Biogenesis of the Signal Recognition Particle (SRP) Involves Import of SRP Proteins into the Nucleolus, Assembly with the SRP-RNA, and Xpo1p-mediated Export✪

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Abstract. The signal recognition particle (SRP) targets nascent secretory proteins to the ER, but how and where the SRP assembles is largely unknown. Here we analyze the biogenesis of yeast SRP, which consists of an RNA molecule (scR1) and six proteins, by localizing all its components. Although scR1 is cytoplasmic in wild-type cells, nuclear localization was observed in cells lacking any one of the four SRP "core proteins" Srp14p, Srp21p, Srp68p, or Srp72p. Consistently, a major nucleolar pool was detected for these proteins. Sec65p, on the other hand, was found in both the nucleoplasm and the nucleolus, whereas Srp54p was predominantly cytoplasmic. Import of the core proteins into the nucleolus requires the ribosomal protein import receptors Pse1p and Kap123p/Yrb4p, which might, thus, constitute a nucleolar import pathway. Nuclear export of scR1 is mediated by the nuclear export signal receptor Xpo1p, is distinct from mRNA transport, and requires, as evidenced by the nucleolar accumulation of scR1 in a *dis3/rrp44* exosome component mutant, an intact scR1 3′ end. A subset of nucleoporins, including Nsp1p and Nup159p (Rat7p), are also necessary for efficient translocation of scR1 from the nucleus to the cytoplasm. We propose that assembly of the SRP requires import of all SRP core proteins into the nucleolus, where they assemble into a pre-SRP with scR1. This particle can then be targeted to the nuclear pores and is subsequently exported to the cytoplasm in an Xpo1p-dependent way.

Key words: SRP • XPO1 • nuclear pore complex • nucleocytoplasmic transport • nucleolus

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Introduction

Nucleocytoplasmic transport of proteins and RNAs occurs through the nuclear pore complexes (NPCs)[1] and is usually an active, carrier-mediated process (for review see Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999; Ryan and Wente, 2000). Proteins destined to enter the nucleus are bound in the cytoplasm by receptors (importins or karyopherins) that mediate targeting and translocation through the NPC, followed by release of the cargo inside the nucleus. All the importins identified so far are members of the importin β family and contain a characteristic, conserved Ran-GTP binding domain. Importin β family members (exportins) are also involved in nuclear export processes. One of them, CRM1 (Xpo1p in yeast), is the export receptor for the leucine-rich nuclear export signal (NES) found in many different proteins (for review see Görlich and Kutay, 1999). NES-containing proteins that bind to RNA can also serve as adapters in RNA nuclear export as has been shown for viral mRNAs and snRNAs. A central role in nucleocytoplasmic transport is played by the small GTPase Ran and its effectors (Azuma and Dasso, 2000). According to the current view, nuclear Ran is in the GTP-bound form (Ran-GTP), whereas in the cytoplasm Ran-GDP prevails due to the asymmetric subcellular distribution of the Ran nucleotide exchange factor RCC1 (Prp20p in yeast) and the GTPase-activating protein RanGAP1 (Rna1p in yeast). Binding of Ran-GTP to an importin or an exportin in the nucleus regulates the release or the association, respectively, of the cargo, whereas hydrolysis of the Ran-bound GTP in the cytoplasm can cause dissociation of an exportin–cargo complex. In the case of nuclear export of mRNA, the involvement of known exportins and Ran appears not to be direct (Görlich and Kutay, 1999; Strässer and Hurt, 1999). Instead, several RNA-binding proteins such as TAP1/Mex67p or ALY/Yra1p have recently been identified that function as

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[1] *Abbreviations used in this paper: BIB,* beta-like import receptor binding; GFP, green fluorescent protein; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; SRP, signal recognition particle.

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export factors (Cole, 2000; Strässer et al., 2000; Zhou et al., 2000). Unlike mRNA, tRNA is capable of actively exiting the nucleus by associating directly with the exportin Los1p/Xpo-t. However, a Los1p-independent but aminoacylation-dependent nuclear tRNA export pathway has also been identified in yeast (for review see Grosshans et al., 2000b).

Here we study the biogenesis and nuclear export of another conserved ribonucleoprotein complex, the signal recognition particle (SRP). In eukaryotes, SRP mediates the cotranslational targeting of secretory and membrane proteins to the ER membrane (for review see Brodsky, 1998; Kalies and Hartmann, 1998; Bui and Strub, 1999; Stroud and Walter, 1999). The SRP binds to the hydrophobic ER–targeting (signal) sequences of nascent proteins and targets the ribosome–nascent chain complex to the SRP receptor, which is anchored in the ER. Subsequently, the SRP is released and the ribosome–nascent chain complex is delivered to the ER translocation machinery. Translation then resumes with concomitant translocation of the nascent chain through the lipid bilayer of the ER membrane. Mammalian SRP is composed of a 7S RNA (SRP-RNA) and six proteins which associate with the RNA as monomers (SRP19 and SRP54) or heterodimers (SRP9/14 and SRP68/72). The best characterized component is SRP54, a GTPase which recognizes the signal sequence and also mediates the interaction with the SRP receptor. SRP19 is thought to facilitate the association of SRP54 with the SRP-RNA. These two proteins, together with the SRP68/72 dimer and the core sequence of the SRP-RNA, constitute the S domain of the SRP. SRP9 and SRP14 bind to the 5′ and 3′ sequences of the RNA to form the Alu domain, which is involved in elongation arrest. Yeast SRP resembles its mammalian counterpart in that it also consists of six proteins (Srp72p, Srp68p, Srp54p, Sec65p, Srp21, and Srp14p) and a single RNA molecule called scR1 (Hann and Walter, 1991; Brown et al., 1994; Mason et al., 2000). All the proteins are homologous to those of mammalian SRP (Sec65p being the homologue of SRP19) except Srp21p which is yeast specific. No yeast homologue of SRP9 exists, but the function of this protein is most likely carried out by a second copy of Srp14p, which binds to an Alu-like sequence as a homodimer (Strub et al., 1999; Mason et al., 2000). Four of the protein subunits of yeast (Srp14p, Srp21p, Srp68p, and Srp72p) were shown to be required for the stable expression of the SRP (Brown et al., 1994), suggesting that together with scR1, they build up a stable "core" particle to which Sec65p and Srp54p can subsequently bind.

In contrast to the wealth of functional and structural data on SRP (Stroud and Walter, 1999), relatively little is known about its biogenesis and assembly in vivo. The SRP-RNA is transcribed by RNA polymerase III and, in the case of mammalian SRP-RNA, undergoes limited processing at the 3′ end: three uridylates are removed and a single adenylate is added (Chen et al., 1998; Sinha et al., 1998, 1999). This processing is thought to require an intact Alu domain, i.e., binding of the SRP9/14 heterodimer. SRP-RNA microinjected into the nuclei of mammalian cells localizes transiently in the nucleolus before it appears in the cytoplasm, suggesting a nucleolar phase in the biogenesis of the SRP (Jacobson and Pederson, 1998). In agreement with this observation, endogenous SRP-RNA has been detected in the nucleoli of rat fibroblasts together with three green fluorescent protein (GFP)-tagged transfected SRP proteins, SRP19, SRP68, and SRP72 (Politz et al., 2000). Microinjection studies in *Xenopus* oocytes further showed that nuclear export of SRP-RNA is a carrier-mediated and -facilitated process that also depends on the presence of the Alu domain (He et al., 1994).

To analyze the biogenesis of the SRP in yeast, we localized both its protein components (as GFP fusion proteins) and scR1 (by FISH) in wild-type and mutant yeast cells. Our results suggest that assembly of a nuclear export–competent SRP takes place in the nucleolus and requires the four "core" SRP proteins, which are actively imported into the nucleus by the ribosomal import pathway, as well as an intact scR1 3′ end. Subsequent transport into the cytoplasm involves the nuclear export factor Xpo1p and the nucleoporin Nsp1p (provided by O. Gadal, BZH, Heidelberg, Germany).

Materials and Methods

Yeast Strains and Plasmids

The yeast strains carrying deletions in the SRP genes were a gift from P. Walter (University of California at San Francisco, San Francisco, CA) and are listed together with the other strains used in this study in Table I. The following plasmids were used: pUN100 (CEN/ARS, *LEU2*; Elledge and Davis, 1988); pRS315 (*CEN/ARS*, *LEU2*), pRS316 (*CEN/ARS*, *URA3*; Sikorski and Hieter, 1989); pSCR1 (Ogg and Walter, 1995); pRS313-XPO1 and pRS313-xpo1-1 (Stade et al., 1997); pRS315-NOP1::GFP (Hellmuth et al., 1998), peGFP-L25 (Gadal et al., 2001), pGFP-L25$_{NLS}$ (Nehrbass et al., 1993), pGAD-GFP (GFP-NLS$_{SV-40}$; Shulga et al., 1996), pRS425-GFP-YRB1 (Künzler et al., 2000), pGFP-YAP1 (Yan et al., 1998), and pUN100-DsRed-NOP1.

Cloning and GFP Tagging of SRP Proteins

The genes coding for the SRP protein subunits were amplified by PCR from yeast genomic DNA using oligonucleotides containing appropriate restriction sites, and hybridizing 200–300 nucleotides upstream (for the sense oligonucleotide) or downstream (for the antisense oligonucleotide) of the corresponding ORFs. The amplified genes were cloned into vectors pRS315 or pRS316 and their functionality was tested by complementation of the slow growth phenotype of the corresponding yeast disruption mutants. To fuse the GFP reporter protein at the NH_2 termini of the SRP proteins, the sense oligonucleotides used for PCR amplification were hybridized at the beginning of the ORFs immediately after the start ATG codons. These amplified products were cloned into vector pRS315-NOP1::GFP, thus creating GFP-SRP protein fusions under the control of the constitutive *NOP1* promoter. The expression and stability of the corresponding full-length fusion proteins were checked by Western blot analysis of total yeast cell extracts using an antibody against GFP (CLONTECH Laboratories, Inc.) and their functionality was confirmed as described above.

FISH

The yeast SRP-RNA (scR1) was localized by FISH essentially as described for tRNA, except that *Escherichia coli* 5S rRNA in the hybridization buffer was replaced with the same concentration of tRNA (Grosshans et al., 2000a). Hybridizations were performed in hybridization buffer containing 50% formamide (for further details see Amberg et al., 1992) with a mixture of three Cy3-labeled oligonucleotide probes (SRP1, 5′-AATTCTCAACGTATCCCATCCCACC-3′; SRP2, 5′-CACTTCAG-AACGGACTCTCCCGCCT-3′; and SRP3, 5′-TGCCTTAACCAAC-TGGGCCAAGAG-3′) at 4 pmol/μl each at 37°C overnight. DNA was stained with 50 ng/ml DAPI and the slides were mounted with Mowiol. Poly(A)$^+$ RNA was localized using an FITC- or Cy3-labeled oligo(dT)$_{50}$ probe and tRNA was localized using a mixture of 5 pmol/μl each of the

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Table I. Yeast Strains

Strain	Genotype	Reference
BHY116	α, trp1, lys2, his3, ura3, ade2, [rho −], srp54::HIS3	Hann and Walter, 1991
BHY133	α, trp1, lys2, his3, ura3, ade2, [rho −], scr1::HIS3	Brown et al., 1994
CSY186	α, trp1, lys2, his3, ura3, ade2, [rho −], sec65::HIS3	Stirling and Hewitt, 1992
D348	a, ura3-52, lys2, trp1, ade2, rrp44-1	Bousquet-Antonelli et al., 2000
JDY100	α, trp1, lys2, his3, ura3, ade2, [rho −], srp72::HIS3	Brown et al., 1994
JDY105	α, trp1, lys2, his3, ura3, ade2, [rho −], srp21::HIS3	Brown et al., 1994
JDY66	α, trp1, lys2, his3, ura3, ade2, [rho −], srp14::HIS3	Brown et al., 1994
JDY98	α, trp1, lys2, his3, ura3, ade2, [rho −], srp68::HIS3	Brown et al., 1994
mex67-5	α, ura3, ade2, his3, leu2, trp1, mex67::HIS3 <mex67-5, LEU2, CEN>	Segref et al., 1997
MNY7	a, leu2, his3, trp1, ura3, xpo1::KAN^r <XPO1-HA, LEU2, CEN>	Neville and Rosbash, 1999
MNY8	a, leu2, his3, trp1, ura3, xpo1::KAN^r <xpo1-T539C-HA, LEU2, CEN>	Neville and Rosbash, 1999
nsp1-L640S	a, ade2-1, his3-11,15, ura3-52, leu2-3,112, trp1-1, can1-100, nsp1::HIS3 <nsp1-L640S, TRP1, CEN>	Wimmer et al., 1992
nsp1-ala6	a, ade2-1, his3-11,15, ura3-52, leu2-3,112, trp1-1, can1-100, nsp1::HIS3 <nsp1-ala6-Δrep, LEU2, CEN>	Wimmer et al., 1992
nup49-313	α, ade2, ade3, his3, leu2, lys2, ura3, trp1, nup49::TRP1 <nup49-313, LEU2, CEN>	Doye et al., 1994
nup82-27	a, ade2-1, his3-11,15, ura3-52, leu2-3,112, trp1-1, can1-100, nup82::HIS3 <PtA-nup82-27, LEU2, CEN>	Bailer et al., 2000
nup85-ΔN	ura3, leu2, ade2, trp1, nup85::HIS3 <nup85-ΔN, TRP1, CEN>	Siniossoglou et al., 1996
P54	a, ade1-100, his4-519, leu2-3,112, ura3-52, rrp4-1	Mitchell et al., 1996
prp20-1	ade2, his3, leu2, trp1, ura3, prp20-1	Hurt et al., 1999
PSY1042	a, ura3, leu2, trp1, pse1-1, kap123::HIS3	Seedorf and Silver, 1997
PSY1200	a, ura3-52, leu2Δ1, his3Δ200, trp1Δ63, sxm1::HIS3	Seedorf and Silver, 1997
PSY1201	a, pse1-1, ura3-52, trp1Δ63, leu2Δ2	Seedorf and Silver, 1997
PSY967	α, ura3-52, leu2Δ1, his3Δ200, kap123::HIS3	Seedorf and Silver, 1997
rat7-1	a, his3-200, ura3-52, leu2-1, rat7::HIS3 <rat7-1, LEU2, CEN>	Gorsch et al., 1995
rna1-1	ade2, his3, leu2, trp1, ura3, rna1-1	Hurt et al., 1999
RS453	a/α, ade2-1, his3-11,15, ura3-52, leu2-3,112,trp1-1, can1-100	Hellmuth et al., 1998
srp1-31	a, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, srp1-31	Loeb et al., 1995
srx1-1	α, ura3-52, his3Δ200, leu2Δ1, srx1-1	Hann and Walter, 1991
TR3	α, trp1, lys2, his3, ura3, ade2	D. Lau, derived from Neville and Rosbash, 1999
XPO1	a, leu2, his3, trp1, ura3, xpo1::KAN^r <HIS3, XPO1, CEN>	D. Lau, derived from Neville and Rosbash, 1999
xpo1-1	a, his3, leu2, ura3, ade3, trp1, xpo1::KAN^r <HIS3, xpo1-1, CEN>	Neville and Rosbash, 1999
Y1171	α, ade2-1, his3-11,15, ura3-52, leu2-3,112, trp1-1, can1-100, mtr10::HIS3 <mtr10-7, TRP1, CEN>	Senger et al., 1998

previously described probes against $tRNA^{Glu(UUC)}$ and $tRNA^{Gly(GCC)}$ (Grosshans et al., 2000a).

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from yeast cells as described (Sharma et al., 1996), separated on a 6% urea-polyacrylamide denaturing gel and transferred to a Hybond XL membrane. Hybridization with radioactively labeled oligonucleotides with identical sequences to the probes used for FISH was performed at 37°C in 6× SSPE (900 mM NaCl, 60 mM NaH_2PO_4, 0.3 mM EDTA) overnight.

Leptomycin B Treatment of Leptomycin B–sensitive Yeast

Yeast cells expressing the *XPO1* wild-type allele or the leptomycin B (LMB)-sensitive *xpo1-T539C* point mutant allele (Neville and Rosbash, 1999) were grown in minimal medium to an OD_{600} of 0.6, 10 ml was spun down and resuspended in 1 ml medium, and 10 μl of 10 μg/ml LMB in ethanol was added. As a control, 10 μl of ethanol alone was added. Cells were incubated for 20 min at 30°C before fixation. The LMB-sensitive *XPO1* mutant strain and LMB were gifts from M. Rosbach (Brandeis University, Waltham, MA) and M. Yoshida (University of Tokyo, Tokyo, Japan), respectively.

Screen for SRP-RNA Export Mutants

A collection of yeast *ts* mutant cells used previously to identify mRNA export mutants (Amberg et al., 1992) was screened for temperature-dependent intranuclear accumulation of SRP-RNA. Cells were grown at 23°C and shifted to 37°C for 4 h. The complementing gene for *srx1* mutant cells (SRP-RNA export), which accumulated SRP-RNA inside the nucleus at 37°C, but not at 23°C, was cloned by complementation of the *ts* phenotype through transformation with a yeast genomic DNA library on a centromeric vector (Bergès et al., 1994). Sequencing of two of the complementing plasmids, which showed similar but not identical restriction patterns, revealed *NSP1* as the only complete ORF present on both plasmids. Complementation by *NSP1* was confirmed using plasmids pSB32-NSP1 containing full-length *NSP1* (Wimmer et al., 1992) and pNOP::ProtA-NSP1-C, respectively, containing the essential COOH terminus of NSP1 under control of the NOP1-promoter (provided by S. Bailer, BZH, Heidelberg, Germany).

scR1 3′ End Determination

The 3′ ends of scR1 from wild-type and *rrp44-1* mutant cells, respectively, were determined by a modified 3′ rapid amplification of cDNA ends (RACE) approach (Li et al., 1998). In brief, 100 pmol of a 3′ end-blocked DNA oligonucleotide (sequence: GATTGACAGGATCCTAAGTTCC*, C* being cordycepin, i.e., 3′ deoxyoadenosine, from NAPS) was annealed to the 3′ ends of 5 μg of total RNA by incubation with 15 U of T4 RNA ligase at 0°C on ice overnight in 50 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 10 mM DTT, 0.01 mg/ml BSA, and 1 mM ATP. The annealed RNA was reverse transcribed and a 282-bp fragment of the scR1 3′ end was amplified using the Titan One Tube reverse transcription PCR kit (Roche Diagnostics) according to the manufacturer's instructions and oligos scR1-3′-complement (GAACTTAGGATCCTGTCAATC) and scR1-For(261) (TTTCTCGAGGCGTGAGGAATCCGT), the latter introducing an XhoI site at the 5′ end of the fragment. Resulting amplification products were cloned into pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions. Sequencing of several randomly selected, positive clones was performed by TopLab (Martinsried). The sequences obtained were, with the exceptions mentioned in Results, identical to the previously predicted 3′ end of scR1 (Felici et al., 1989). However, in contrast to the sequence published previously (Felici et al., 1989), and in agreement with the sequence in the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/), we found an additional two nucleotides, C and A, at positions +363/4, whereas G at position +404 was absent.

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Sequence Analysis

Comparison of the SRP core protein sequences to the nuclear localization signals (NLSs) of yeast ribosomal protein L25 characterized previously (amino acids 1–41; Schaap et al., 1991) and mammalian ribosomal protein L23a [beta-like import receptor binding (BIB)] domain, amino acids 32–74; Jäkel and Görlich, 1998) was performed by pairwise sequence alignments using the Baylor College of Medicine search launcher (http://searchlauncher.bcm.tmc.edu). BLOSUM 62 was used as comparison matrix, gap open penalty was set at 12, and gap extension penalty was set at 4.

Miscellaneous

Cells were examined using a fluorescence microscope (Axioskop; ZEISS) equipped with a CCD camera (Microimager; Xillix). Data were processed using the Improvision Openlab and Adobe Photoshop® softwares. All experiments were performed on exponentially growing cells. All shifts to 37°C were performed in glucose-containing rich medium (YPD). DNA manipulations such as restriction analysis, PCR amplification, and ligation were performed according to standard protocols (Sambrook et al., 1989). The anti-SRP antibodies were a gift from P. Walter (University of California at San Francisco, San Francisco, CA).

Online Supplemental Material

The expression level and functionality of the GFP-tagged SRP proteins were analyzed by Western blot and complementation of the srp null strains, respectively. Yeast GFP-tagged SRP proteins were localized in the *mtr10-7*, *sxm1*, *srp14*, and *srp68* mutant yeast strains. Two-hybrid analysis was performed using Xpo1p as bait and the individual SRP proteins as preys. The two hybrid plasmids with XPO1 were a gift from F. Stutz (Microbiology Institute, Lausanne, Switzerland). These results and the relevant experimental details are available at http://www.jcb.org/cgi/content/full/153/4/745/DC1.

Results

Detection of the SRP-RNA by FISH

Yeast SRP-RNA (scR1), which is predicted to be 519 nucleotides long, represents 0.2% of the total yeast RNA (Felici et al., 1989). Because of this abundance, we sought to detect scR1 by fluorescence in situ hybridization using a method that recently allowed us to detect single yeast tRNA species (Grosshans et al., 2000a). As probes, we used a mixture of three synthetic deoxyoligonucleotides labeled at their 5′ ends with the fluorochrome Cy3, which are complementary to positions 19–43, 190–214, and 336–360 of scR1, respectively (Fig. 1 A). To test their specificity, we performed Northern blot analysis on total yeast RNA using the same oligonucleotides labeled with ^{32}P. As shown in Fig. 1 B, the probes reacted with a single band corresponding to scR1, which was absent when RNA was isolated from a yeast strain with deleted scR1 gene. Transformation of the *scR1*$^-$ strain with a plasmid containing the scR1 gene led to the reappearance of this band, showing that the oligonucleotide probes were specific for yeast SRP-RNA. When these fluorescent SRP-RNA probes were used for in situ hybridization, a strong cytoplasmic labeling with nuclear exclusion was observed (Fig. 1 C). Apparently, the signal was often stronger around the nuclear periphery, which might reflect the preferential localization of SRP at the ER membrane. Similar results were obtained when the single probes were used, although the overall intensity of the signal was weaker (data not shown). Finally, we performed the same experiment with the cells lacking the scR1 gene. This experiment revealed only background staining in the *scR1*$^-$ cells (Fig. 1 D). Thus, we can specifically localize by in situ hybridization yeast SRP-RNA, which is predominantly found in the cytoplasm and concentrated around the nuclear membrane.

The Core SRP Proteins, Srp14p, Srp21p, Srp68, and Srp72, Are Required for Efficient Nuclear Export of the SRP-RNA

SRP is not essential for cell growth in yeast, but cells lacking any of the genes coding for the SRP components grow poorly and are impaired in ER targeting of proteins that contain strongly hydrophobic signal sequences (Ng et al., 1996). Moreover, absence of any one of Srp14p, Srp21p, Srp68p, or Srp72p causes a strong decrease in the cellular amounts of scR1, probably by reducing its stability (Brown et al., 1994). The absence of Sec65p or Srp54p, in contrast, does not influence the expression of scR1, which is assembled together with the other four subunits into a stable "core" SRP. To test whether the lack of any of the SRP proteins affects the subcellular distribution of scR1, we localized it in the corresponding disruption mutants. In cells lacking Sec65p or Srp54p, scR1 was distributed normally with a strong signal in the cytoplasm and nuclear exclusion (Fig. 2). This distribution was similar to wild-type cells (see above), but the perinuclear staining was less prominent. Therefore, in the absence of either Sec65p or Srp54p, scR1 can be efficiently exported into the cytoplasm. In contrast, in cells lacking any of the other four SRP proteins, the intensity of the signal was reduced and the residual scR1 was located predominantly inside the nucleus with very little staining in the cytoplasm (Fig. 2). We conclude that absence of any of Srp14p, Srp21p, Srp68p, or Srp72p not only reduces the stability of scR1 but also inhibits its export into the cytoplasm.

The Core SRP Proteins Concentrate in the Nucleolus

The requirement of the four SRP core proteins for the export of scR1 into the cytoplasm suggests that these proteins assemble with scR1 inside the nucleus to form a transport-competent particle. To localize the SRP protein subunits in living cells, they were tagged at their NH_2 termini with GFP and expressed in the corresponding disruption strains. Functionality of the tagged proteins was shown by complementation of the slow-growth phenotype of the disruption mutants and expression levels were confirmed by Western blot analysis (data not shown). When inspected by fluorescence microscopy, GFP-Srp54p localized only to the cytoplasm, whereas the other five SRP proteins concentrated in the nucleus exhibiting, in addition, a cytoplasmic staining which was strongest for Sec65p and weakest for Srp14p (Fig. 3 A). Closer inspection revealed that GFP-tagged Srp14, Srp21, Srp68p, and Srp72p accumulated in the nucleolus as shown by colocalization with the nucleolar marker Nop1p tagged with the red fluorescent protein DsRed (Fig. 3 B). Similar results were obtained when GFP-tagged SRP proteins were localized in wild-type or *scR1*$^-$ cells (data not shown). Therefore, the steady-state nucleolar localization of the tagged SRP proteins is not affected by the presence of endogenous copies and, importantly, by the absence of the SRP-RNA component.

To address the question of whether the SRP proteins are imported into the nucleus independent of one another

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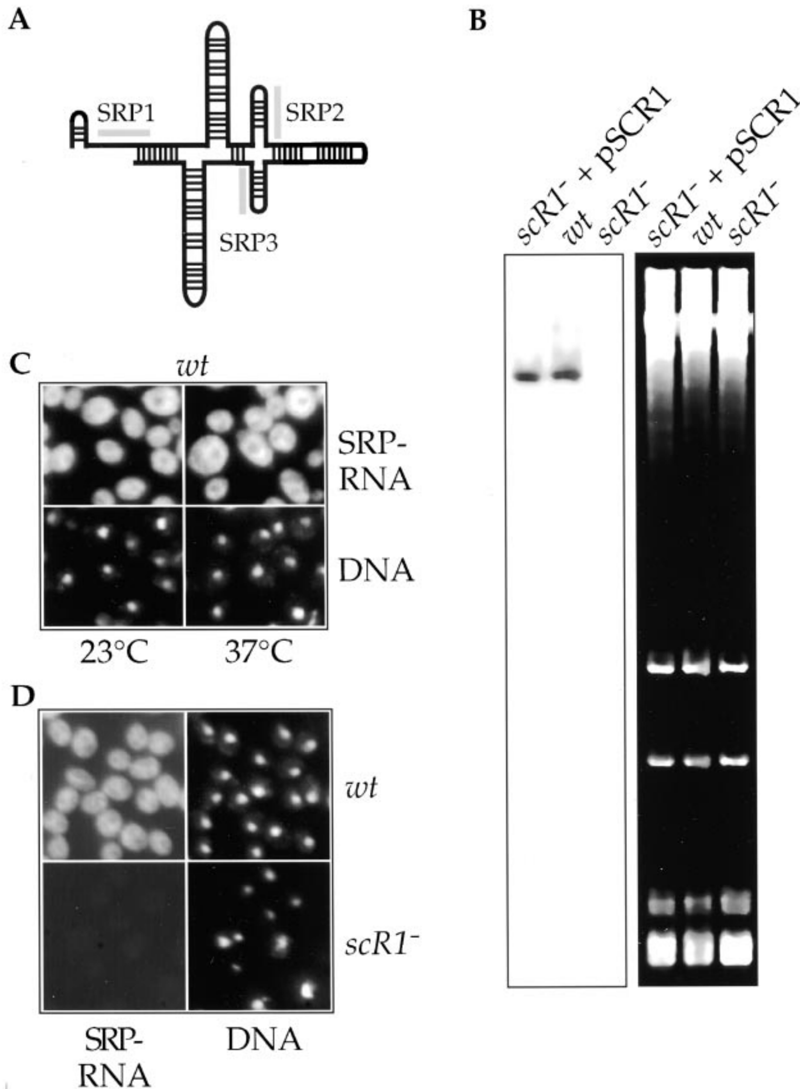

Figure 1. Localization of the yeast SRP-RNA by fluorescence in situ hybridization. (A) A highly schematic representation of the yeast SRP-RNA, scR1 (adapted from Felici et al., 1989). The gray lines represent the areas where the probes used in this study hybridize. (B) Detection of scR1 by Northern blot analysis. Total RNA was extracted from *scrR1*⁻ cells with an empty plasmid or a centromeric plasmid harboring the *SCR1* gene (pSCR1), or the isogenic wild-type strain TR3 (wt) grown at 30°C and separated on a 6% denaturing urea-polyacrylamids gel. (Left) Autoradiography after hybridization with a mixture of the three radioactive probes depicted in A. (Right) Corresponding ethidium bromide staining of the gel before transfer. (C) Localization of scR1 in wild-type RS453 cells grown at 23°C or shifted to 37°C for 4 h by in situ hybridization using a mixture of the three fluorescently labeled probes depicted in A. DNA was stained by DAPI. (D) Localization of scR1 in *scR1*-Δ2 (scrR1⁻) or isogenic wild-type TR3 cells grown at 30°C. Pictures were taken at identical exposure times. DNA was stained by DAPI.

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or as heterodimers or -oligomers, we analyzed SRP protein localizations in mutants lacking a given SRP protein, e.g., Srp14p, Srp21p, Srp68p, and Sec65p in *srp72*⁻ cells. No difference in localization was found for any of these proteins in any combination (data not shown), suggesting that the SRP proteins are imported into the nucleus independent of one another.

The Core SRP Proteins Use the Same Nuclear Import Receptors as the Ribosomal Proteins

To investigate the nuclear import route of the SRP proteins, their localization was examined in different nucleocytoplasmic transport mutants. A classical nuclear transport mutant is *rna1-1*, which affects the yeast Ran cycle (Corbett et al., 1995). When these cells were grown at 23°C (the permissive temperature) GFP-tagged SRP proteins exhibited a wild-type localization (i.e., nuclear and cytoplasmic for Sec65p and predominantly nucleolar for the core SRP proteins), but when shifted for 2 h to 37°C the nuclear or nucleolar accumulation was largely abolished with a concominant increase in the cytoplasmic labeling (Fig. 4 A and data not shown). Similarly, nuclear accumulation of SRP proteins was inhibited in the *prp20-1* mutant, which is defective in the yeast RanGEF (data not shown). This shows that nuclear import of the SRP proteins requires a functional Ran cycle.

We next tested which import receptors are involved in nuclear uptake of the SRP proteins. Although nuclear accumulation of GFP-Sec65p was drastically reduced in the *srp1-31* mutant (defective in the yeast importin α), the nucleolar localization of Srp14p, Srp21p, Srp68p, and Srp72p was not affected, showing that the SRP core proteins are not imported by the classical NLS-mediated pathway (Fig. 4 B and data not shown).

To identify the potential import receptors, we analyzed the SRP core protein distribution in several importin mutants. Interestingly, nuclear uptake of the core SRP proteins was strongly impaired only in the double mutant strain *pse1-1 kap123*⁻ (Fig. 4 C and data not shown), which is also defective in the nuclear import of ribosomal proteins (Rout et al., 1997; Schlenstedt et al., 1997). To find out which of the two proteins, Kap123p or Pse1p, is more important for this import route, we also tested SRP protein localization in the single mutant strains *pse1-1* or *kap123*⁻ (Fig. 4, D and E, and data not shown). Except for Srp68p, which was mislocalized in both the *pse1-1* or *kap123* single

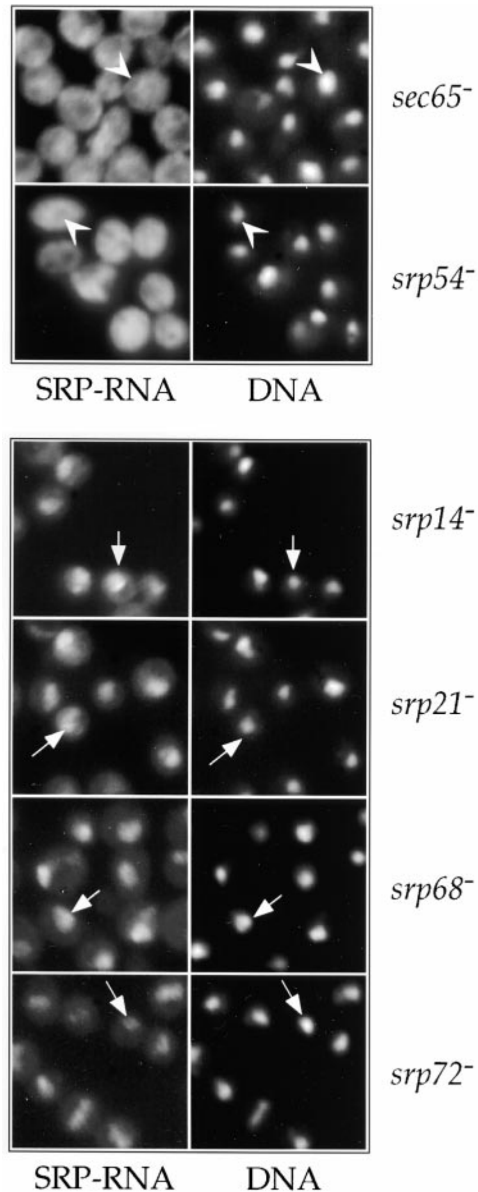

Figure 2. Intranuclear accumulation of yeast SRP-RNA in the absence of a subset of SRP proteins. scR1 was localized in cells disrupted for the indicated genes and grown at 30°C. Representative cells are labeled by arrows (in the case of scR1 accumulation) or arrowheads (lack of accumulation) pointing to the nucleoplasmic space as judged by DAPI staining.

mutants, only weak effects were seen for the SRP core proteins in the single mutants, suggesting that both Kap123 and Pse1p are required for efficient nucleolar import of SRP proteins. Normal nucleolar accumulation was observed in other importin mutants, such as the *sxm1*$^-$ (Rosenblum et al., 1997) and *mtr10-7* (Senger et al., 1998) strains (data not shown). Nuclear accumulation of Sec65p was also affected in the *pse1-1 kap123*$^-$ mutant and, to a lesser degree, in the *sxm1*$^-$ and *mtr10-7* mutants (Fig. 4 C and data not shown). These results suggest that all the core SRP proteins, Srp14p, Srp21p, Srp68, and Srp72p, follow the same transport route as the ribosomal proteins (Rout et al., 1997; Schlenstedt et al., 1997), which is mediated by the importins/karyopherins Pse1p and Kap123p.

The Nuclear Export of the SRP-RNA Is Distinct from mRNA Export and Mediated by Xpo1p

To identify the nuclear export route of scR1, we localized SRP-RNA in strains with an impaired Ran cycle, i.e., the above mentioned *rna1-1* and *prp20-1* mutant cells (Aebi et al., 1990; Corbett et al., 1995). At permissive temperature, scR1 is exported normally in these cells. In contrast, incubation at the restrictive temperature leads to a nuclear export defect which is only weak in *rna1-1* cells, but extensive in *prp20-1* cells (Fig. 5 A). These data show that an intact Ran cycle is required for scR1 nuclear export. This might be due to the impairment of SRP core protein nuclear import under these conditions. Alternatively, (an) exportin(s) might be involved in scR1 nuclear export. To address this question, we analyzed mutants in which one of the four yeast exportins, Xpo1p (Stade et al., 1997), Cse1p (Hood and Silver, 1998; Künzler and Hurt, 1998; Solsbacher et al., 1998), Msn5p (Kaffman et al., 1998), or Los1p, the tRNA exportin (Hellmuth et al., 1998), was absent or mutated. In the *los1*$^-$, *cse1-1*, and *msn5*$^-$ mutant cells, the localization of scR1 was normal and indistinguishable from that in wild-type cells, suggesting that the corresponding proteins are not involved in the nuclear export of scR1 (data not shown). However, when scR1 distribution was analyzed in cells expressing the *xpo1-1 ts* mutant allele, nuclear accumulation of scR1 could be observed in few cells already at the permissive temperature (Fig. 5 B). This defect was dramatically enhanced after shift to 37°C for 5 min, and after 30 min virtually all cells accumulated scR1 inside their nuclei. No mislocalization of scR1 could be detected when an isogenic strain expressing the wild-type *XPO1* gene was incubated at 37°C (Fig. 5 B).

The *xpo1-1* mutation has been shown previously to cause nuclear accumulation of polyadenylated RNA (Stade et al., 1997). Therefore, it is possible that the scR1 nuclear export defect seen in this mutant is caused indirectly by the poly(A)$^+$ RNA nuclear accumulation, or even reflects the use of identical export pathways for both scR1 and mRNA. To address this possibility we colocalized scR1 and poly(A)$^+$ RNA in *xpo1-1* cells using Cy3-labeled anti-scR1 and FITC-labeled oligo-dT probes. Although after 30 or 60 min of incubation at the restrictive temperature most cells accumulated both types of RNA inside their nuclei, we could readily find cells that accumulated scR1 but not poly(A)$^+$ RNA, suggesting that the scR1 nuclear accumulation was not simply a consequence of the export defect for mRNA (data not shown). More importantly, no nuclear accumulation of scR1 could be observed in the *mex67-5 ts* mutant incubated at 37°C (Fig. 5 C), i.e., under conditions where poly(A)$^+$ RNA export is strongly impaired (Segref et al., 1997). This, and the data presented below, strongly argue for distinct nuclear export mechanisms for mRNA and scR1.

Recently, a *Saccharomyces cerevisiae* strain has been constructed, which contains an *XPO1* allele sensitive to the drug LMB due to a single amino acid substitution (Neville and Rosbash, 1999). In this strain, nuclear export of an NES-containing reporter protein is rapidly inhibited

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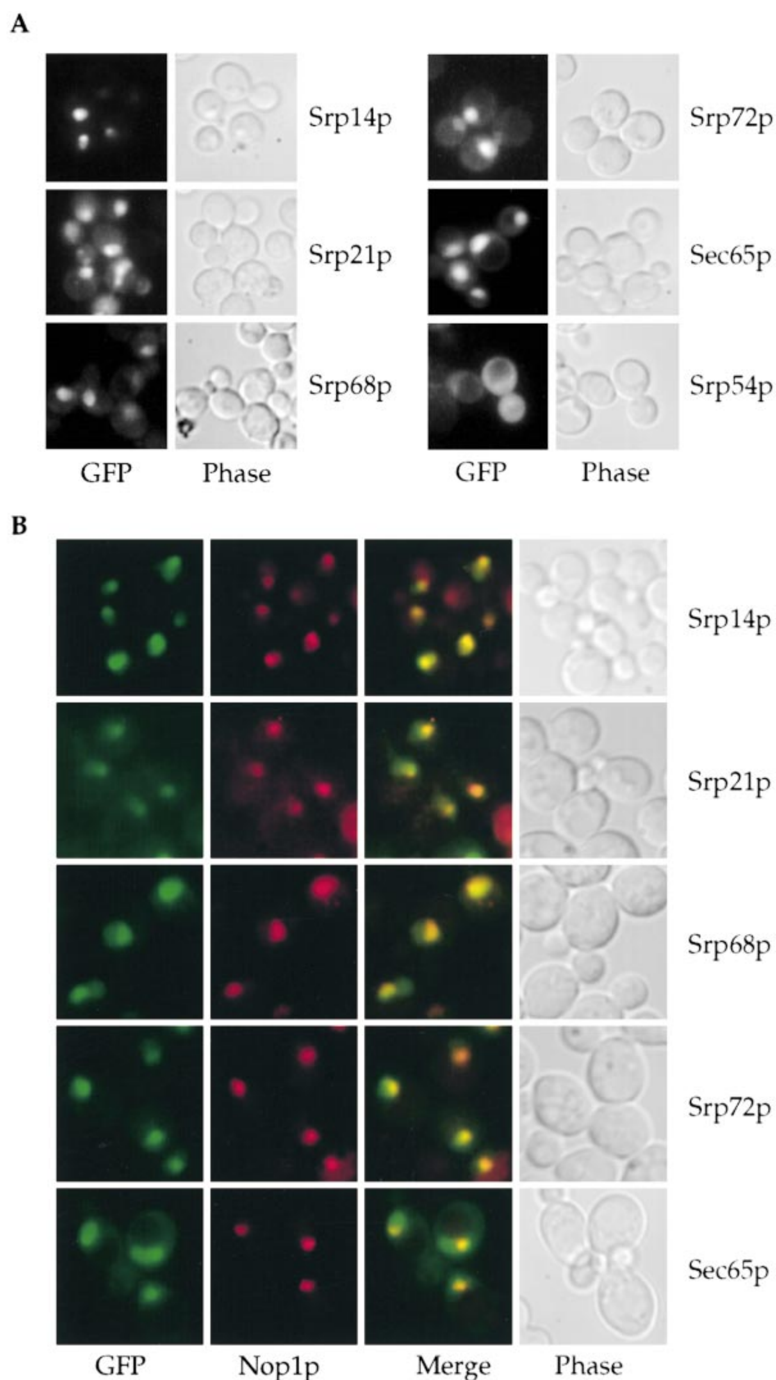

Figure 3. The yeast SRP core proteins are detected in the nucleolus. (A) The indicated GFP-tagged fusion proteins were localized in the corresponding disruption strains grown at 30°C. (B) GFP-tagged fusion proteins were localized as in the legend to A, except that cells carried a plasmid coding for the nucleolar marker DsRed-Nop1p. Superimposition (Merge) is shown for cells that have been pseudocolored so that the GFP signal is green, DsRed signal red. Colocalization results in yellow color.

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upon LMB addition, whereas nuclear accumulation of poly(A)$^+$ RNA occurs with a delay. This, together with other findings, was taken as evidence to suggest that Xpo1p is not directly involved in the nuclear export of mRNA and that the nuclear accumulation of poly(A)$^+$ RNA in *xpo1* mutants is an indirect consequence of impaired nuclear protein export (Hodge et al., 1999; Neville and Rosbash, 1999). Therefore, we analyzed nuclear export of scR1 in the LMB-sensitive strain. Strikingly, these cells displayed a prominent nuclear accumulation of scR1 20 min after addition of LMB, whereas under these conditions only weak and infrequent nuclear accumulation of poly(A)$^+$ RNA was observed (Fig. 6). No significant nuclear accumulation could be observed in mock-treated or wild-type cells (Fig. 6). We conclude that nuclear export of scR1 directly involves the NES-export receptor Xpo1p and does not overlap with the mRNA export pathway.

A Mutation in an Exosome Component Causes 3′ End Truncation of SRP-RNA and Its Accumulation in the Nucleolus

Findings mainly from the mammalian system suggest that the SRP-RNA undergoes limited 3′ end processing; i.e., removal of three uridylates and addition of a single adenylate (Chen et al., 1998; Sinha et al., 1998, 1999). A prominent machinery for exonucleolytic 3′-processing of RNA

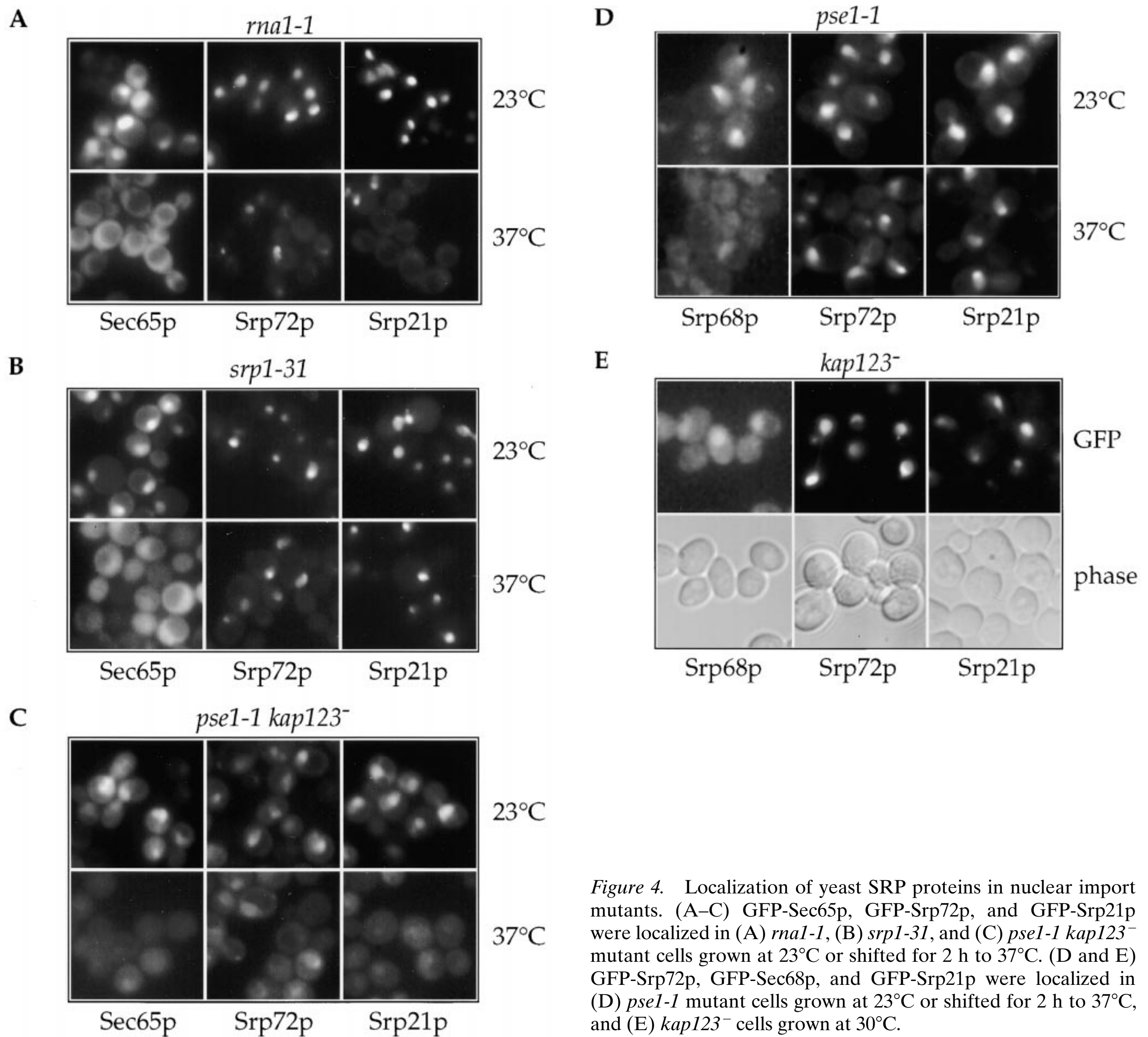

Figure 4. Localization of yeast SRP proteins in nuclear import mutants. (A–C) GFP-Sec65p, GFP-Srp72p, and GFP-Srp21p were localized in (A) *rna1-1*, (B) *srp1-31*, and (C) *pse1-1 kap123⁻* mutant cells grown at 23°C or shifted for 2 h to 37°C. (D and E) GFP-Srp72p, GFP-Sec68p, and GFP-Srp21p were localized in (D) *pse1-1* mutant cells grown at 23°C or shifted for 2 h to 37°C, and (E) *kap123⁻* cells grown at 30°C.

is the exosome complex consisting of at least 10 proteins and required for rRNA as well as snoRNA and snRNA maturation (for review see Mitchell and Tollervey, 2000). In particular, the exosome component Rrp6p was shown to mediate the trimming of the final three nucleotides from the 3′ end of several of these RNAs (Allmang et al., 1999a; van Hoof et al., 2000). To test whether inhibition of the exosome function can cause a defect in the biogenesis of scR1, we analyzed its subcellular distribution in three exosome mutants: *rrp6Δ*, *rrp4-1*, and *rrp44-1*. No aberrant localization could be detected in either *rrp6Δ* or *rrp4-1* mutants (data not shown). However, incubation of the *rrp44-1* cells at the restrictive temperature yielded a strong intranuclear accumulation of scR1 in most cells (Fig. 7 A). Strikingly, the site of accumulation did not coincide with the DNA signal but rather appeared to be located directly adjacent to it, suggesting that scR1 might accumulate in the nucleolus. This notion was confirmed when scR1 was localized in *rrp44-1* cells expressing GFP-Nop1p: the two signals largely overlapped (Fig. 7 B). Combined with the preferential nucleolar localization of the SRP core proteins, the nucleolar accumulation of scR1 in *rrp44-1* cells is suggestive of a role of the nucleolus in SRP biogenesis.

To identify the reason for the scR1 nuclear export defect in *rrp44-1* cells, we first performed Northern blot analysis. No significant change in migration was observed when scR1 extracted from *rrp44-1* mutant cells incubated at restrictive temperature was compared with the RNA from the same cells grown at permissive temperature or wild-type cells (Fig. 7 C), showing that integrity and stability of scR1 is not grossly affected in the exosome mutant. To track down more subtle differences, we analyzed the 3′ ends of scR1 molecules from wild-type and mutant cells using a modified RACE approach. To this end, we extracted total RNA from the cells, annealed a DNA oligonucleotide to the RNA 3′ ends, and reverse transcribed the annealed RNAs using a specific primer. A 282-bp fragment, corresponding to the 3′ end of scR1, was amplified

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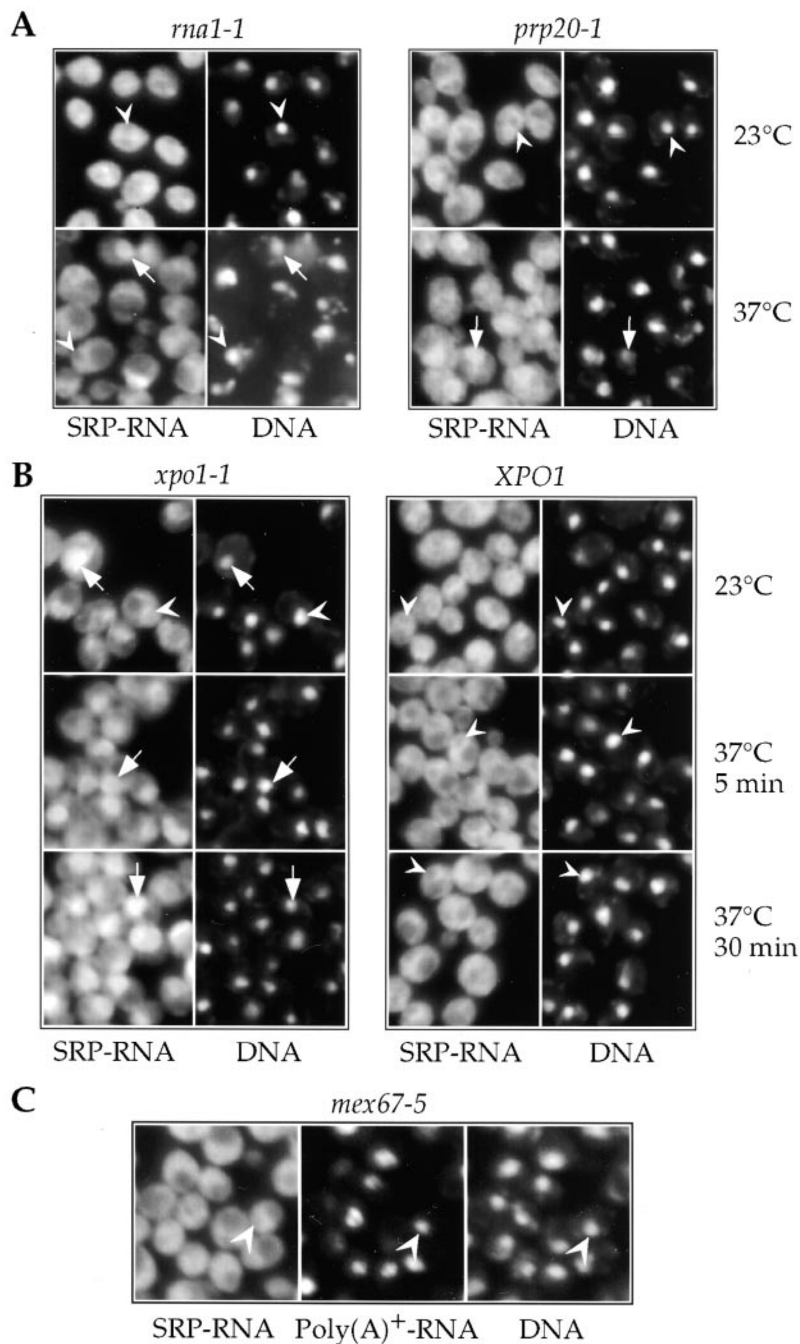

Figure 5. Yeast SRP-RNA nuclear export requires an intact Ran cycle and Xpo1p. (A) Localization of scR1 in RanGAP and RanGEF mutant strains *rna1-1* and *prp20-1*, respectively, grown at 23°C or shifted to 37°C for 4 h. (B) Localization of scR1 in a strain lacking the endogenous *XPO1* gene and carrying a centromeric plasmid with either the *XPO1* wild-type or the *xpo1-1* mutant allele. Cells were grown at 23°C or shifted for the indicated times to 37°C. (C) Colocalization of scR1 and poly(A)$^+$ RNA in *mex67-5* mutant cells shifted for 1 h to 37°C using probes labeled with Cy3 and FITC, respectively. Representative cells are labeled by arrows (in the case of scR1 accumulation) or arrowheads (lack of accumulation, accompanied by poly(A)$^+$ RNA accumulation in panel C) pointing to the nucleoplasmic space as judged by DAPI staining.

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by PCR and cloned into a vector. Sequencing of several independent plasmid clones showed that both in wild-type and *rrp44-1* mutant cells grown at the permissive temperature the scR1 3′ end was either identical to the sequence predicted previously (Felici et al., 1989) or shortened by one or two thymidylates (Table II). In two cases, and in agreement with previous predictions (Sinha et al., 1999), we could also detect 3′ terminal adenylates (Table II) that must have been added posttranscriptionally. In striking contrast, scR1 from *rrp44-1* mutant cells incubated for 4 h at the restrictive temperature displayed longer truncations of the 3′ end in six out of the nine clones sequenced. In two cases, as much as 22 nucleotides were missing from the 3′ end (Table II).

Taken together, these data suggest that the mutation in the *rrp44-1* cells does not affect the overall stability of scR1, but leads to aberrant processing of scR1 3′ ends, thus possibly rendering the RNA incompetent for nuclear export.

The Essential Nucleoporin Nsp1p Is Required for SRP-RNA Nuclear Export

To find other factors involved in scR1 nuclear export, we started screening a collection of random yeast *ts* mutants using the scR1 FISH assay. We identified a *ts* mutant strain (called *srx1-1* for SRP-RNA export), which strongly accumulated scR1 inside the nucleus after the shift to the nonpermissive temperature (Fig. 8 A). The mutated gene

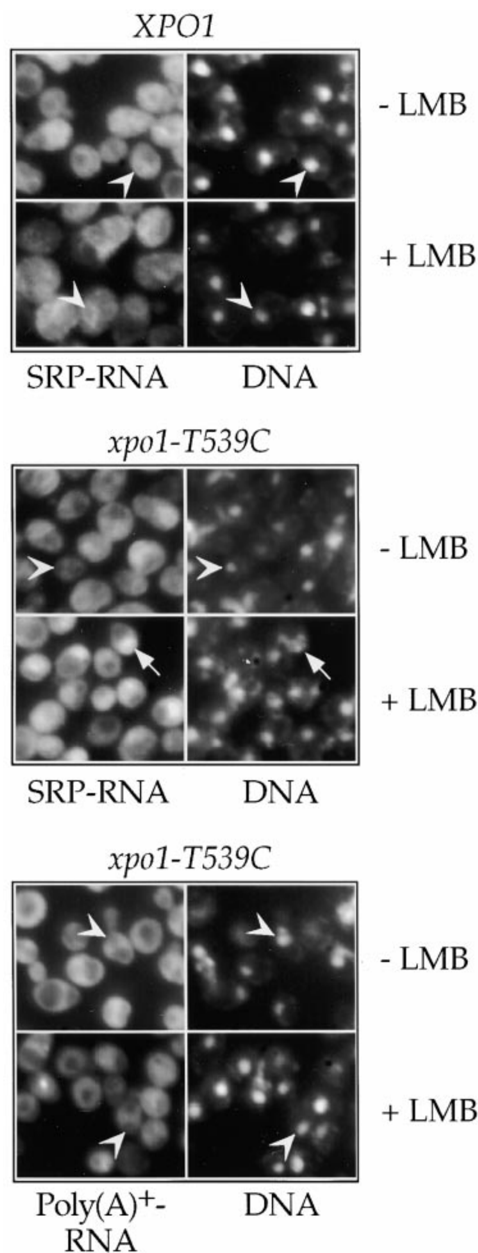

Figure 6. LMB treatment impairs yeast SRP-RNA nuclear export in an LMB-sensitive mutant. Localization of scR1 or poly(A)$^+$-RNA in a strain lacking the endogenous *XPO1* gene and carrying a centromeric plasmid with the *XPO1* wild-type or the LMB-sensitive *xpo1-T539C* mutant allele after 20 min in the presence of ethanol (− LMB) or 100 ng/ml LMB in ethanol (+ LMB). Representative cells are labeled by arrows (in the case of RNA accumulation) or arrowheads (lack of accumulation), respectively, pointing to the nucleoplasmic space as judged by DAPI staining.

in this *ts* strain which causes inhibiton of scR1 nuclear export was cloned by complementation and found to correspond to the essential nucleoporin Nsp1p (Fig. 8 A and data not shown).

The observed nuclear export defect of SRP-RNA in the *srx1-1 ts* mutant might be secondary to impaired nuclear import of components required for nuclear SRP assembly. Therefore, we analyzed in the *srx1-1* cells the localization

*Table II. scR1 3′ Ends**

Sequence	wt (TR3)	*rrp44-1* 23°C	*rrp44-1* 37°C
GGAGTGTGTCCTGAACCATATTTTT	2	4	0
GGAGTGTGTCCTGAACCATATTTT	2	3	2
GGAGTGTGTCCTGAACCATATTT(A)	1‡	1	1
GGAGTGTGTCCTGAACCATATT	0	1	0
GGAGTGTGTCCTGAACCATAT(AAAA)	0	1§	2
GGAGTGTGTCCTGAACCAT	0	0	2
GGA	0	0	2

*3′ sequences determined from cDNA clones.
‡One adenylate present in the clone derived from wild-type(wt) cells.
§Four adenylates present in the clone derived from *rrp44-1* mutant cells at the permissive temperature.

of the GFP-tagged SRP proteins. All of these proteins efficiently accumulated in the nucleus after 4 h incubation of *srx1-1* cells at 37°C, suggesting that their nuclear import is not significantly affected (Fig. 8 B and data not shown). Consistently, no mislocalization was observed for a reporter–GFP construct carrying the Pse1p-Kap123p–dependent L25-NLS (Fig. 8 C; Nehrbass et al., 1993). Finally, normal localization was also observed for GFP reporters containing the importin α/β–dependent SV-40–NLS (pGAD-GFP; Shulga et al., 1996) or full-length Srp1p (yeast importin α, Fig. 8 C; Künzler and Hurt, 1998). Thus, Nsp1p is required for the nuclear export of scR1.

To analyze additional export pathways in this mutant, we localized polyadenylated RNA and tRNA by FISH. Only weak nuclear accumulation of polyadenylated RNA was observed in a subset of *srx1-1* mutant cells upon shift to the restrictive temperature, whereas $tRNA^{Glu}$ and $tRNA^{Gly}$ did not accumulate (Fig. 8 D). Similarly, no accumulation of GFP-tagged ribosomal protein L25 (Gadal et al., 2001) was observed at the restrictive temperature, suggesting that ribosomal export proceeds unaffected in this mutant (Fig. 8 E).

To find out whether export of other Xpo1p cargoes is also affected in the *srx1-1* mutant strain, we analyzed the distribution of two other Xpo1p transport substrates, Yap1p (Yan et al., 1998), a transcription factor for antioxidative genes, and Yrb1p (Künzler et al., 2000), the yeast homologue of mammalian RanBP1. GFP-Yap1p continued to be cytoplasmic and excluded from the nucleus upon shift to the restrictive temperature (Fig. 8 E). GFP-Yrb1p, in contrast, was found to accumulate in the nuclei of a subset of cells after 4 h at the restrictive temperature, a defect that was increased after prolonged incubation at the nonpermissive temperature (Fig. 8 E and data not shown).

In summary, our data suggest that the *srx1-1* mutant cells are defective in several nuclear export pathways, including two Xpo1p substrates, whereas nuclear import proceeds normally.

A Subset of Nucleoporins Is Involved in scR1 Nuclear Export

The finding that Nsp1p is required for scR1 nuclear export prompted us to investigate the requirement of NPC components in this process. To confirm our initial finding, we first analyzed two previously described mutant alleles of *NSP1*, *nsp1-L640S*, and *nsp1-ala6-Δrep* (Wimmer et al.,

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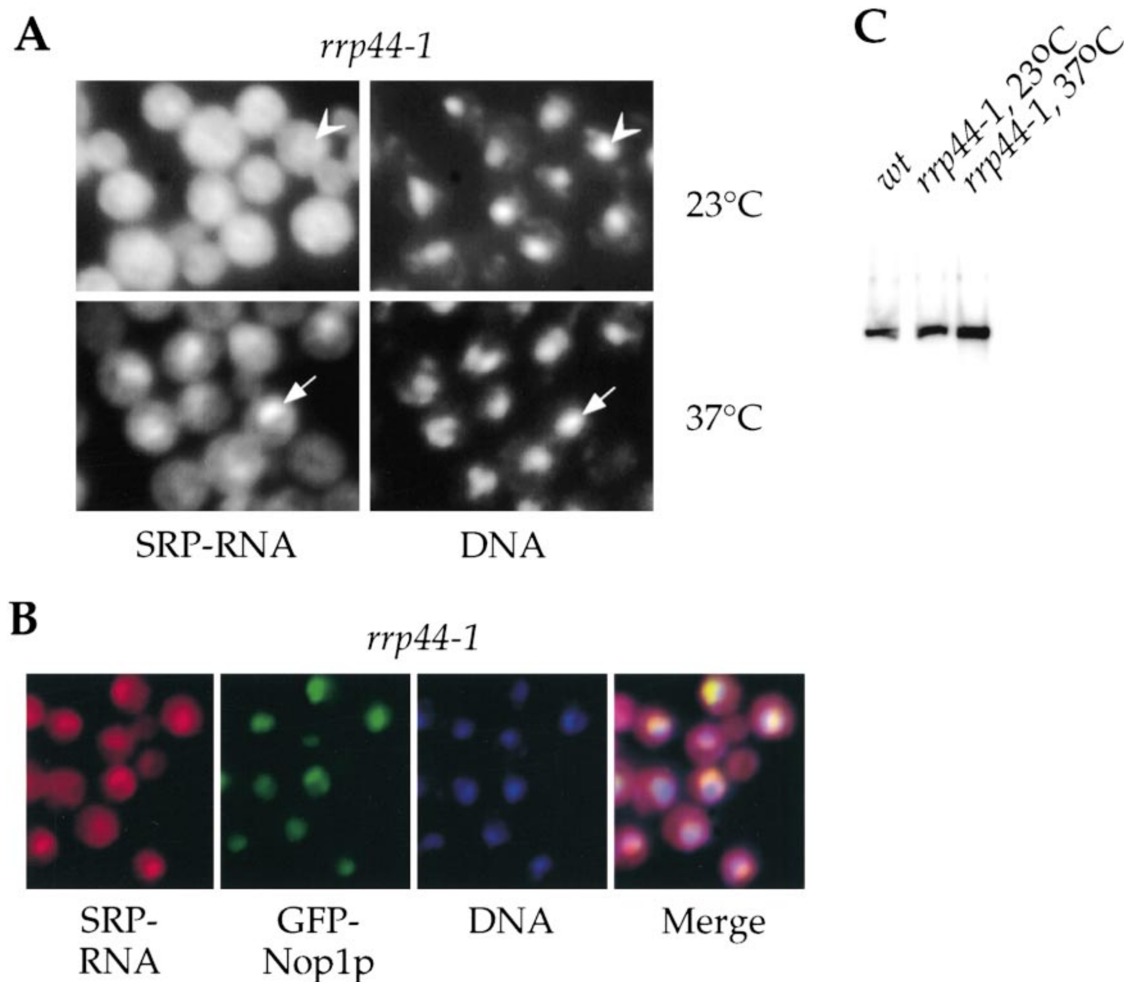

Figure 7. Nucleolar accumulation of the yeast SRP-RNA in an *rrp44* mutant. (A) Localization of scR1 in *rrp44-1* mutant cells grown at 23°C or shifted for 4 h to 37°C. Representative cells are labeled by an arrow (in the case of SRP-RNA accumulation) or an arrowhead (lack of accumulation) pointing to the nucleoplasmic space as judged by DAPI staining. (B) Colocalization of scR1 and GFP-Nop1p in *rrp44-1* mutant cells shifted for 2 h to 37°C. Cells were pseudocolored to allow superimposition (Merge) of scR1 signal (red), GFP-Nop1p signal (green), and DNA signal (blue). (C) Detection of scR1 by Northern blot analysis. Total RNA was extracted from wild-type strain TR3 (wt) grown at 30°C and *rrp44-1* mutant cells grown at 23°C or shifted for 4 h to 37°C, and separated on a 6% denaturing urea-polyacrylamide gel. scR1 was detected using the three radioactive probes depicted in Fig. 1 A. The autoradiograph shows another part of the gel shown in Fig. 1 B; the wild-type lane is identical.

1992, 1993). Although *nsp1-L640S* accumulated scR1 in a subset of cells at the permissive temperature, this accumulation was lost upon shift to the restrictive temperature (Fig. 9 A and data not shown). The *nsp1-ala6-*Δ*rep* mutant strain did not accumulate scR1 at either temperature. As these mutations in *nsp1* are known to affect nuclear protein import (Doye and Hurt, 1997; Fabre and Hurt, 1997), we reasoned that a shift to the restrictive temperature might cause termination of scR1 production, thus accounting for the lack of observable RNA accumulation under these conditions. Therefore, we decided to analyze scR1 distribution in this and the other nucleoporin mutant strains after a shift to the semipermissive temperature of 30°C. Under these conditions, we could see strong nuclear accumulation of scR1 in *srx1-1* mutant cells (Fig. 9 A). A similar defect was observed in *nsp1-L640S* and, less pronounced, in *nsp1-ala6-*Δ*rep* mutant cells (Fig. 9 A and data not shown).

Nsp1p occurs in two subcomplexes in the NPC, the Nup82p complex consisting further of Nup82p, Nup159p, and Nup116, and the Nup49 complex, comprising in addition Nsp1p, Nup49p, Nup57p, and Nic96 (Grandi et al., 1995a,b; Belgareh et al., 1998; Bailer et al., 2000). We analyzed strains bearing mutant alleles of other members of these subcomplexes, i.e., *rat7-1* (*nup159*; Gorsch et al., 1995), *nup49-313* (Doye et al., 1994), and *nup82-27* mutant cells (Bailer et al., 2000). Although only little accumulation was observed in *nup49-313* and *nup82-27* mutant cells, at the permissive, semi-, and nonpermissive temperatures, scR1 readily accumulated in the nuclei of *rat7-1* mutant cells incubated at the semipermissive temperature (Fig. 9 B and data not shown).

Another major NPC subcomplex in the cell is the Nup84p complex, consisting of the nucleoporins Nup85p, Nup120p, Sec13p, Seh1p, and Nup145Cp (Siniossoglou et al., 1996, 2000). We chose *nup85-*Δ*N* mutant cells to analyze scR1 distribution upon mutation of a representative member of this complex. No accumulation of scR1 was observed in these cells at the permissive, semi-, or nonper-

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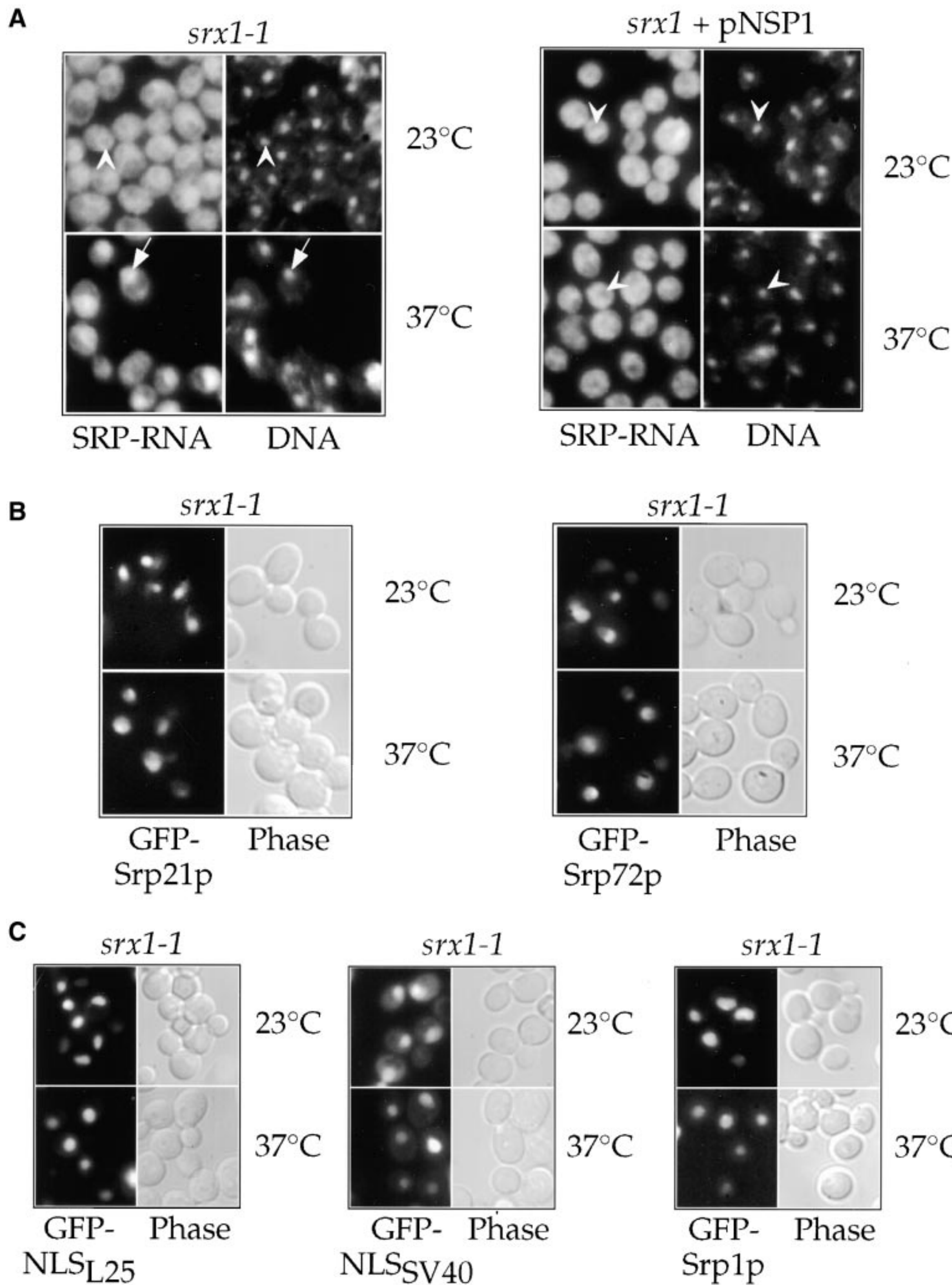

Figure 8. Nsp1p is required for the efficient nuclear export of yeast SRP-RNA. (A) Localization of scR1 in *srx1* mutant cells with or without a centromeric plasmid harboring the *NSP1* gene grown at 23°C or shifted for 4 h to 37°C. Representative cells are labeled by arrows (in the case of scR1 accumulation) or arrowheads (lack of accumulation) pointing to the nucleoplasmic space as judged by DAPI staining. (B–E) Localization of GFP-Srp21p and GFP-Srp72, (C) GFP-NLS_{L25}, GFP-NLS_{SV40}, and GFP-Srp1p, (D) poly(A)$^+$-RNA and tRNAs tRNA$_{Glu(UUC)}$ and tRNA$^{Gly(GCC)}$, and (E) GFP-L25, GFP-Yap1p, and GFP-Yrb1p in *srx1* mutant cells grown at 23°C or shifted for 6 h (Yrb1p) or 4 h (all other cases) to 37°C. In D, representative cells are labeled by arrows (in the case of poly(A)$^+$-RNA accumulation) or arrowheads (lack of accumulation of poly(A)$^+$ RNA or tRNA, respectively) pointing to the nucleoplasmic space as judged by DAPI staining.

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missive temperatures, although polyadenylated RNA was found to accumulate under the latter two conditions (Fig. 9 B and data not shown).

Taken together, these data provide evidence that a subset of nucleoporins, consisting of Nsp1p and Nup159p, is important for scR1 nuclear export, whereas other nucleoporins, such as Nup85p, seem to be less important. Moreover, different mutant alleles of the nucleoporin Nsp1p show preferential effects on nuclear export and import, respectively.

Discussion

In this study we analyzed the localization of both the RNA and the protein components of yeast SRP under wild-type, steady-state conditions and in several nucleocytoplasmic transport and RNA-processing mutants. Our findings support the SRP biogenesis model shown in Fig. 10. According to this model, discussed in more detail below, the SRP core proteins Srp14p, Srp21p, Srp68, and Srp72p are imported via Kap123p and Pse1p into the nucleolus, where assembly of a pre-SRP occurs. The nuclear export of this particle requires the presence of all four SRP core proteins and an intact scR1 3′ end, and is mediated by the exportin Xpo1p and the nucleoporins Nsp1p (Srx1p) and Nup159p (Rat7p).

The strong nucleolar accumulation of the core proteins came as a surprise, considering the established role of SRP in the cytoplasm. However, our data are in agreement with the recently published localization of the mammalian SRP19 (the homologue of Sec65p), SRP68, SRP72, and

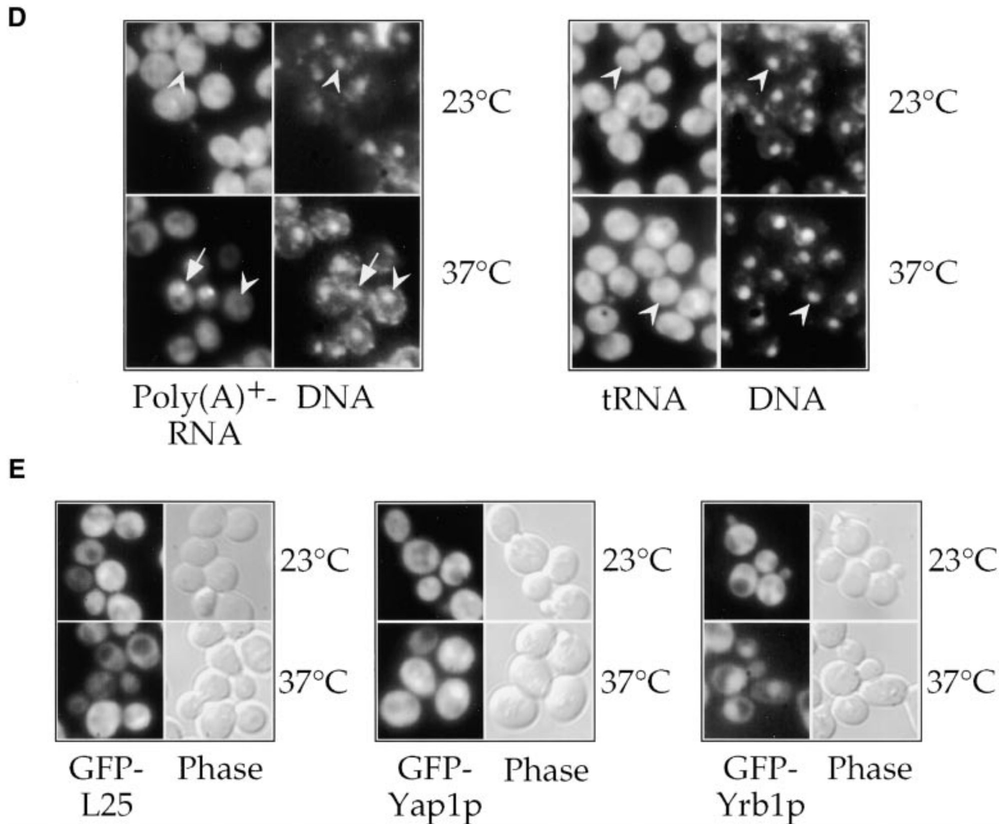

Figure 8 (Continued)

SRP-RNA that were shown to be present in both the cytoplasm and the nucleolus (Politz et al., 2000).

The nucleolar localization of the four yeast core SRP proteins, together with the fact that disruption of any of these proteins causes destabilization as well as nuclear accumulation of scR1, strongly suggest that an SRP subparticle (pre-SRP) containing these proteins and scR1 is assembled in the nucleolus, although direct evidence supporting this hypothesis based on localization data is currently lacking. In fact, it is also likely that the core SRP proteins act as RNA chaperones that aid correct folding and stabilization of scR1. The subparticle can only be exported from the nucleus if assembly of scR1 in the pre-SRP is successful.

It is not clear whether Sec65p is a component of the pre-SRP. Although Sec65p is found in both the nucleus and the cytoplasm, its absence affects neither scR1 stability nor export. Thus, the nuclear role of Sec65p in the assembly of an export-competent pre-SRP may only be auxiliary.

An interesting question is how the pre-SRP proteins reach the nucleolus. The protein components of the pre-SRP resemble the ribosomal proteins not only in their overall basic charge and their function in the assembly of a ribonucleoprotein particle, but also in the fact that they use the same nuclear import receptors, Pse1p and Kap123p. Pse1p and Kap123p might, thus, be components of a nucleolar, rather than merely a nuclear, import pathway. Nucleolar factors, or even scR1 or the rRNA nascent transcripts themselves, respectively, may facilitate the release of cargo destined for this compartment, similar to the stimulated release of the yeast protein Npl3p from its nuclear import receptor Mtr10p through RNA binding (Senger et al., 1998).

To identify similarities between the SRP proteins and the NLSs of ribosomal import cargoes characterized previously, we compared the SRP protein sequences to the bipartite NLS of the yeast ribosomal protein L25 (Schaap et al., 1991) and the BIB domain, i.e., NLS, of mammalian ribosomal protein L23a (Jäkel and Görlich, 1998) by pairwise sequence alignments. Short, lysine-rich stretches of ~20 residues were found for each of the core proteins that exhibited between 24 and 32% identity to the NLS_{L25} (data not shown). Although comparison to the BIB domain yielded generally lower homologies, a slightly better match was found when amino acids 22–60 of L25 were used, a sequence stretch that itself displays the highest homology to the BIB domain (59% identity over 39 residues).

The import route for Sec65p is less obvious. Mislocalization of this protein to the cytoplasm in multiple importin mutants suggests that this protein requires the concerted action of several nuclear import pathways for efficient transport. However, it is likewise possible that at least some of the defects are indirect, e.g., in the case of the *pse1-1 kap123⁻* mutant due to impaired SRP core protein import.

If the nucleolus is the site of pre-SRP assembly, it should also contain scR1. The fact that we could not detect scR1 in the nucleoli of wild-type cells suggests that the assembly of scR1 into pre-SRP and its subsequent exit from the nucleolus is a rapid event facilitated by the presence of an excess of pre-SRP proteins. In agreement with this idea, certain mutations, e.g., a mutation in an exosome component,

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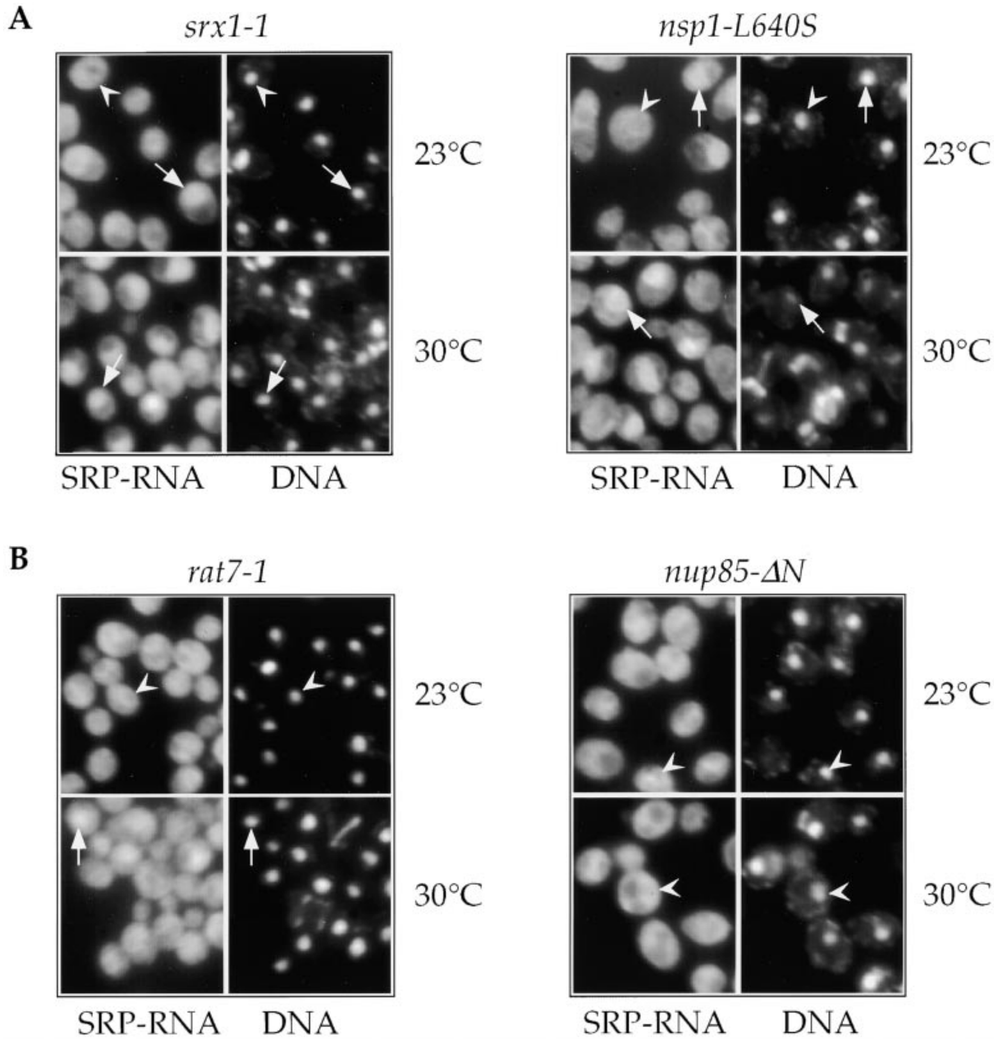

Figure 9. Nucleoporin mutant cells accumulate scR1 at the semipermissive temperature. Localization of scR1 (A) *srx1-1* (left) and *nsp1-L640S* (right), and (B) *rat7-1* (left) and *nup85*-ΔN (right) mutant cells grown at 23°C or shifted for 4 h to 30°C. Representative cells are labeled by arrows (in the case of scR1 accumulation) or arrowheads (lack of accumulation) pointing to the nucleoplasmic space as judged by DAPI staining.

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can indeed cause accumulation of scR1 in the nucleolus (see also below). Transient nucleolar localization was also observed for mammalian SRP-RNA that had been microinjected into rat kidney cells (Jacobson and Pederson, 1998), suggesting that the SRP-RNA found in the nucleolus is not a dead-end product but an intermediate along the SRP-biogenesis pathway. The SRP-RNA is not the only RNA polymerase III transcript found in the nucleo-

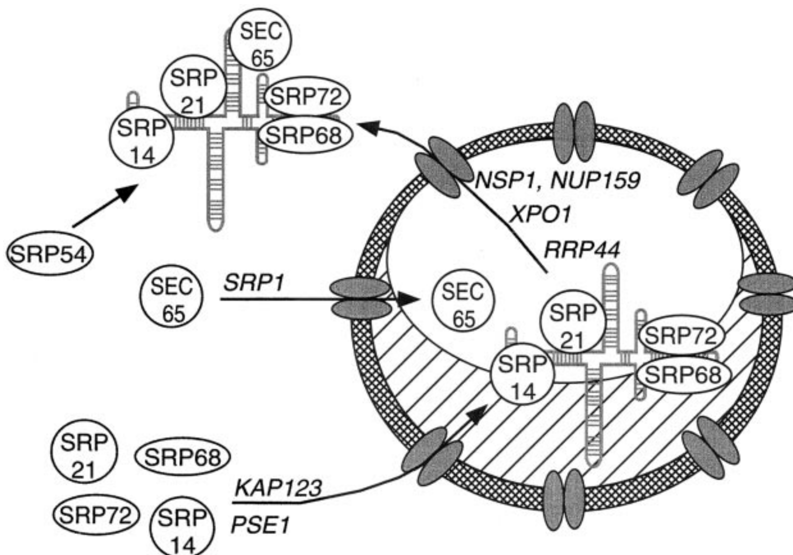

Figure 10. A schematic model for SRP biogenesis in yeast. The SRP core proteins Srp14p, Srp21p, Srp68p, and Srp72p are imported into the nucleolus in a Kap123p- and Pse1p-dependent manner. Import of Sec65p into the nucleus requires Srp1p, but is also defective in other importin mutants. Assembly of a pre-SRP occurs in the nucleolus (hatched): nuclear export of this particle requires the presence of the four SRP core proteins and depends on the function of Rrp44p (Dis3p), Xpo1p, and the nucleoporins Nsp1p (Srx1p) and Nup159p (Rat7p). Srp54p assembles onto the particle in the cytoplasm. See main text for details. Cross-hatched area, nuclear envelope.

lus. Pre-tRNAs as well as components of the tRNA-processing machinery have also been shown to localize within the nucleolus (Bertrand et al., 1998), which also coincided with the site of mature tRNA accumulation upon inhibition of nuclear tRNA export (Grosshans et al., 2000a). It is thus possible that the nucleolus is not only the site of ribosomal biogenesisl, but also presents a stage for Pol III transcript maturation as well as other important biological activities (for review see Lewis and Tollervey, 2000; Olson et al., 2000; Pederson and Politz, 2000).

Rrp44p (also called Dis3p) is a component of the exosome, a large protein complex that is involved in RNA processing and consists of 10 or 11 proteins in its cytoplasmic and nuclear form, respectively (Noguchi et al., 1996; Mitchell et al., 1997; Allmang et al., 1999b). Most of these proteins have predicted $3' \rightarrow 5'$ exonuclease activity, and for some of them, including Rrp44p, this has also been experimentally proven. Interestingly, Rrp44p has also been localized in the nucleolus of exponentially growing yeast cells (Shiomi et al., 1998). Rrp44p might thus account for or contribute to the end-trimming of scR1. Indeed, our data are consistent with the hypothesis that mutation of Rrp44p leads to aberrant processing of scR1, i.e., 3′ end truncation, possibly due to a deregulation of the exosome, an intriguing observation that we will continue to study.

The correlation between this 3′ end shortening and nucleolar accumulation of scR1 in the *rrp44-1* mutant cells suggests that an intact 3′ end is required for scR1 nuclear export. In both *Schizosaccharomyces pombe* and mammalian cells, the 3′ end of the SRP-RNA is part of the Alu domain, which is required for binding of the SRP9/14 heterodimer. Although it is not clear at present whether the 3′ end of scR1 is likewise part of the Alu-like domain to which an Srp14p homodimer was shown to bind (Strub et al., 1999; Mason et al., 2000), the excessive 3′ end-trimming we observed in the *rrp44-1* mutant might prevent Srp14 from binding. This would ultimately lead to a defect in pre-SRP assembly and nuclear export. In agreement with this idea, the Alu domain of vertebrate SRP-RNA was shown by microinjection experiments to be required for the nuclear export of SRP-RNA (He et al., 1994).

After the nucleolar phase of maturation and assembly into the pre-SRP particle, scR1 has to be exported into the cytoplasm. Our data indicate that Xpo1p is the receptor responsible for this transport step. CRM1, the vertebrate homologue of Xpo1p, mediates export of viral HIV RNA and U snRNAs using NES-containing, RNA-binding proteins as adapters (for review see Görlich and Kutay, 1999). Therefore, the SRP-RNA would represent another cellular RNA (the first one in yeast) that can be exported via Xpo1p/CRM1. To mediate SRP nuclear export Xpo1p would have to bind to one of the proteins of the pre-SRP in a Ran-GTP–dependent way. All SRP proteins are quite leucine rich and Srp68p in particular contains two sequences that match perfectly the NES consensus motif $L(X)_{2\text{-}3}L(X)_{2\text{-}3}LXL$ (Mattaj and Englmeier, 1998): ^{177}**L**EH**L**KN**L**S**L**^{185} and ^{409}**L**CK**L**YFQ**L**F**L**^{418} (one letter code, leucines shown in bold). Therefore, these areas could be targets for Xpo1p. However, the fact that absence of any one of the pre-SRP proteins leads to nuclear accumulation of scR1 suggests that SRP export is more complex than simple NES export. Using two-hybrid analysis with Xpo1p as bait and SRP proteins as prey or vice versa, we were unable to detect an interaction between Xpo1p and any individual SRP protein (our unpublished data), suggesting that, indeed, assembly of the core particle is required to expose an active NES. This would efficiently safe-guard against futile rounds of export of SRP proteins not bound to the RNA. Alternatively, an adapter protein, similar to the recently identified PHAX, which is required for CRM1-mediated U snRNA nuclear export (Ohno et al., 2000), might be necessary for scR1 nuclear export.

The involvement of Xpo1p in scR1 nuclear export was also suggested in another report that appeared shortly before this manuscript was submitted (Ciufo and Brown, 2000). In agreement with the data presented here, these authors also observed similar localizations for the SRP proteins which they localized by immunofluorescence. In contrast to our observations, these authors, however, reported lack of scR1 mislocalization in *rna1-1*, *prp20-1*, or *rat7-1* mutant cells. This might be due to the different incubation conditions and/or the detection method for scR1 which these authors used.

As a last step on its way to the cytoplasm, the pre-SRP associated with its export receptor must be translocated through the NPCs. This translocation step requires interactions between the transport factors and nucleoporins (Nups), most often with those that contain FG (Phe-Gly) repeats (Ryan and Wente, 2000). Our results suggest that Nsp1p, an phe-x-phe-Gly repeat–containing nucleoporin, might be one of the nucleoporins that mediate the translocation of pre-SRP. Most other known mutant alleles of *NSP1* cause only a defect in nuclear protein import (Doye and Hurt, 1997; Fabre and Hurt, 1997). However, Nsp1p is known to be part of at least two different complexes in yeast, the Nup82p–Nup159p and the Nup49p–Nup57p complexes (Grandi et al., 1995a,b; Belgareh et al., 1998; Bailer et al., 2000). Although the latter seems to be more important for protein import, mutations in the Nup82p complex components Nup82p, Nup159p, or Nup116p lead to strong nuclear mRNA export, but not protein import, defects. Thus, it is possible that the mutation in the *srx1-1* strain is more detrimental to the function of the Nsp1p–Nup82p–Nup159–Nup116p complex. This idea is supported by our observation of a weak intranuclear accumulation of poly(A)$^+$ RNA in the *srx1-1* cells at the restrictive temperature, as well as the strong scR1 nuclear export defect present in *nup159* (*rat7*) mutant cells. Interestingly, Nup159p localizes, unlike most other FG repeat nucleoporins, exclusively to the cytoplasmic side of the NPC (Rout et al., 2000). Nup159p might, thus, provide the terminal interaction site between the export receptor and the NPC before release of the export complex into the cytoplasm.

In summary, our data show that biogenesis of the yeast SRP is a complex process that necessitates crossings of the nuclear membrane in both directions. Using the methodology described in this work and in particular our FISH-based assay, we should be able to identify further components of these transport steps. In combination with biochemical analysis, which should address processing of scR1 as well as composition of an export-competent pre-SRP, this should help to fill in the details of the picture outlined here.

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