Nuclear and nucleolar localization of *Saccharomyces cerevisiae* ribosomal proteins S22 and S25

Antonius C.J. Timmers[1], Rogier Stuger[2], Peter J. Schaap[3], Jan van 't Riet*, Hendrik A. Raué

Faculty of Sciences, Division of Chemistry, Department of Biochemistry and Molecular Biology, Institute for Molecular Biological Sciences, BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

Received 30 March 1999; received in revised form 4 May 1999

Abstract Nuclear import usually relies on the presence of nuclear localization sequences (NLSs). NLSs are recognized by NLS receptors (importins), which target their substrates to the nuclear pore. We identified the NLSs of the yeast ribosomal proteins S22 and S25 and studied the former by mutational analysis. Furthermore, in S25 the nucleolar targeting information was found to overlap with its NLS. Comparison with previously published data on yeast ribosomal protein NLSs and computer analysis indicates the existence of a novel type of ribosomal protein-specific NLS that differs from the classical Chelsky and bipartite NLSs. The existence of such a ribosomal protein-specific NLS is in accordance with the recent identification of ribosomal protein-specific importins.

Key words: Nuclear localization sequence; Consensus sequence; Nucleolus; Ribosomal protein; Yeast

1. Introduction

In eukaryotes the nucleolus is the site for synthesis of rRNAs and their assembly with ribosomal proteins (RPs) into ribosomes (reviewed in [1,2]). Since RPs are synthesized in the cytoplasm, they must be imported into the nucleus and subsequently find their way into the nucleolus. The common mechanistic theme in nuclear import of macromolecules is its dependence on the presence of nuclear localization signals (NLSs). These NLSs are recognized by NLS receptors, which target their substrates to the nuclear pore. A considerable variety of NLSs as well as NLS receptors has been show to exist (for a review see [3]).

The two best defined classes of nuclear import signals are the monopartite and bipartite NLS, the prototypes of which are PKKKRKV found in the SV40 large T antigen [4] and the nucleoplasmin sequence KR[PAATKKAGQA]KKKK [5], respectively. Both signals are recognized by importin α [6], also named karyopherin α. Importin α in turn contains a bipartite NLS that is specifically recognized by importin β, also called p97 or karyopherin β [7,8], which can be considered the actual import mediator. Although it is difficult to define clear consensus sequences for these two types of NLSs, basic and hydrophobic residues predominate, and many members contain a helix-breaking amino acid (G or P) adjacent to or within the basic cluster [9]. Chelsky et al. [10] proposed the consensus sequence $K\text{-}^{K}/_{R}\text{-}X\text{-}^{K}/_{R}$ for monopartite NLSs. The bipartite NLS is characterized by two short basic clusters separated by about 10 residues [11]. A Chelsky sequence may, therefore, be part of a bipartite NLS.

Various other types of NLS, many of which are still ill-defined, have also been reported (see [3] for a review). Examples are the acidic M9 domain of hnRNP A1 [12,13], the sequence KIPIK found in the yeast transcription repressor Matα2 [14] and the complex signals of U snRNPs [15]. Most of these NLSs appear to be recognized directly by specific receptors belonging to the importin β family without the intervention of an importin α-like protein. So far 13 members of the importin β family have been identified in yeast. Most of these have been shown to possess a vertebrate counterpart (reviewed in [16]). Thus, a number of alternative, specialized nuclear import pathways appear to co-exist.

Recently it was demonstrated that such specialized pathways play a major role in nuclear import of eukaryotic RPs. Nehrbass et al. [7] reported that nuclear import of L25 was not inhibited by mutations in the nuclear pore protein Nsp1, which prevented import of non-ribosomal nuclear proteins. Rout et al. [17] found that the NLSs of many yeast RPs are recognized by the importin β homologue Kap123p/Yrb4p but not Kap95, the yeast counterpart of mammalian importin β. The importin β homologues Kap121p (Pse1p) and Sxm1p have also been implicated in nuclear import of a number of specific yeast RPs [17–19]. In mammalian cells the import of rat RPs S7, L5, and L23a is mediated by any of the importin β family members transportin, RanBP5, RanBP7 as well as importin β itself [20].

To date, NLSs of yeast RPs L3, L25, L28, and S17a (according to the nomenclature of [21]) have been published (reviewed in [22]). Furthermore, the NLSs of the human RPs S6 [23,24] and L7a [25], as well as rat L31 [26] and L23a [20] have been identified in greater or lesser detail. Although these NLSs share the preponderance of basic and hydrophobic residues with the 'classical' mono- and bipartite NLSs described above, they often show only poor similarity to the proposed consensus sequences and are more complex [20,22].

An interesting question is why yeast RPs can be imported by various importin β-like receptors but seem to ignore the

*Corresponding author. Fax: (31) (20) 4447553.
E-mail: vantriet@chem.vu.nl

[1] Present address: Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, CNRS-INRA, P.O. Box 27, 31326 Castanet-Tolosan Cedex, France.
[2] Present address: Department of Molecular Cell Physiology, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.
[3] Present address: Section Microbiology of Industrial Microorganisms, Landbouw-universiteit, Dreyenlaan 2, 6703 HA Wageningen, The Netherlands.

0014-5793/99/$20.00
PII: S0014-5793(99)00669-9

'classical' importin β. To shed more light on this issue we studied the nuclear import of yeast RPs S22 and S25 by analyzing the ability of various portions of these RPs to direct nuclear import of the reporter protein β-galactosidase. We identified two structurally different sequences in S25 and a single region in S22 having nuclear targeting activity. Furthermore, we identified a region in S25 showing nucleolar targeting activity, which overlaps with the N-terminal nuclear targeting sequence.

Based on the set of nuclear import signals identified in yeast RPs and mutational analysis of the NLSs of L28 [27] and S22 (this paper), we propose a novel consensus sequence for yeast RP NLSs. Computer analysis revealed the presence of (a) sequence(s) matching this consensus in the majority of yeast RPs.

2. Materials and methods

2.1. Expression and detection of proteins in yeast

DNA manipulation was performed as described in [28]. Plasmids pBMCY135 [29] and pBMCY65 [30] served as the source of (fragments of) the *S22* and *S25* genes, respectively. Gene fragments were created using Bal31 exonuclease and inserted into pLGSD5 [31] to express β-galactosidase fusion proteins carrying the RP fragment at their N-terminus in yeast. Mutant NLS sequences were created synthetically and inserted into pLGSD5 in the same way. YEpmyc81 [32] was used for expression of c-*myc*-tagged S25. Details of the cloning procedures have been published elsewhere [33]. Tagged proteins were expressed in *Saccharomyces cerevisiae* BWG1-7A [31] transformed by the freeze-thaw method of Klebe et al. [34].

Transformed cells were grown in YNB medium containing 160 mM lactic acid and 100 mM sodium lactate (adjusted to pH 4.5 with KOH), supplemented with adenine, histidine, and leucine (50 μg/l each), to an OD_{660} of 0.4. After addition of 2% (w/v) galactose to induce the *RP-βGal* fusion gene growth was continued for either 1, 2.5, or 5 h. Cells were harvested by centrifugation and fixed for 10 min at room temperature with a solution of 3% formaldehyde (freshly prepared from paraformaldehyde) in the growth medium, followed by 1 h fixation in a solution of 3% formaldehyde in phosphate-buffered saline (PBS) at room temperature. Cells were attached to polylysine-coated coverslips. Immunofluorescent staining was performed according to Adams and Pringle [35]. To improve the efficiency of antibody labeling, cells were air-dried before applying antibodies. Cells were labeled using either mouse anti-β-galactosidase (Promega) or anti-c-*myc* (Santa Cruz Biotechnology), followed by fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma). Cells were mounted in glycerol containing 1,4-diazabicyclo[2,2,2]octane (Jansen) as anti-fading agent and 4′,6-diamino-2-phenylindole (DAPI) to stain DNA, and examined by fluorescence microscopy.

2.2. Database search

Yeast RP sequences retrieved from the SwissProt database were scored for the presence of putative NLSs of the S22 type (YRP-NLS). Search criteria were $[(^{K}/_{R})_3\ B_{1-4}]$ for the YRP-NLS. Amino acids in square brackets are in random order; B is any non-acidic amino acid. Sequences were scored using the BLAST algorithm according to [36]. YRP-NLS sequences with internal or flanking acidic amino acids and YRP-NLS sequences lacking a flanking helix-breaking amino acid were manually deleted.

3. Results and discussion

3.1. The nuclear localization sequence of S22

To identify NLSs in yeast RPs S22 and S25 we genetically fused (portions of) the two RPs to the N-terminus of *Escherichia coli* β-galactosidase. This protein is an excellent reporter as it normally resides in the cytoplasm and due to its large size eliminates the possibility of NLS-independent diffusion across the nuclear envelope [37]. The fusion proteins were visualized by indirect immunofluorescence, using a commercial antibody against β-galactosidase.

The caveat in this approach derives from the possibility that RPs could already associate in the cytoplasm. Thus, a region identified as having nuclear targeting activity might not be an NLS, i.e. a sequence recognized by a nuclear import receptor, but might be required for the incorporation of the protein into such a 'pre-assembly complex' whose import depends upon another, NLS-bearing RP ('piggy-back' import). It should be noted, however, that the existence of RP 'pre-assembly complexes' is so far entirely hypothetical. Furthermore, because RPs are synthesized in stoichiometric amounts [1,2] a sequence involved in 'piggy-back' import would be expected to cause less efficient nuclear localization than a bona fide NLS, when present in a fusion protein that is overexpressed.

As shown in Fig. 1 the fusion protein containing full-length S22 (residues 1–126) is located in the nucleus, indicating the presence of nuclear targeting information in this RP. Analysis of fusions containing either the N- or the C-terminal half of S22 indicates that the nuclear targeting information resides in

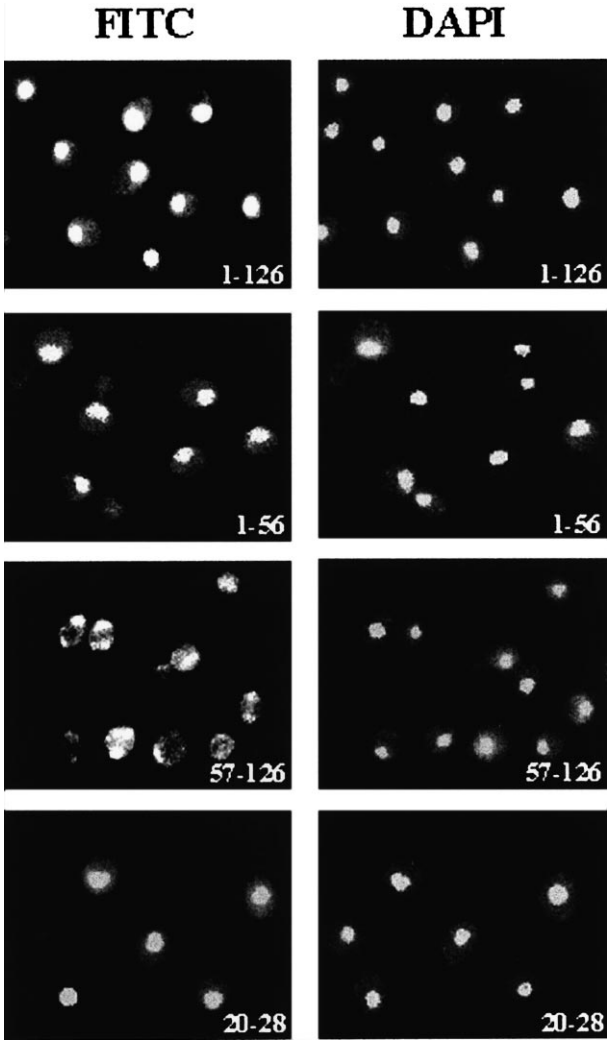

Fig. 1. Immunocytochemical localization of S22/β-galactosidase fusions containing (from top to bottom) amino acids 1–126, 1–56, 57–126, or 20–28 of S22. Left column: indirect immunofluorescence using anti-β-galactosidase antiserum. Right column: corresponding DAPI image showing the position of the nuclei.

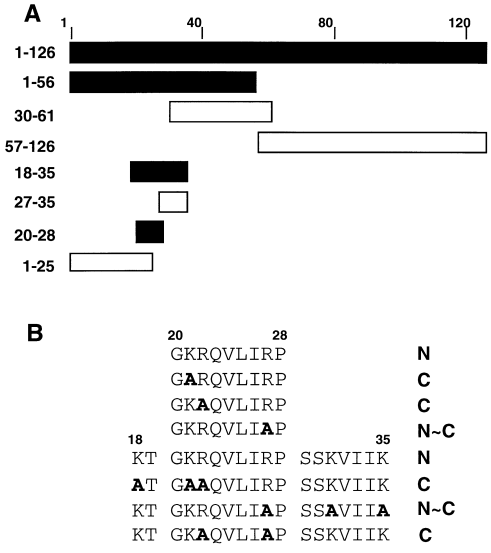

Fig. 2. NLS activity of various portions of yeast RP S22 and mutant forms of the S22 NLS. A: Summary of the S22/β-galactosidase fusions used to identify the NLS. Black bars represent fragments showing NLS activity; white bars correspond to fragments having no detectable NLS activity. The first and last residue present in each of the fragments is indicated. B: Mutational analysis of the S22 NLS. Mutated amino acids are in bold-face type. The numbers indicate the positions of the amino acids in the S22 sequence. N, nuclear localization. C, cytoplasmic localization.

the former region (residues 1–56; Fig. 1). A detailed analysis of this region mapped the NLS of S22 to the sequence GKRQVLIRP encompassing amino acids 20–28 (Figs. 1 and 2A). To investigate the importance of the basic amino acids in this sequence we synthesized various oligonucleotides encoding mutant versions of the GKRQVLIRP sequence, as well as a slightly longer fragment spanning residues 18–35, in which one or more basic residues were replaced by alanine. We then studied the effect of these sequences on the cellular localization of the β-galactosidase reporter (Fig. 2B). Replacement of either of the two N-proximal basic residues by alanine completely abolished nuclear localization of the reporter, whereas a similar replacement of the C-proximal Arg had a significant, but less dramatic negative effect. Thus, both Lys-21 and Arg-22 are essential for NLS activity whereas Arg-27 has an important stimulatory role. Mutational analysis of the longer fragment confirmed these conclusions and also shows that the basic residues located upstream and downstream of the GKRQVLIRP sequence do not detectably contribute to the nuclear targeting information (Fig. 2B).

We conclude that yeast RP S22 possesses a single, N-terminally located nuclear targeting region containing three important basic residues flanked by the helix-breaking amino acids Gly-20 and Pro-28. Although this sequence is basic in nature it does not easily conform to any prototypic or consensus NLS sequences. The very efficient accumulation of the fusion protein carrying the S22 sequence, its resemblance to other yeast RP NLSs and the results of the mutational analysis, however, strongly argue that amino acids 20–28 indeed constitute a true NLS. Interestingly, the KVIIK motif resembling the NLS of the yeast Matα2 protein (amino acids 31–35) does not promote nuclear import (Fig. 2B). A striking difference with the Matα2 NLS (KIPIK) is the absence of a helix-breaking amino acid within the sequence, which suggests that such a residue might be required for nuclear targeting activity.

3.2. Nuclear targeting sequences in S25

As shown in Fig. 3 yeast RP S25 also contains nuclear targeting information since a β-galactosidase fusion protein carrying almost the complete S25 sequence (amino acids 5–100) was located to the nucleus. Further analysis demonstrated that S25 contains at least two regions that can efficiently direct a reporter to the nucleus, a situation not uncommon in yeast RPs [27,37]. One of these regions consists of amino acids 11–36 (AKAAAALAG**GKKSKKKW**SKKSMKDRA), the other of amino acids 35–100 (Figs. 3 and 4). The former region contains a sequence (highlighted) that closely resembles both the Chelsky consensus [10] and the prototypic SV40 large T NLS [4]. Remarkably, however, this sequence, encompassing amino acids 20–27, did not show any NLS activity (Figs. 3 and 4). The most likely candidate for the N-terminal NLS of S25, therefore, is the C-terminal portion

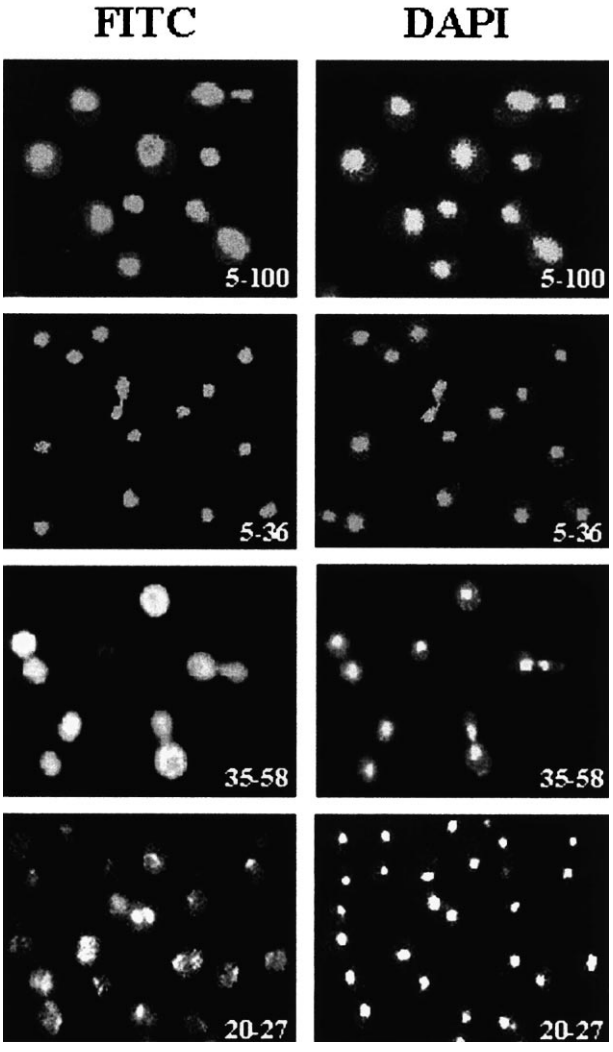

Fig. 3. Immunocytochemical localization of S25/β-galactosidase fusions containing amino acids 5–100, 5–36, 35–58, or 20–27. Left column: indirect immunofluorescence using anti-β-galactosidase antiserum. Right column: corresponding DAPI image showing the position of the nuclei.

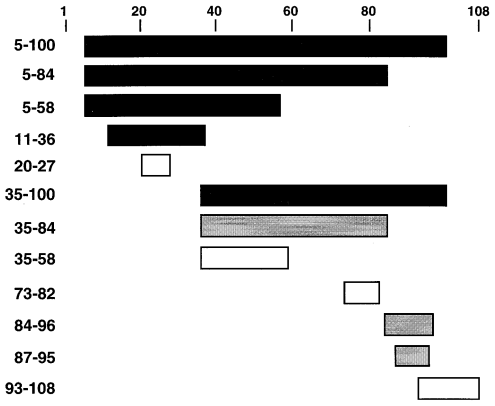

Fig. 4. NLS activity of various portions of yeast RP S25. Black bars represent fragments showing strong NLS activity (exclusive nuclear staining). Gray bars correspond to fragments showing weaker NLS activity (distinct cytoplasmic staining but stronger staining of the nucleus). White bars represent fragments having no NLS activity (uniform staining). The first and last residue present in each of the fragments is indicated.

of the fragment which conforms to the consensus of the 'classical' bipartite NLS (KKSKKKWSKKSMK). In this respect S25 resembles L3, whose NLS (RK[X]$_{12}$PRKRA) also conforms to the bipartite consensus (see Table 1). The resemblance to the bipartite consensus NLS and the high efficiency with which amino acids 11–36 direct nuclear localization of the reporter argue in favor of the conclusion that this region functions as a true NLS. Further experiments, however, are required to substantiate this conclusion.

The nature of the nuclear targeting information present in residues 35–100 appears to be rather complex. This region, which as a whole shows strong independent nuclear targeting activity (Fig. 4), could be divided into two separate portions, each of which was still capable of directing the reporter to the nucleus (Fig. 4), albeit less efficiently as judged from a lower ratio of nuclear to cytoplasmic staining. The shortest fragment showing nuclear targeting activity spans amino acids 87–95 (GII**KPISK**H), a sequence that contains a Matα2-like motif [14]. The only potential candidate for an NLS present in the other portion (residues 35–84) that can be identified by visual inspection is the sequence RIALR (residues 77–81) that is distantly related to the Matα2 motif. However, a shorter region containing the RIALR sequence (amino acids 73–82) has no nuclear targeting capacity (Fig. 4). Further analysis has to be carried out to determine whether the 35–100 region of S25 contains two relatively weak NLSs, whose activity is additive, or whether it could be involved in 'piggy-back' import as discussed above.

3.3. A novel consensus NLS for yeast ribosomal proteins?

The results reported above, together with previous data on yeast RP NLSs (see Section 1) and Table 1), suggest the existence of at least two types of efficient NLSs in yeast RPs. On the one hand there are the bipartite signals found in L3 and S25. On the other hand, the nuclear targeting information of L25, L28, S17a and S22 is contained in short sequences that show an obvious mutual resemblance but are only distantly related to the classical monopartite NLS (Table 1). This 'S22-type' NLS might be responsible for the nuclear import of at least some yeast RPs by a pathway different from that used by non-ribosomal nuclear proteins, as indeed shown for RP L25 and other yeast RPs [17,18]. This prompted us to search the sequences of the complete set of yeast RPs for the occurrence of an 'S22-type' signal. The criteria for this search were based on the experimentally determined NLS sequences of L25, S17a, L28 and S22 and the results of the mutational analysis of the latter two sequences. Furthermore, we noted that the C-terminal portion of L25 contains two sequences (I**KKAVK**E and T**KKAYVR**L) that, despite their close resemblance to the 'S22-like signal', do not have NLS activity [37]. The striking difference between these inactive sequences and the actual NLSs of L25 is the absence of a helix-breaking glycine or proline flanking the basic region, as well as the presence of a flanking acidic residue in the first sequence. Thus, the 'S22-type' YRP-NLS should consist of up to seven residues, at least three of which are basic, flanked by one or more helix-breaking residues. In addition, no acidic amino acids should be present within or adjacent to the signal (Table 1). A sequence satisfying these criteria could be found in a large majority (54 out of 78) of the yeast RPs, supporting our suggestion that it may indeed represent an RP-specific nuclear import signal. The detailed analysis of the complete set of yeast RP-NLS sequences will be published elsewhere (Van 't Riet et al., in preparation).

3.4. Nucleolar localization of S25

Once inside the nucleus, ribosomal proteins have to be further transported to the nucleolus for assembly into ribosomal subunits. As this does not involve crossing a membrane it has been proposed that direct interaction with nucleolar components is responsible for accumulation of proteins in the nucle-

Table 1
NLSs identified in yeast ribosomal proteins

Protein		NLS	Type	Ref.
L3		1-SHRKYEAPRHGHLGFLPRKRA-21	bipartite	[41]
L25	NLS1	11-KKAVVKG-17	'S22'	[37]
	NLS2	18-TNGKKALKVRT-28	'S22'	
L28	NLS1	6-KTRKHRG-12	'S22'	[27]
	NLS2	23-KHRKHPG-29	'S22'	
S17a		2-GRVRTK-7	'S22'	[38]
S22		20-GKRQVLIRP-28	'S22'	this study
S25	NLS1	11-AKAAAALAGGKKSKKKTSKKSMKDRA-36	bipartite	this study
	NLS2	87-GIIKPISKH-95	Matα2-like	

The minimal sequences identified as able to direct a reporter protein to the nucleus are shown. Numbers indicate the position of the first and last residue in the complete sequence of the protein.

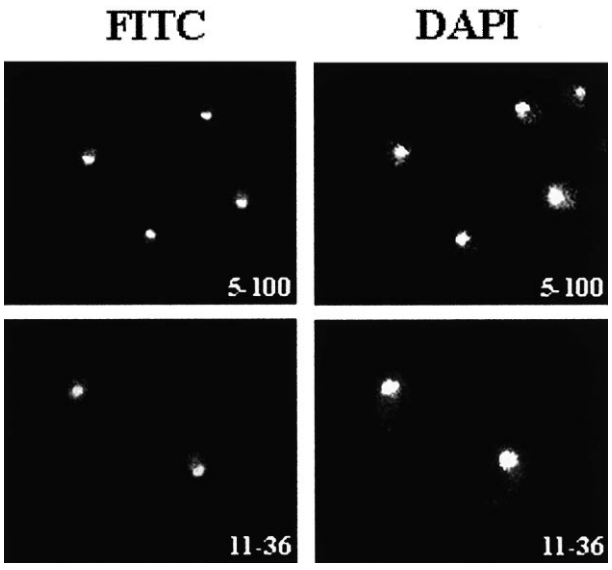

Fig. 5. Immunocytochemical localization of c-*myc*-tagged portions of S25 containing amino acids 5–100 or 11–36. Left column: immunocytochemical staining using a c-*myc* antibody. Right column: corresponding DAPI image showing the position of the nuclei. The FITC staining overlaps with the part of the nuclei only weakly stained with DAPI indicating the nucleolar localization.

olus [39,40]. So far, however, only a few sequences responsible for nucleolar localization have been identified. In rat RP L31 the sequences responsible for nuclear import and nucleolar localization overlap [26], whereas in human RP S6 these sequences were found to reside in different parts of the protein [23]. In order to identify sequences in S25 responsible for nucleolar trafficking we constructed an epitope-tagged version of the protein containing the c-*myc* epitope fused to amino acids 5–100 of S25. The tag itself does not contain information required for nucleolar localization [32]. We reasoned that the small c-*myc* epitope (11 amino acids) would not interfere with binding of S25 to any binding site in the nucleolus or with access of the fusion protein to the nucleolus. The latter may be a problem for the RP/β-galactosidase fusions, which did not accumulate in the nucleolus (Fig. 3). As shown in Fig. 5, c-*myc*-tagged S25 did indeed accumulate in the nucleolus. We did not detect cytoplasmic staining, indicating either that the tagged protein is not incorporated into ribosomal subunits or that upon its incorporation the epitope is shielded. To delineate the sequence responsible for nucleolar targeting we constructed c-*myc*-tagged versions of the same fragments used to identify the NLS of S25 (Fig. 4). Strikingly, we found that the shortest fragment accumulating in the nucleolus is the one encompassing residues 11–36 (Fig. 5), the same region that also shows strong nuclear targeting activity (Figs. 3 and 4). From this we conclude that information for efficient nuclear import of S25 and that ensuring its subsequent translocation to the nucleolus resides in the same region of the protein. To our knowledge this is the first example of a nucleolar targeting region in a yeast RP. Whether nucleolar localization of S25 is the result of a specific targeting signal or of binding to (pre)-rRNA, other RPs, or acidic nucleolar proteins involved in ribosome assembly [39] remains an open question.

Acknowledgements: The authors thank Pauline Perquin-Jäger for expert help in elucidating the NLSs of S25, Jan Boesten for preparing the oligonucleotides, Dr. Conrad L. Woldringh for help with the fluorescence microscopy, Dr. Jaap Venema for critical reading the manuscript, and the Netherlands Scientific Organization (NWO) for granting a 'Stimulans' postdoctoral fellowship to A.C.J.T.

References

[1] Tollervey, D. (1996) Exp. Cell Res. 229, 226–232.
[2] Planta, R.J. (1997) Yeast 13, 1505–1518.
[3] Mattaj, I.W. and Englmeier, L. (1998) Annu. Rev. Biochem. 67, 265–306.
[4] Kalderon, D., Roberts, E.L., Richarson, W.D. and Smith, A.E. (1984) Cell 39, 499–509.
[5] Robbins, J., Dilworth, S.M., Laskey, R.A. and Dingwall, C.M. (1991) Cell 64, 615–623.
[6] Görlich, D., Vogel, F., Mills, A.D., Hartmann, E. and Laskey, R.A. (1995) Nature 377, 246–248.
[7] Nehrbass, U., Fabre, E., Dihlmann, S., Herth, W. and Hurt, E.C. (1993) Eur. J. Cell Biol. 62, 1–12.
[8] Adam, E.J.H. and Adam, S.A. (1994) J. Cell Biol. 125, 547–555.
[9] Dang, C.V. and Lee, W.M.F. (1989) J. Biol. Chem. 264, 18019–18023.
[10] Chelsky, D., Ralph, R. and Jonak, G. (1989) Mol. Cell. Biol. 9, 2487–2492.
[11] Dingwall, C.M. and Laskey, R.A. (1991) Trends Biochem. Sci. 16, 478–481.
[12] Pollard, V.W., Michael, W.M., Nakielny, S., Siomi, M.C., Wang, F. and Dreyfuss, G. (1996) Cell 86, 985–994.
[13] Aitchison, J.D., Blobel, G. and Rout, M.P. (1996) Science 274, 624–627.
[14] Hall, M.N., Hereford, L. and Herskowitz, I. (1984) Cell 36, 1057–1065.
[15] Fischer, U., Sumpter, V., Sekine, M., Satoh, T. and Lührmann, R. (1993) EMBO J. 12, 573–583.
[16] Wozniak, R.W., Rout, M.P. and Aitchison, J.D. (1998) Trends Cell Biol. 8, 184–188.
[17] Rout, M.P., Blobel, G. and Aitchison, J.D. (1997) Cell 89, 715–725.
[18] Schlenstedt, G., Smirnova, E., Deane, R., Solsbacher, J., Kutay, U., Gorlich, D., Ponstingl, H. and Bischoff, F.R. (1997) EMBO J. 16, 6237–6249.
[19] Rosenblum, J.S., Pemberton, L.F. and Blobel, G. (1997) J. Cell Biol. 139, 1655–1661.
[20] Jäkel, S. and Görlich, D. (1998) EMBO J. 17, 4491–4502.
[21] Mager, W.H., Planta, R.J., Ballesta, J.P.G., Lee, J.C., Mizuta, K., Suzuki, K., Warner, J.R. and Woolford, J. (1997) Nucleic Acids Res. 25, 4872–4875.
[22] Fried, H.M. (1993) in: Protein Targeting in Yeast (Brown, A.J.P., Tuite, M.F. and McCarthy, J.E.G., Eds.) NATO-ASI Series, Vol. 71, pp. 257–267, Springer-Verlag, Berlin.
[23] Schmidt, C., Lipsius, E. and Kruppa, J. (1995) Mol. Biol. Cell 6, 1875–1885.
[24] Annilo, T., Karis, A., Hoth, S., Rikk, T., Kruppa, J. and Metspalu, A. (1998) Biochem. Biophys. Res. Commun. 249, 759–766.
[25] Russo, G., Ricciardelli, G. and Pietropaolo, C. (1997) J. Biol. Chem. 272, 5229–5235.
[26] Quaye, I.K.E., Toku, S. and Tanaka, T. (1996) Eur. J. Cell Biol. 69, 151–155.
[27] Underwood, M. and Fried, H.M. (1990) EMBO J. 9, 91–99.
[28] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[29] Bollen, G.H.P.M., Molenaar, C.M.T., Cohen, L.H., Van Raamsdonk-Duin, M.M.C., Mager, W.H. and Planta, R.J. (1982) Gene 18, 29–37.
[30] Nieuwint, R.T.M., Molenaar, C.M.T., Van Bommel, J.H., Van Raamsdonk-Duin, M.M.C., Mager, W.H. and Planta, R.J. (1985) Curr. Genet. 10, 1–5.
[31] Guarente, L., Yocum, R.R. and Gifford, P. (1982) Proc. Natl. Acad. Sci. USA 79, 7410–7414.
[32] Reisdorf, P., Maarse, A.C. and Daignan-Fornier, B. (1993) Curr. Genet. 23, 181–183.

[33] Schaap, P.J. (1993) Nuclear Import of Yeast Ribosomal Proteins, Thesis, Vrije Universiteit, Amsterdam.
[34] Klebe, R.J., Harriss, J.V., Sharp, Z.D. and Douglas, M.G. (1983) Gene 25, 333–341.
[35] Adams, A.E.M. and Pringle, J. (1984) J. Cell Biol. 98, 934–945.
[36] Vodkin, M.H., Novak, R.J. and McLaughlin, G.L. (1996) Bio-Techniques 21, 1116–1117.
[37] Schaap, P.J., Van 't Riet, J., Woldringh, C.L. and Raué, H.A. (1991) J. Mol. Biol. 221, 225–237.
[38] Gritz, L., Abovich, N., Teem, J.L. and Rosbash, M. (1985) Mol. Cell. Biol. 5, 3436–3442.
[39] Yan, C. and Mélèse, T. (1993) J. Cell Biol. 123, 1081–1091.
[40] Schmidt-Zachmann, M.S. and Nigg, E.A. (1993) J. Cell Sci. 105, 799–806.
[41] Moreland, R.B., Nam, H.G., Hereford, L.M. and Fried, H.M. (1985) Proc. Natl. Acad. Sci. USA 82, 6561–6565.