Calbiochem, San Diego, CA
Nikkol ($C_{12}E_8$)	Calbiochem, San Diego, CA

2.1.10 Lipids

Asolectin	Avanti Polar Lipids Inc., Alabaster. AL

2.1.11 Protease Inhibitors

Aprotinin	Roche, Indianapolis, IN
Complete EDTA-free	Roche, Indianapolis, IN
Leupeptin	Roche, Indianapolis, IN
Pefabloc SC	Roche, Indianapolis, IN
Pepstatin A	Roche, Indianapolis, IN

2.1.12 Antibodies

purified rabbit IgG	ICN Biochemicals, Cleveland OH
anti-rabbit Ig, horseradish peroxidase linked. Whole antibody from donkey	Amersham Bioscience Inc., NJ
anti-mouse Ig, horseradish peroxidase linked. Whole antibody from sheep	Amersham Bioscience Inc., NJ

2.1.13 Nucleic Acids

5'-DPAP: 5’-ATT TAG GTG ACA CTA TAG AAA CCA AAC AAA ACA AAT AAA ACA AAA ACA CAA GTT CTA CCC ATA CGA TGT TCC AGA TTA CGC TGA AGG TGG CGA AGA AGA AGT TG-3’

3'-DPAP: 5’-ATC GTA GAC AGA TTT AAC AAC GTA-3’

5'-SRP68: 5'-AAT GAG CAG ACG GAA GGA GAG CCT AAA AAG AAG CGT GGT TTC TTG GGC CTA TTT GGT CGT ATT GAA GGT AGA GGT GAA GCT CAA AAA CTT ATT-3'

3'-SRP68: 5'-AAG ATG TGT TAA GTA ATG CAG TTC GTT ATT TGG CTA CTA TAG TTG GCA ATC ATC ATT TGT GTC GAC GGT ATC GAT AAG CTT-3'

5'-SRbeta: GTG GTG TC CATATG GGG ATC AAG CAA AAA AGT TAT C

3'-SRbeta: GCA GCA AGC TTA CAG TTT TTC ATC TAT CCA TTC

5'-Sbh1: 5'-GGA ATT CCC ATG TCA AGC CCA ACT CCT CCA G

3'-Sbh1: 5'-ATA AGA ATG CGG CCG CCT AGG AGT CGA CTC TGA ATC CAT TGG

5'-Sbh2: 5'-TAG CAG AAT TCA TGG CAG CTT CAG TTC CAC CAG G-3'

3'-Sbh2: 5'-CTA GCT ACA GAA GCG CGG CCG CGG AFT CGA CTC TGA ATC CAT TGG-3'

All other materials have been acquired at the highest quality possible from standard vendors.

2.1.14 Equipment

Äkta purifier	Amersham Bioscience Inc., NJ
BioComp Gradient Master	Biocomp Instruments Inc., Fredericton, New Brunswick, Canada
BioComp Piston Gradient Fractionator	Biocomp Instruments Inc., Fredericton, New Brunswick, Canada
Carbon Evaporator	Denton, Moorestown, NJ
Cell Centrifuges	Sorvall, Newtown, CT
CM12 Transmission Electron Microscope	FEI/Phillips, Eindhoven, Netherlands
CM200 Field Transmission Electron Microscope	FEI/Phillips, Eindhoven, Netherlands
Glow Discharging Unit	University of Basel, Basel Switzerland
HiScan	Eurocore, Saint-Denis, France
Robocycler	Stratagene, La Jolla, CA
SGI Octane	Silicon Graphics, Mt. View, CA
SGI Origin 2000	Silicon Graphics, Mt. View, CA
Table-top Centrifuges	Eppendorf, Hamburg, Germany
Ultra Centrifuges	Beckman Coulter, Fullerton, CA

2.1.15 Computer Software

Data processing:	
SPIDER	Health Research Inc., Albany, NY
Graphics:	
DINO, Visualizing Structural Biology (2002)	http://www.dino3d.org
gnuplot	http://www.gnuplot.info/
IRIS explorer	NAG Inc., Downers Grove, IL
Kooka	http://www.kde.org/apps/kooka/
Origin 6.0	OriginLab Corporation, Northampton, MA
POVRAY, Persistence of Vision Raytracer	www.povray.org
the GIMP	www.gimp.org
Sequence alignments:	
ClustalW	http://searchlauncher.bcm.tmc.edu
DNAstar	DNASTAR, Inc. Madison, WI
JavaShade applet	http://industry.ebi.ac.uk/JavaShade
Other:	
IRIX 6.5	Silicon Graphics, Mt. View, CA
KDE 3.1.3	http://www.kde.org
Mandrake Linux 9.1	MandrakeSoft S.A., Paris, France
OpenOffice 1.1	http://www.openoffice.org
WINE	http://www.winehq.com

2.2 Molecular Biological and Biochemical Methods

2.2.1 Molecular Biology

2.2.1.1 Polymerase Chain Reaction (PCR)

PCR reaction (50 µl)

Template DNA (50 ng/µl)	4 µl
Primer I (1 µM)	5 µl
Primer II (1 µM)	5 µl
10x PCR buffer	5 µl
10x dNTP (2.5 mM/each)	5 µl
dH_2O	ad 50 µl
Pfu DNA Polymerase	2.5 U

After mixing the sample, the template was denatured at 94 °C for 5 minutes. The following temperature program was used for 30 cycles:

denaturation	94 °C	45 seconds
primer annealing	53 °C	45 seconds
primer extension	72 °C	2 minutes/kb
final extension	72 °C	10 minutes

2.2.1.2 Desalting of PCR Products

To desalt and concentrate the PCR product, the DNA was precipitated using ethanol. The reaction was mixed with 1/10 of the volume of 3M sodium acetate (pH 5.2) and 2 ½ times the volume of 100% Ethanol. The precipitation reaction was incubated for 30 minutes at -20 ºC and then spun for 30 minutes at x g in a table top centrifuge at 4ºC. The pellet was washed with 70% ethanol and spun again for 15 minutes at x g in a table top centrifuge at 4ºC. The DNA pellet was resuspended in water and stored at -20ºC.

2.2.1.3 Plasmid Preparation

Plasmid preparations were carried out using the GFX Micro Plasmid Prep Kit (Amersham) according to the manufacturer's instructions.

2.2.1.4 Restriction Digestion

Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and reaction conditions applied as recommended by the supplier.

2.2.1.5 Gel Purification of DNA

DNA fragments were purified after restriction digestion on horizontal agarose gels in 1x TAE buffer. The bands were visualized under UV light after staining the gel in an ethidium bromide solution. Appropriate bands were cut out from the gel and the DNA isolated using a GFX DNA and Gel Band Purification Kit (Amersham) according to the manufacturer's instructions.

2.2.1.6 Ligation

Reaction mix:

restriction digested vector	100 ng
restriction digested PCR fragment	30-300 ng
10x ligase buffer	1.5 µl
dH_2O	ad 14 µl
T4-DNA ligase	1 µl

Three ligation reactions with varying amounts of PCR fragment and a constant amount of digested vector were set up in parallel. In addition a negative control without any PCR fragment was set up. Reactions were incubated at 24 °C for 2 h and sometimes for additional 12 h at 16 °C.

2.2.1.7 Transformation

Frozen aliquots of competent *E. coli* cells were thawed on ice and 3 µl of a ligation reaction added. Gently mixed, the cells were kept on ice for 30 min and then incubated at 42 °C for 45 sec. The cells were incubated another 2 min on ice, then 400 µl SOC medium were added and the culture incubated at 37 °C for 60 minutes. The cells were then plated on LB selection plates and the plates incubated at 37 °C until colonies appeared.

2.2.1.8 Colony PCR

To identify colonies that contain the transformed plasmid with the desired insert of ligated DNA, colonies are used as templates in a PCR colony screening.

2x PCR mix (for 5 reactions):

dH_20	15.0 µl
10x buffer	10.0 µl
dNTPs	2.0 µl
3'	10.0 µl
5'	10.0 µl
taq	1.0 µl

5 colonies per plate were picked using a sterile tooth-pick and each was resuspended in 50 µl of water. 10 µl of this cell resuspension was mixed with 10 µl of a 2x PCR-mix.

A 30-cycle PCR reaction was carried out and the products were analyzed on horizontal agarose gels. The remaining cell resuspension of 2-3 clones with correct vector inserts were transferred each into 5 ml LB liquid cultures and grown over night at 37 ºC in a thermal shaker. Plasmid DNA from these cultures was isolated and further analyzed by DNA sequencing.

2.2.2 Purification of Recombinant Proteins

2.2.2.1 Overproduction of Recombinant Proteins

A single colony harboring the expression vector was picked from a fresh LB-master plate and used to inoculate 25 ml of LB medium containing 50 µg/ml of the appropriate antibiotic. After incubation overnight at 37 °C in a thermal shaker the pre-culture was added to 1 l LB medium supplemented with 0.4 % (w/v) glucose and 50 µg/ml of the appropriate antibiotic. Expression of the recombinant gene was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an optical density at 600 nm of 0.8. The

cells were incubated 2-3 hours at 30 ºC in a thermal shaker and harvested by centrifugation (5000 rpm, GS-3 rotor, 10 min, at 4 ºC).

2.2.2.2 Protein Purification using a 6xHIS Tag

A pellet obtained from a 2 liter culture of cells was resuspend cells in 15 ml of lysis buffer A [20 mM Tris·HCl, pH 8.0, 250 mM NaCl, 5mM β-mercaptoethanol] The cells were lysed using an Avestin cell disrupter. The lysate was centrifuged for 20 minutes at 15.500 rpm in a SS34 rotor at 4 ºC. The cleared supernatant was incubated for 30 minutes with 2 ml of equilibrated TALON™ Metal Affinity Resin (Clontech) at 4 °C and additional 15 minutes at room temperature (RT). The resin material was washed 3 times with 40 ml of buffer A and once with 40 ml of buffer A containing 10 mM imidazole. The protein was eluted using 5-6 resin volumes of buffer A containing 150 mM imidazole. The imidazole was removed by dialysis over night at 4 ºC against buffer B [20 mM Hepes (pH 7.5), 300 mM KOAc and 1 mM DTT). The protein was stored at -80 ºC.

2.2.2.3 Protein Purification using a Glutathione Affinity Tag

A pellet obtained from a 2 liter culture of cells was resuspend cells in 15 ml of lysis buffer C [20 mM Hepes, pH=7.5, 250 mM KOAc, 1 mM DTT and 0.5 mM PMSF]. The cells were lysed using an Avestin cell disrupter. The lysate was centrifuged for 20 minutes at 15.500 rpm in a SS34 rotor. The cleared supernatant was incubated for 30 minutes with 750 µl of equilibrated glutathione sepharose (Amersham). The resin was washed twice with 30 ml and twice with 15 ml of buffer B. The protein was eluted using 6 resin volumes of buffer C containing 20 mM glutathione. The protein was stored in aliquots at -80 ºC.

2.2.3 Loading of purified SRβ with mantGDP

The cytosolic GTPase domain of SRβ from S. cerevisiae (residues 31-244) was expressed in BL21(DE3) cells (Stratagene). SRβ31-244 was purified by Cobalt-affinity chromatography (TALON™ Metal Affinity Resin, Clontech) from two liters of cell culture and eluted in 15 ml of buffer A [20 mM Tris·HCl, pH 8.0, 250 mM NaCl, 5mM β-mercaptoethanol] containing 150 mM imidazole. To remove endogenous nucleotide bound to SRβ31-244, EDTA (0.5 mM) was added to the eluate, followed by an incubation at 4 ºC for 30 minutes. The eluate was then subjected to size exclusion chromatography in the presence of 0.5 mM EDTA in buffer D [20 mM Hepes, pH 7.5, 250 mM NaCl, 1 mM DTT] (Superdex-75, Amersham). Fractions of a single A_{280} peak representing SRβ31-244 were pooled and stored at -80 ºC.

A 10 µM solution of SRβ31-244 was incubated for 30 minutes at 4ºC with 200 µM of 3'-(N-Methyl-anthraniloyl)-2'-deoxy-guanosine-5'-diphosphate (mantGDP) in buffer E [20 mM Hepes, pH 7.5, 350 mM KOAc, 1 mM DTT and 5 mM $Mg(OAc)_2$]. The excess of unbound mantGDP was removed by dialysis against buffer E at 4 ºC for four hours.

2.2.4 *SRβ Nucleotide Exchange Assay*

Steady-state fluorescence intensity was measured using a SPEX Fluorolog FL3-11 photon-counting spectrofluorimeter with a single grating excitation monochromator and a 450-watt xenon lamp. Samples were exited at 352nm (1 nm bandpass) and emission was detected at 440nm (5nm bandpass). All spectral measurements were done at 25 ºC in buffer J [20 mM Hepes (pH 7.5), 350 mM KOAc, 5 mM $Mg(OAc)_2$, 10% glycerol, 1 mM DTT, 100 µM GDP and 0.3% DeoxyBigCHAP] using a 4 mm x 4 mm quartz cuvette. The release of mantGDP from SRβ31-244 was measured in real time as a decrease in fluorescence.

2.2.5 Yeast genetics

2.2.5.1 Protein A-Tagging of Yeast Proteins

Yeast is an eukaryotic organism in which it is possible to modify genes of interest using standard molecular biology methods. To facilitate the purification of proteins from yeast, affinity tags can be fused to yeast proteins that bind to affinity columns. An efficient and well established method to purify proteins from yeast is the protein A tag (see Beckmann et al., 1997).

Homologous Recombination

The method of “tagging by homologous recombination” is based on the introduction of a piece of double stranded DNA behind the target gene into the yeast genome that codes for an additional tag (used for purification) and a selection gene (used for selection of positive colonies on minimal media). The DNA is flanked by a 5'-end that is homologous to the end of the target gene and a 3'-end that is homologous to the region within the yeast chromosome that follows the target gene with a gap of 50 nucleotides between the two homologous sites (Figure 2.1). The DNA, that is used for homologous recombination, is generated by PCR using a template plasmid (pBXaHis5) that contains the DNA coding for the affinity tag and the gene used as selection marker.

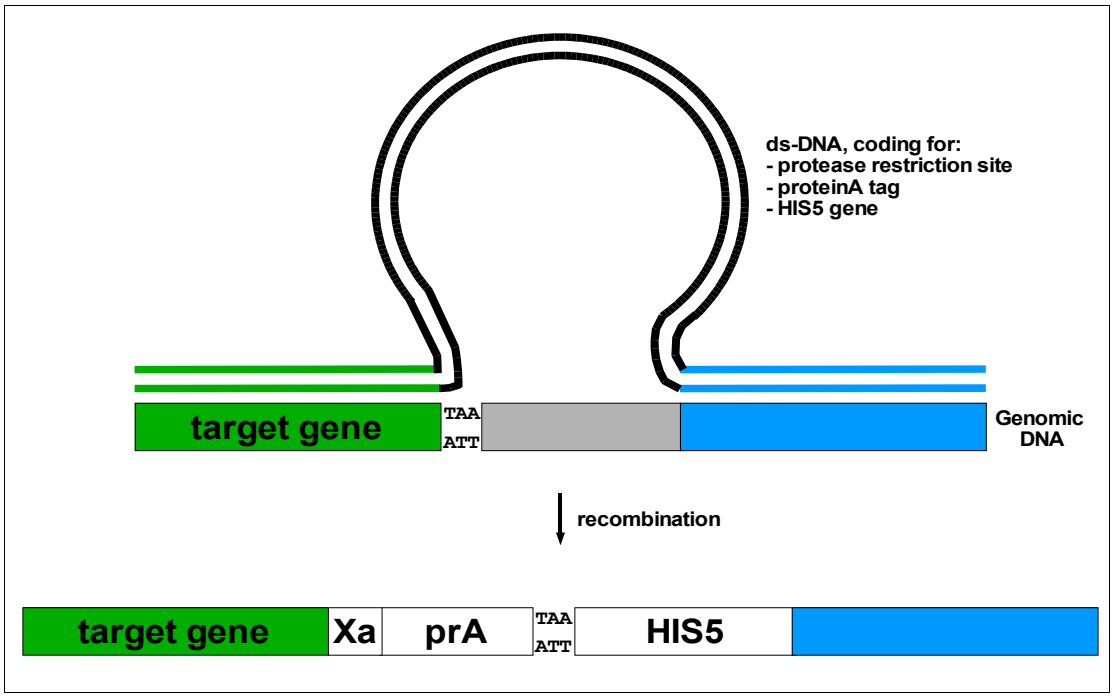

Figure 2.1: Schematic illustration of the COOH-terminal tagging of yeast proteins by homologous recombination. A double stranded DNA is generated by PCR of a template coding for the IgG binding domains of protein A , a protease site, and the HIS5 gene from *S. pombe.* The primers used for the PCR include flanking regions homologous for the C-terminus of the target gene. The DNA is transformed into wild type yeast cells and the C-terminus of the endogenous target gene is replaced by homologous recombination leading to a new gene, coding for a "target protein-Factor Xa recognition site-prA fusion protein".

Design of Primers

A 5'-primer was designed, that was homologous to the last 60 nucleotides of the target gene but omitted the stop codon. The 3'-end of the 5'-primer was homologous to the 21 nucleotides that code for the beginning of the gene of the immunoglobulin G (IgG)-binding domains of protein A in the template plasmid pBXaHis5. To facilitate the future protein purification, the primer included a recognition site for the Factor X (Xa) protease between the target gene and the protein A tag, that allowed to proteolytically cleave off the affinity tag during the purification procedure. The 3'-primer was homologous to the beginning of the HIS5 gene from S. pombe in the template plasmid pBXaHis5 and was flanked by 60 nucleotides that were homologous to the DNA following the target gene.

2.2.5.2 Generation of Double Stranded DNA for Recombination

The double stranded DNA needed for homologous recombination was generated by PCR using the pair of primers specific for the target gene (see above) and a template plasmid (pBXaHis5, see (Beckmann et al., 1997). The PCR product was checked by horizontal agarose gel electrophoresis for purity and stored at -20 ºC.

2.2.5.3 Introduction of DNA into Yeast Cells

A 10 ml culture of wild type yeast at A_{600}=1.0 was sedimented for 5 minutes at 2.000 rpm in a table top cell centrifuge at 4ºC and resuspended in 200 µl of lithium acetate-TE buffer. Per transformation reaction, 50 µl of this cell resuspension, 3 µl of herring sperm DNA solution (Promega), 3 µl of double stranded DNA (PCR product) and 300 µl of lithium acetate-TE-PEG solution (1:1:8) were incubated for 30 minutes at 30 ºC while shaking. The reaction was then incubated for 15 minutes at 42 ºC and the cells sedimented in a table top centrifuge. The cell pellet was resuspended in 100 µl of sterile water and plated on selective media (-HIS). The plates were incubated at 30 ºC until colonies appeared. Each colony was replated onto a fresh selection plate (-HIS) and incubated again at 30 ºC until colonies appeared.

2.2.5.4 Verification of Proper Gene Integration

Although the primers designed for homologous recombination are specific for only one target gene, it is still possible that the double stranded DNA randomly integrates into the yeast genome during the homologous recombination. To verify the proper integration of the DNA into the yeast genome, a crude cell extract was prepared from strains that had been positively identified on selective plates. The cell extract was used to identify tagged target proteins by gel shift due to the addition of the affinity tag:

3 ml of YPD each were inoculated with a single colony of the strains growing on selective media and incubated over night at 30 ºC. The cells were sedimented and 2 µl of the loose cell pellet were resuspended in 20 µl of 1x sample buffer at 95 ºC. The resuspension was heated at 95ºC for 5 minutes and briefly vortexed. After short centrifugation in a table top centrifuge to sediment any unlysed cells, 15 µl of the supernatant was analyzed by SDS-PAGE electrophoresis, transferred to nitro-cellulose and the target protein identified by immuno blotting using an antibody specific for the Protein A tag fused to the target protein (IgG, Cappel) followed by ECL detection (Pierce).

2.2.6 Purification of the Signal Recognition Particle (SRP)

The SRP complex was immunopurified from yeast strain *Srp68prA*, in which the gene coding for SRP68p was tagged by in-frame integration of a DNA fragment encoding for a Xa protease site and the immunoglobulin G (IgG)-binding domains of protein A, yielding a fusion protein of SRP68p-Xa site-Protein A.

A cell pellet obtained from a 36 liter culture of yeast strain Srp68prA was resuspended in 200 ml of lysis buffer [20 mM Hepes·KOH pH=7.5, 100 mM KOAc, 7.5 mM $Mg(OAc)_2$, 1 mM DTT, 0.5 mM EDTA, 0.5 mM PMSF, and 0.02% Nikkol] containing 6 ml of protease mix [11 protease inhibitor tablets (Complete EDTA-free), 2 mg Leupeptin, 10 mg Pefablock, 500 µl Pefablock protection solution and 400 µg Pepstatin]. The cells were lysed at 13.000 psi using an Avestin cell disrupter. After the lysis the KOAc-, Nikkol- and PMSF-concentrations were adjusted to 500 mM KOAc, 0.02% Nikkol, and 0.5 mM PMSF and the lysate spun at 8000 rpm for 20 min at 4ºC in a GS-3 rotor. The supernatant was collected and adjusted to 10% glycerol. To obtain a membrane- and ribosome-free supernatant the cleared lysate was subjected to an ultra-centrifugation at 125.000 x g for 2.5 hours at 4ºC. The supernatant was incubated with 1 ml IgG-sepharose at 4 ºC overnight.

The resin was collected by a centrifugation for 5 minutes at 500 x g for 5 minutes at 4 ºC and the supernatant carefully removed. The resin was transferred into a 10 ml chromatography column and washed with 9 ml washing buffer [20 mM Hepes·KOH pH 7.5, 500 mM KOAc, 7.5 mM $Mg(OAc)_2$, 1 mM DTT, 0.5 mM EDTA, 0.01% Nikkol, and 10% Glycerol] and 5 ml of elution buffer [20 mM Hepes·KOH pH=7.5, 100 mM KOAc, 7.5 mM $Mg(OAc)_2$, 1 mM DTT, 0.01% Nikkol, and 5% Glycerol]. The SRP complex was proteolytically cleaved off the resin material by incubation for 5 hours in 1 ml of elution buffer containing 1 mM of $CaCl_2$ and 5 µg of factor X-protease at room temperature. The SRP complex was eluted using 4 x 250 µl and 2 x 500 µl of elution buffer.

The elution fractions were pooled and subjected to an ultra-centrifugation for 14.5 hours at 200.000 x g at 4ºC using a sucrose gradient [20-40% sucrose, 20 mM HepesKOH pH 7.5, 100 mM KOAc, 7.5 mM $Mg(OAc)_2$, 1 mM DTT, 0.5 mM EDTA, 0.5 mM PMSF, 0.01% Nikkol, and 50ug/ml tRNA (from yeast)]. The gradients were fractionated in 5 mm steps from the top of the gradient using a piston gradient fractionator (BioComp). The peak fractions containing the SRP were pooled and the SRP concentrated using a 50 kDa cutoff protein concentrator.

2.2.7 Purification of the Heptameric- and the Sec61p complex

The heptameric complex was immunopurified from yeast strain *Sec63prA*, in which the gene coding for Sec63p was tagged by in-frame integration of a DNA fragment encoding for a Xa protease site and the immunoglobulin G (IgG)-binding domains of protein A, yielding a fusion protein of Sec63p-Xa site-Protein A.

Cells from a 36 liter culture were lysed in lysis buffer [20 mM Hepes, pH 7.5, 100 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, 0.5 mM PMSF, 125 mM sucrose and protease inhibitors (Complete EDTA-free, Roche)] at 13.000 psi using a French-Press. The cell lysate was cleared by low speed centrifugation at 6.000 x g for 15 minutes at 4 ºC and

then centrifuged at 100.000 x g for 38 minutes at 4 ºC to obtain a crude membrane pellet. Aliquots of the crude membrane pellet were stored at -80 ºC. A 10^{th} part of the crude membrane pellet (5 ml) was solubilized by resuspending it in 46 ml solubilisation buffer [20 mM Hepes, pH 7.5, 400 mM sucrose, 750 mM KOAc, 2.5 mM $Mg(OAc)_2$, 0.5 mM EDTA, 3 % digitonin (Calbiochem), 5 mM DTT and 0.5 mM PMSF] and incubation on ice for 30 minutes. Insoluble material was pelleted by centrifugation at 100.000 x g for 30 minutes at 4 ºC. The supernatant was diluted 1:1 with dilution buffer [20 mM Hepes, (pH 7.5), 400mM Sucrose, 2.5 mM, $Mg(OAc)_2$] and incubated over night at 4 ºC with 1 ml IgG sepharose (Cappel Durham, NC). The resin was washed using washing buffer WLX [20 mM Hepes (pH 7.5), 100 mM KOAc, 3 mM $Mg(OAc)_2$, 10% glycerol, 1 mM DTT] containing 0.5% digitonin and 5mg/ml asolectin (Avanti Polar Lipids), and the heptameric complex eluted by incubating with 5µg/ml Xa protease in 1 ml WLX buffer for 5 hours at 25 ºC. To exchange detergents, pooled fractions containing the heptameric complex were incubated with Q-sepharose (Pharmacia), equilibrated and eluted with buffer WLX containing 0.3% DeoxyBigChap (Calbiochem).

The trimeric Sec61p complex was essentially purified as described (Beckmann et al., 1997). Briefly, the immuno-immobilized heptameric complex (see above) was incubated with buffer WLX containing 1% Triton-X100, thereby dissociating the trimeric Sec61p complex from the heptameric complex. To exchange detergent, the trimeric Sec61p complex was then bound to SP-sepharose, equilibrated and eluted with buffer WLX containing 0.3% DeoxyBigChap.

2.2.8 Purification of Non-Translating Ribosomes

A cell pellet obtained from a 6 liter culture of a wild type yeast strain (DF5a) was resuspended in 20 ml of lysis buffer [20 mM Hepes, pH 7.5, 200 mM KOAc, 7.5 mM $MgOAc_2$, 1mM DTT and 0.5 mM PMSF] and the cells were lysed at 13.000 psi using an Avestin cell disrupter. The lysate was cleared by low-speed centrifugation at 6.000 x g for

15 minutes at 4 ºC. The supernatant was subjected to an ultra-centrifugation step at 100.000 x g for 38 minutes at 4 ºC. To sediment the ribosomes and prevent any contamination with membrane vesicles, the supernatant of the ultracentrifugation-step was loaded on two 3 ml cushions of 2.0M and 1.5 M sucrose in lysis buffer and the ribosomes sedimented at 125.000 x g for 14.5 hours at 4 ºC.

The resulting ribosomal pellets were resuspended in 1-2 ml of water and for further purification loaded on sucrose gradients [10-30 % sucrose in lysis buffer] and subjected to zonal ultracentrifugation at 100.000 x g for 4.5 hours at 4 ºC. A single peak representing 80S ribosomes was identified by light scattering and collected using a piston gradient fractionator (BioComp). The ribosomes were finally pelleted in an ultra-centrifugation step at 225.000 x g for 2 hours at 4 ºC. The resulting ribosomal pellets were resuspended in ribosome dilution buffer [5 mM Hepes (pH 7.5), 10 mM KOAc and 5 mM $MgOAC_2$] and stored at -80 ºC.

2.2.9 Purification of Programmed Ribosome Nascent Chain Complexes

For the generation of RNCs we used a cell-free yeast translation system, which was programmed with truncated synthetic mRNA coding for the 120 N-terminal amino acids of the type II membrane protein dipeptidylpeptidase B (DPAP-B). An additional N-terminal HA-tag (YPYDVPDYA) was used for the immunopurification of the stalled RNCs.

Capped mRNA was synthesized using a SP6 polymerase based Message Machine kit (Ambion Inc, Tx) with DNA fragments as templates. The DNA fragments were generated by PCR with genomic DNA (Promega) as a template. The primers coded for the SP6 site, an high initiation efficiency 5'-untranslated region, an optimized AUG context, and in case of the tagged construct for the additional 9 amino acids of the HA-tag (5'-DPAP) together

with a reverse primer determining the length of the nascent chain (3'-DPAP). The amounts of mRNA in translation reactions were optimized for each batch of yeast extract. The yeast translation extracts were prepared haploid strain derived from DF5α and used as previously described (Waters and Blobel, 1986). Gel filtration of the extract was carried out in buffer A (20 mM Hepes, pH 7.5, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 0.5 mM PMSF, 10 % w/v glycerol). The extract was treated with nuclease S7, frozen in liquid nitrogen and stored at –80ºC.

A 100 µl translation reaction contained 33% yeast extract (~ 100 OD_{260}/ml) ,20 mM Hepes, pH 7.5, 150 mM KOAc, 2.5 mM $Mg(OAc)_2$, 2 mM DTT, 20 mM creatine phosphate, 0.1 mg/ml creatine kinase, 1 mM ATP, 0.5 mM GTP, 0.1 mg/ml yeast tRNA, 0.2 mM amino acids, 3.3 % glycerol, 0.8 U/µl RNasin (Promega) and ^{35}S- methionine (NEN). Reactions were incubated for 70 or 90 minutes at 17ºC and terminated by addition of 2µl of 10mg/ml cycloheximide. Aliquots were analyzed by SDS-PAGE. After subsequent semidry-blotting onto nitrocellulose membranes, proteins were visualized with amido-black. The membranes were dried and exposed to X-ray film or probed with α-anti-HA antibody followed by ECL detection (Pharmacia).

Analytical immunopurification of RNCs was performed by using 100 µl reactions as starting material for each type of nascent chain. The purification was done as described below for the preparative purification except that the amounts of reagents and volumes were 5 to 8-fold smaller. Preparative immunopurification was performed from eight 100 µl translation reactions. After addition of cycloheximide, two reactions were combined and spun through a 800 µl high-salt sucrose cushion (50 mM Tris.Cl, pH 7.0, 500 mM KOAc, 25 mM $Mg(OAc)_2$, 2 mM DTT, 1 M sucrose, 10 µg/ml 125 mM KOAc, 26.25 mM $Mg(OAc)_2$, 1.5 mM DTT, 250 mM sucrose, 100 µg/ml cycloheximide, 0.1 % Nikkol, 0.1 % pill/ml [1 pill complete protease mix/ml H_2O], 0.4 U/µl RNasin) at 335.000 x g for 45 minutes. Each of the resulting four pellets was resuspended in 200 µl of ice-cold buffer B (32.5 mM Tris•Cl (pH 7.0), 125 mM KOAc, 26.25 mM $Mg(OAc)_2$, 1.5 mM DTT, 250 mM sucrose, 100 µg/ml cyclohexemide, 0.1 % Nikkol, 0.1 % pill/ml, and 0.4 U/µl RNasin). 4 µg of a

biotinylated anti-HA antibody (clone 12CA5 from Boehringer Mannheim) was added, followed by incubation for 60 minutes on ice and 30 minutes at RT. Each suspension was then added to 3 mg of equilibrated (2x 200 µl buffer B) streptavidin-coupled magnetic beads (Dynabeads M-280 Strepavidin, Dynal) and incubated for 30 minutes at RT. The beads were washed with 2x 400 µl of ice cold buffer B and 2x 400 µl ice cold buffer C (50 mM Tris.Cl, pH 7.0, 500 mM KOAc, 25 mM $Mg(OAc)_2$, 1 mM DTT, 250 mM sucrose, 100 µg/ml cycloheximide, 0.1 % Nikkol, 0.1 % pill/ml, 0.4 U/µl RNasin). To elute the bound RNCs the beads were incubated with 200 µl of buffer C including 1 mg/ml HA peptide (Boehringer Mannheim) for 30 minutes on ice and 15 minutes at RT. After rinsing the beads with 100 µl buffer C, eluates were combined in two fractions of 600 µl and each spun through a 400 µl high salt sucrose cushion as described above. The resulting pellets were slowly resuspended in 50-100 µl of buffer G (20 mM Tris.Cl, pH 7.0, 50 mM KOAc, 10 mM $Mg(OAc)_2$, 1 mM DTT, 125 mM sucrose, 100 µg/ml cycloheximide, 0.05 % Nikkol, 0.5 % pill/ml, 0.2 U/µl RNasin), shock frozen and stored at -80ºC.

The concentration of the final RNC fraction was determined to be between 2.5 and 5 OD_{260}/ml corresponding to an overall yield of about 5-10 pmol. Aliquots of the various purification steps were precipitated with 7 % trichloroacetic acid in the presence of deoxycholate for analysis by SDS-PAGE.

2.2.10 Reconstitution of RNC-Sec61 Complexes

RNC-Sec61 complexes were reconstituted by incubating 0.25 pmol of RNCs in a volume of 25 µl with 4-40 fold molar excess of Sec61 in 0.2 % DBC or 1 % Triton X-100. The incubation was done in the presence and absence of lipids (0.4 mg/ml of phosphatidylcholine and phosphatidylethanolamine, 4:1) for 10 minutes on ice and 15 minutes at RT in 30 mM Tris.Cl, pH 7.0, 100 mM KOAc, 10 mM $Mg(OAc)_2$, 1.5 mM DTT, 12.5 mM sucrose, 8% glycerol, 20 µg/ml cycloheximide and 0.005 % Nikkol.

Protease protection was assayed by subsequent addition of 2.7 µl of 0.1 mg/ml proteinase K solution and incubation on ice for 15 minutes. The reaction was stopped by addition of 2.5 µl 0.1 M PMSF, precipitated with trichloroacetic acid and analyzed by SDS-PAGE followed by autoradiography.

For Cryo-EM, 1 pmol RNCs were reconstituted with a 10-fold excess of DBC-Sec61 complex in a volume of 20 µl under the described conditions without lipids. Empty ribosome-Sec61 complexes were generated by a mock translation reaction in the absence of mRNA and the presence of 0.5 mM puromycin. The ribosomes were spun through a high salt sucrose cushion as described above, resuspended in buffer G and reconstituted with the DBC-Sec61 complex for cryo-EM as described for RNCs.

2.3 Cryo Electron Microscopy

The information that can be obtained from a cryo-EM specimen results from the contrast between protein and water. To minimize the background and to support the specimen, the standard 300 µm copper palladium grids were coated with a perforated carbon film. The holes in this carbon film were covered with an extremely thin film of ultra pure carbon supporting the specimen during the preparation of the grid and during the experiment.

2.3.1 Preparation of Holey EM Grids

The preparation of holey film grids is based on the creation of a thin film of perforated cellulose-acetate-butyrate polymer which is replaced in a subsequent step with a thin film of carbon.

Glass microscopy slides were cleaned by soaking in tetra-chloride overnight. The cleaned slides were incubated in 0.02% benzalkonium chloride solution for 30 minutes,

rinsed with water and air dried. The slides were cooled for 5-10 minutes at -20 ºC in a freezer on a pre-cooled aluminum block. Depending on the air humidity fine drops of condensed water formed when the slides were brought to room temperature. A solution of 0.3% cellulose-acetate-butyrate was flowed over the slides using a Pasteur pipette. The cellulose-acetate-butyrate solution polymerized around the water drops on the slide covering it with a thin layer of perforated cellulose-acetate-butyrate polymer. The size of the holes in the polymer was evaluated under a light microscope and areas with holes of unusable size were removed with a fresh razor blade.

To use the polymer film, the slides were soaked for 45-60 minutes in a 0.008% di-octyl-sulfo-succinate solution and the film was floated on dust free water. 300 µm mesh copper palladium grids were placed onto the film and the patch of grid covered film removed from the water with parafilm paper and air dried. The grids were place into the vacuum of a a carbon evaporator, and coated with a film of ultra pure carbon (Ted Pella). The polymer film was then dissolved by soaking the grids twice with ethyl-acetate over night. To seal pseudo holes, the grids were re-coated with second layer of carbon and stored dust free at room temperature.

2.3.2 Preparation of Cryo EM Grids

Cryo-EM grids were prepared essentially as described (Wagenknecht et al., 1988). To obtain a clean, dust free and hydrophilic surface, a sheet of ruby red mica (EMS) was cleaved, placed in the vacuum of a carbon evaporator and coated with a very thin layer of ultra pure carbon. The coated mica sheet was cut to 1cm x 1cm pieces and the carbon film floated off the mica onto dust free water. Holey EM grids were placed coated side down onto the carbon film and the grids were carefully removed from the water resulting in a thin supporting film of carbon on the holey EM grids.

To increase the hydrophilicity of the supporting film of carbon, the carbon covered holey EM grids were placed in the vacuum of a glow discharging device and exposed to 45-60 seconds of plasma discharges.

5 µl of sample solution were applied to the grid and incubated for 30 seconds at room temperature. Excess of buffer was blotted away and the grid was rapidly plunged into liquid ethane at liquid nitrogen temperature using a plunging device. Cryo EM grids were stored under liquid nitrogen.

2.3.3 Data Collection in the Cryo Electron Microscope

To maintain the low temperature and prevent the contamination of the grid surface with ice, vitrified cryo-EM grids were transferred into a cooled cryo electron microscope using a special cryo-transfer holder (Gatan). The quality of the ice was assessed by manual inspection and electron diffraction. Only grids with an even spread of particles at the right density were selected for data collection. Images were recorded on 8.3 cm x 10.2 cm fine grain SO-163 electron image film (Kodak) at a magnification of about 45.000x using a CM12 or at a magnification of about 49.000x using a CM200 field emission gun electron microscope. Micrographs were exposed for one second and developed according to the manufactures recommendations.

2.4 Scanning of Micrographs

Micrographs were scanned using a HiScan drum scanner (Eurocore) and saved as 16 bit gray scale TIFF-files.

2.4.1 Three Dimensional Reconstruction Methods

The 3D reconstructions were calculated using projection matching (Penczek et al., 1994). All calculations were done using the "System for Processing Image Data from Electron microscopy and Related fields" (SPIDER - Modular Image Processing System, Albany, NY).

2.4.1.1 Micrograph Selection

To asses the quality of each recorded and subsequently scanned micrograph, an average of power spectra from each micrograph was calculated (See Figure 2.2).

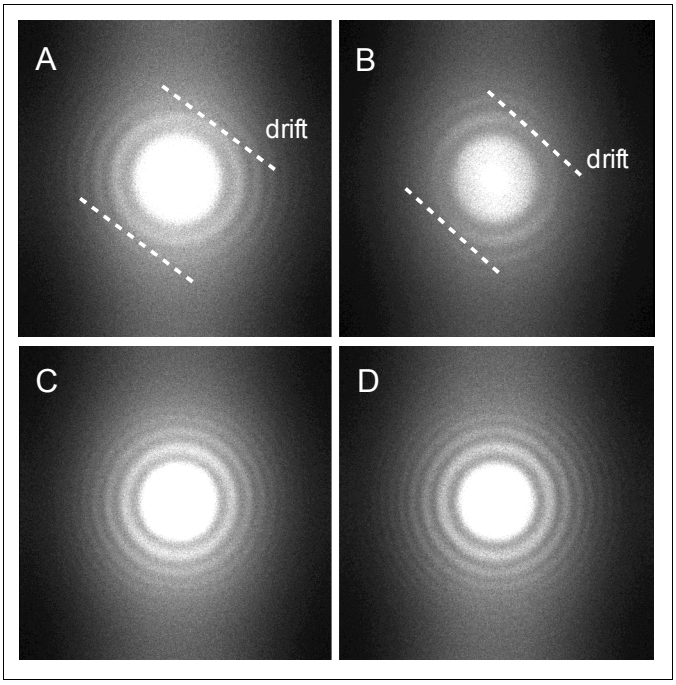

Figure 2.2: Series of averaged power spectra. (A) Averaged power spectra of a micrographs showing signs of drift. (B) Averaged power spectra of a micrographs showing signs of drift and astigmatism. (C and D) Averaged power spectra of two micrographs showing high resolution Thon rings.

Micrographs showing signs of either astigmatism (Thon rings with elliptical, or even hyperbolic shape) or drift (Thon rings which were cut off unidirectionally) were discarded. Only micrographs with Thon rings extending to high resolution were selected for further processing.

To allow the CTF correction of the calculated structure, the defocus of each micrograph was determined by rotationally averaging the power spectra of each micrograph and comparing the spectra to the CTF at varying defocus values. The zeros in the CTF were aligned with the minima of the 1D rotationally averaged power spectra to determine the defocus value for each micrograph.

2.4.1.2 Particle Selection

To obtain a single raw image for each particle, the images of the recorded micrographs were decimated by 1/4, Fourier filtered with a Gaussian low pass filter and automatically searched for contrast peaks over regions corresponding to particle dimensions (56 pixels). Peak coordinates were used to obtain a 95x95 pixel raw image of each particle. The histogram of each raw image was equalized and the particles centered.

Particles resembling the ribosome in shape and size were manually selected by categorizing them in a sequential montage of all images: raw images were sequentially displayed, temporarily adjusted for maximum contrast and good particles selected to creating a selection file containing the file numbers of selected images.

2.4.1.3 Initial Alignment

All selected particles were aligned against reference projections of a reference structure. The initial coarse alignment resulted in translational and rotational parameters as well as

a cross-correlation parameter for the best matching reference projection. The raw images were aligned and segregated into groups according to their defocus.

2.4.1.4 Particle Quality Control

If angular directions were overrepresented, the number of images per angular direction was limited to balance the contribution from each angular direction to reconstruction. Further, particles with a low cross-correlation value were eliminated from the reconstruction.

2.4.1.5 Calculation of an Initial 3D-Structure

The image sets of each defocus group were divided into two subgroups (images with even or odd image numbers) and processed separately. The Fourier transforms of all images in each subgroup were back-projected into Fourier space and iteratively aligned. The reverse Fourier transform of all aligned transforms resulted in a 3D-electron density map for each subgroup. The resolution of the initial 3D electron density maps of each defocus group were determined by comparing the two 3D maps of each defocus group. Finally all 3D electron density maps were corrected for the CTF and combined, resulting in an initial 3D electron density map of the structure.

2.4.1.6 Structure Refinement

Each 3D-reconstruction that is computed using projection matching is biased on its initial reference structure. To reduce the bias, the final iterative refinement used the initial 3D-structure that had been build from the raw image data. The initial map was filtered to its resolution and used as a reference structure to obtain new reference projections. For this,

the angular step size was reduced resulting in a larger number of reference projections, allowing the determination of matching projections at higher resolution. The refinement procedure was repeated until no changes in angle and projection assignments were detected.