

Nuclear and Nucleolar Targeting of Human Ribosomal Protein S6

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Submitted August 3, 1995; Accepted September 11, 1995
Monitoring Editor: Joseph Gall

Chimeric proteins were constructed to define the nuclear localization signals (NLSs) of human ribosomal protein S6. The complete cDNA sequence, different cDNA fragments and oligonucleotides of the human ribosomal proteins S6, respectively, were joined to the 5' end of the entire LacZ gene of *Escherichia coli* by using recombinant techniques. The hybrid genes were transfected into L cells, transiently expressed, and the intracellular location of the fusion proteins was determined by their β -galactosidase activity. Three NLSs were identified in the C-terminal half of the S6 protein. Deletion mutagenesis demonstrated that a single NLS is sufficient for targeting the corresponding S6- β -galactosidase chimera into the nucleus. Removal of all three putative NLSs completely blocked the nuclear import of the resulting S6- β -galactosidase fusion protein, which instead became evenly distributed in the cytoplasm. Chimeras containing deletion mutants of S6 with at least one single NLS or unmodified S6 accumulated in the nucleolus. Analysis of several constructs reveals the existence of a specific domain that is essential but not sufficient for nucleolar accumulation of S6.

INTRODUCTION

The biogenesis of functional ribosomal subunits in the nucleolus is a highly complex process requiring the exact control and regulation of a large number of molecular interactions (Hadjiolov, 1985; Warner, 1990; Raué and Planta, 1991). The complexity of ribosome formation in eucaryotic cells is due to the involvement of all three RNA-polymerases in the synthesis of ribosomal components. In addition, synthesis of subunit constituents takes place in different cellular compartments, e.g. rRNA precursor molecules are transcribed in the nucleolus (Sollner-Webb and Mougey, 1991) and mRNAs in the nucleoplasm whereas ribosomal proteins are translated in the cytoplasm. Newly synthesized ribosomal proteins are imported into the nucleus and subsequently accumulate in the nucleoli at the site of ribosome biogenesis (Lastick, 1980; Sommerville, 1986; Warner, 1990). Assembled ribosomal subunits are exported through the nuclear pores into

the cytoplasm (Khanna-Gupta and Ware, 1989; Bataillé *et al.*, 1990) in which they become engaged in protein synthesis.

Ribosomal protein S6 (Chan and Wool, 1988; Heinze *et al.*, 1988; Wettenhall *et al.*, 1988) contains five phosphorylation sites at its C-terminus (Kruppa *et al.*, 1983; Krieg *et al.*, 1988, Ferrari *et al.*, 1991). Higher phosphorylation states are affected by S6-specific kinases that are present in the cytoplasm and the nucleoplasm (Kozma and Thomas, 1992; Reinhard *et al.*, 1992). A regulatory role of S6 phosphorylation in translational control has been postulated (Duncan and McConkey, 1982; Kruppa and Clemens, 1984; Traugh and Pendergast, 1986). A nuclear pool of chromatin-associated S6 that is phosphorylated after hormone treatment has been documented (Franco and Rosenfeld, 1990). Ribosomal protein S6 associates very early during the assembly process with rRNA (Westermann *et al.*, 1981; Todorov *et al.*, 1983).

Import of proteins into the nucleus depends on nuclear localization signals (NLSs), which represent short basic amino acid sequences (Gracia-Bustos *et al.*, 1991). These NLSs enable karyophilic proteins to traverse the nuclear envelope (Dingwall and Laskey, 1992) via the nuclear pore complex (Forbes, 1992; Rout

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and Wenthe, 1994) as a result of specific recognition of the NLS by import receptors (Yamasaki and Lanford, 1992; Adam and Adam, 1994; Görlich *et al.*, 1994). Protein translocation into the nucleus is catalyzed by the small GTP binding protein RAN/TC4 (Moore and Blobel, 1993, 1994a,b; Melchior *et al.*, 1993). The prototype of an NLS is the sequence of SV40 large T-antigen (Kalderon *et al.*, 1984). Although a strict consensus sequence for NLSs has not been found among many nuclear proteins, an impressive subset of signals conforms to the consensus tetrapeptide K-K/R-X-K/R where X may be substituted by the amino acids K, R, V, P, and A but not by N (Chelsky *et al.*, 1989). In addition, bipartite NLSs have been described for many nuclear proteins (Dingwall and Laskey, 1991). Because of its basic nature, ribosomal protein S6 contains several highly basic tetrapeptides that theoretically could provide the karyophilic signals for directing S6 into the nucleus, allowing the subsequent association with the nucleolus.

Recently, nucleolar targeting signals have been discovered within the regulatory genes of HTLV-1 and HIV-1 that code for the transactivating proteins Rev, Rex, and Tat (Siomi *et al.*, 1988, 1990; Dang and Lee, 1989; Kubota *et al.*, 1989; Cochrane *et al.*, 1990; Hatanaka, 1990). These short viral sequences consisting of 13–18 amino acids contain one or two glutamine residues flanked by basic amino acids. A related sequence can be found in ribosomal protein S6 between residues 188 and 203 (Heinze *et al.*, 1988). In human and bovine angiogenin a short nucleolar localization sequence (NuLS) has also been recently described (Moroianu and Riordan, 1994).

In the following report we have characterized the NLSs of S6 by fusing differently sized S6 peptide fragments as well as the complete sequence to β -galactosidase. These chimeric proteins were transiently expressed in L cells and localized in the respective cellular compartments by β -galactosidase hydrolysis of X-gal. Deletion of all three putative NLSs blocked the nuclear uptake of S6 leading to an cytosolic accumulation of the corresponding chimeric protein. By using specific constructs a domain was identified that is essential but not sufficient for nucleolar accumulation of S6.

MATERIALS AND METHODS

Insertion of S6 cDNA Fragments into the Expression Vector pPbxlacZ

The expression vector pPbxlacZ was derived from pPbxnslacZ (Hilberg *et al.*, 1987) by removal of the NLS from SV40 large T antigen. The plasmid pPbxnslacZ was digested with *SacI* and *PstI* and the DNA fragments were separated on an agarose gel. The 5.3-kb *SacI/PstI* and 2.1-kb *SacI/SacI* fragments were excised from the gel and recovered by electroelution. The 2.1-kb fragment was incubated with *HindIII* and the 1.9-kb *HindIII/SacI* fragment was isolated after agarose gel electrophoresis as described above. The

restriction of the plasmid pPbxnslacZ with *PstI* and *EcoRI* generated a 0.8-kb *PstI/EcoRI* fragment. The three fragments of the vector and the S6 restriction fragments, with an *EcoRI* site at the 5' end and a *HindIII* site at the 3' end, were ligated. *EcoRI* and *HindIII* linkers of the S6 fragments were chosen to insert S6 and its fragments in the correct reading frame of the β -galactosidase at the 5' end of the lacZ gene. S6- β -galactosidase fusion proteins were constructed (Sambrook *et al.*, 1989) using commercially obtained enzymes (Boehringer Mannheim, Mannheim, Germany).

Subcloning of S6 cDNA Fragments

S6 cDNA, isolated from the plasmid pEMBL(S6-59) (Heinze *et al.*, 1988) by *EcoRI* restriction, was either partially digested with *AluI* giving rise to the S6-1 fragment or incubated with *AluI* and *BalI*. S6-1 contained the S6 sequence starting from amino acid 3-239; thus, 95% of the total 249 amino acids of S6 were represented by this fragment. Because the amino terminal tripeptide MKL and the carboxy terminal decapeptide STSKSESSQK do not contain a cluster of basic amino acids that are characteristic of NLSs (Garcia-Bustos *et al.*, 1991) it was assumed that these sequences are not important for nuclear targeting. An *EcoRI* linker (12 mer) was added at the 5' end of the S6-1 fragment, and *EcoRI* linkers (10 mer) were ligated to the 5' end of the S6-2, S6-3, and S6-4 fragments. The S6-2, S6-3, and S6-4 fragments correspond to amino acids 33-139, 138-214, and 214-240, respectively (Figure 1). The 3' end of all S6 restriction fragments was modified with *HindIII* linkers (8 mer). The linker-modified fragments were ligated into pPbxlacZ and the resulting vector was transfected into *Escherichia coli* XL1 Blue (Stratagene, La Jolla, CA). The correct sequence of all recombinant constructs was routinely checked by DNA sequencing (Sanger *et al.*, 1977).

Fusion of Putative S6 NLSs to β -Galactosidase

Synthetic oligonucleotides coding for putative NLSs with an *EcoRI* site at the 5' end and a *HindIII* site at the 3' end were inserted into the pPbxlacZ plasmid at the 5' end of the lacZ gene. The sequences used were as follows: oligo1, 5'-AAT TCC ACC AAG AAG AAC AAG GAA-3'/3'-GG TGG TTC TTC TTG TTC CTT TCG A-5'; oligo2, 5'-AAT TCC AAA AAA CCA AGA ACA-3'/3'-GG TTT TTT GGT TCT TGT TCG A-5'; and oligo3, 5'-AAT TCC AAA AGA AGA AGA TTA-3'/3'-GG TTT TCT TCT AAT TCG A-5'. The constructs were expressed in L cells.

Deletion of S6 NLSs by Site-directed Mutagenesis

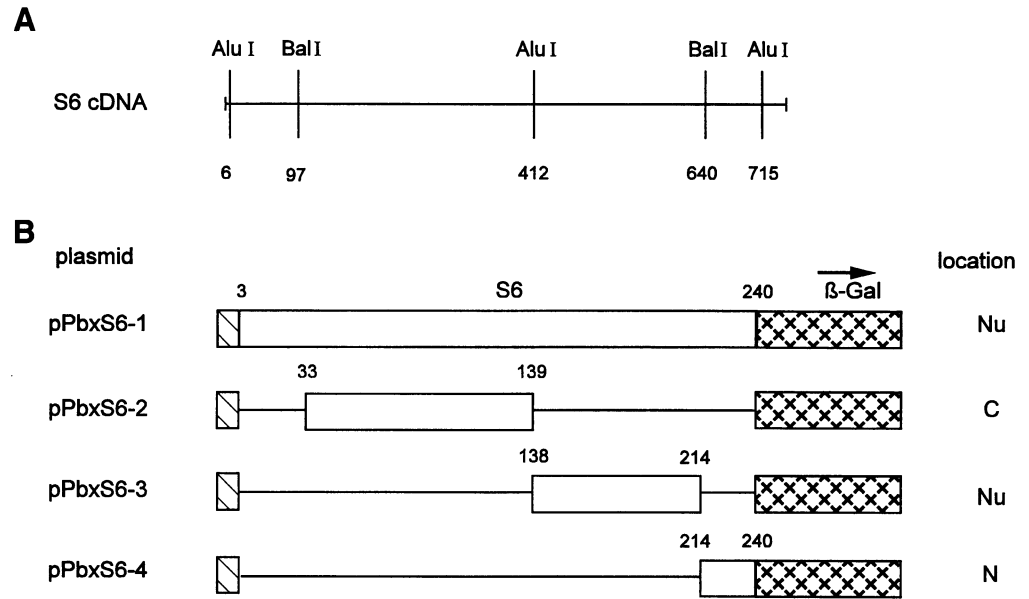
Deletions of the S6 NLSs 1-3 were generated by site-directed mutagenesis using a commercially available in vitro mutagenesis system (Amersham, Buckinghamshire, UK). Deletions were introduced in the S6-1 fragment using single-stranded M13 mp19 DNA as template (Taylor *et al.*, 1985). Mutants were identified by nucleotide sequencing as described above. Inserts carrying the deletions were subcloned into the pPbxlacZ plasmid and expressed in L cells.

Constructs for Analyzing the Nucleolar Targeting Properties of S6

A diagram of the restriction sites and resulting constructs used in the analysis of nucleolar targeting is depicted in Figure 5. The S6(182) fragment was prepared by restricting the 228-nucleotide long fragment of pPbxS6-3 (Figure 1B) with *RSaI* and ligating a 10-mer *HindIII* linker to the newly generated 3' end.

Direct digestion of the S6(182) fragment with *MaeIII* was not possible for technical reasons, thus, a 275-bp long fragment was excised from the DNA of the pBluescript-KS(-)S6(182) with *KpnI* and *SacI*, which was subsequently split into two pieces by *MaeIII*. After cleavage the overhanging 3' ends were filled in with Klenow-fragment. To clone the resulting restriction fragments into the reading frame of β -galactosidase an 8-mer *EcoRI* linker was ligated to the

Figure 1. Fusion proteins between S6 sequences and β -galactosidase. (A) Restriction sites of S6 cDNAs used in the construction of fusion proteins in panel B. Numbers refer to nucleotide positions in the S6 cDNA. (B) S6 protein sequences (blank boxes) were fused to the amino terminus of β -galactosidase (cross-hatched boxes). Numbers refer to the positions of amino acids in the S6 sequence; thin lines represent deleted regions; hatched boxes correspond to an octapeptide sequence derived from the SV40 T antigen and from the *EcoRI* linker. The intracellular location of the fusion proteins after expression of the constructs is indicated as follows: nucleolus, Nu; nucleus, N; and cytoplasm, C.



5' end of the 100-bp long *MaeIII/KpnI* fragment and a 12-mer *HindIII* linker had to be ligated to the 3' end of the 175-bp long *SacI/MaeIII* fragment.

The constructs S6(541) and S6(168) were prepared by *MaeII* restriction of a *SacI/KpnI* fragment that was isolated from a pBlue-script-KS(-)S6(719) clone. After cleavage with *MaeII* the 3'-overhanging ends were filled in with Klenow-fragment. The 199-bp long *MaeIII/KpnI* fragment was ligated to an 8-mer *EcoRI* linker and a 12-mer *HindIII* linker was attached to the 606-bp long *SacI/MaeII* fragment. These linker ligations were essential for inserting the fragments in the correct reading frame of the *lacZ* gene in pPbxlacZ.

Transfection of L Cells and Histochemical Localization of β -Galactosidase Activity

L cells (2×10^4) were seeded on coverslips (12×26 mm) and grown as monolayers in DMEM supplemented with 10% fetal calf serum at 37°C and 5% CO₂. After 72 h, cells were transfected with 0.5–2 μ g plasmid DNA using the DEAE dextran method (Cullen *et al.*, 1987). Forty-eight to seventy-two hours later the cells were washed with phosphate-buffered saline, treated with 0.2% Triton X-100 in phosphate-buffered saline, and incubated for 15 min at 37°C with X-gal (Sanes *et al.*, 1986) to detect β -galactosidase activity. The staining was terminated by rinsing the cells in phosphate-buffered saline. Cells were dehydrated by 1-min incubations in acetone, acetone/xylol (1:1), and xylol, and finally mounted with Eukitt (Riedel de Haen). The cellular location of the fusion proteins was documented at 300 \times magnification with a Zeiss photomicroscope (Oberkochen, Germany).

RESULTS

Transient Expression of S6- β -Galactosidase Fusion Proteins in L Cells

Almost the complete S6 sequence and several restriction fragments (Figure 1A) were modified with appropriate linkers and subsequently fused to the 5' end of the *lacZ* gene in the plasmid pPbxlacZ (as described in

MATERIALS AND METHODS). L cells were transfected with these recombinant plasmids (Figure 1B) employing the DEAE dextran procedure. The corresponding S6- β -galactosidase hybrid proteins were transiently expressed (Figure 2) and the β -galactosidase activity was localized by cleavage of X-gal.

The fusion protein S6-1- β -galactosidase containing almost the complete S6 amino acid sequence (Figure 1B) was imported into the cell nucleus and accumulated preferentially in the nucleoli (Figure 2A). The amino terminal fragment S6-2 is not able to target the resulting chimera into the nucleus because the expressed fusion protein gives rise to a uniformly blue-stained cytosol of the transfected L cells (Figure 2B). Fragment S6-3 can direct the corresponding chimeric protein into the nucleus in which it accumulates in the nucleoli (Figure 2C). The C-terminal fragment S6-4 targets S6-4- β -galactosidase into the nucleus (Figure 2D) but this fusion protein does not accumulate in the nucleoli. This chimera does not appear to be evenly distributed in the nuclei; e.g. the two central cells show regions of lighter staining reminiscent of nucleoli. Because the constructed fusion proteins appear in different intracellular compartments, one has to conclude that the S6 peptide sequence contributes the essential information for targeting. These results indicate that the nuclear localization signals are contained in the C-terminal half of the S6 protein.

Fusion of Selected S6 Tetrapeptide Sequences to β -Galactosidase

By taking Chelsky's proposed consensus sequence K-K/R-X-K/R as a guide for putative NLSs, several tet-

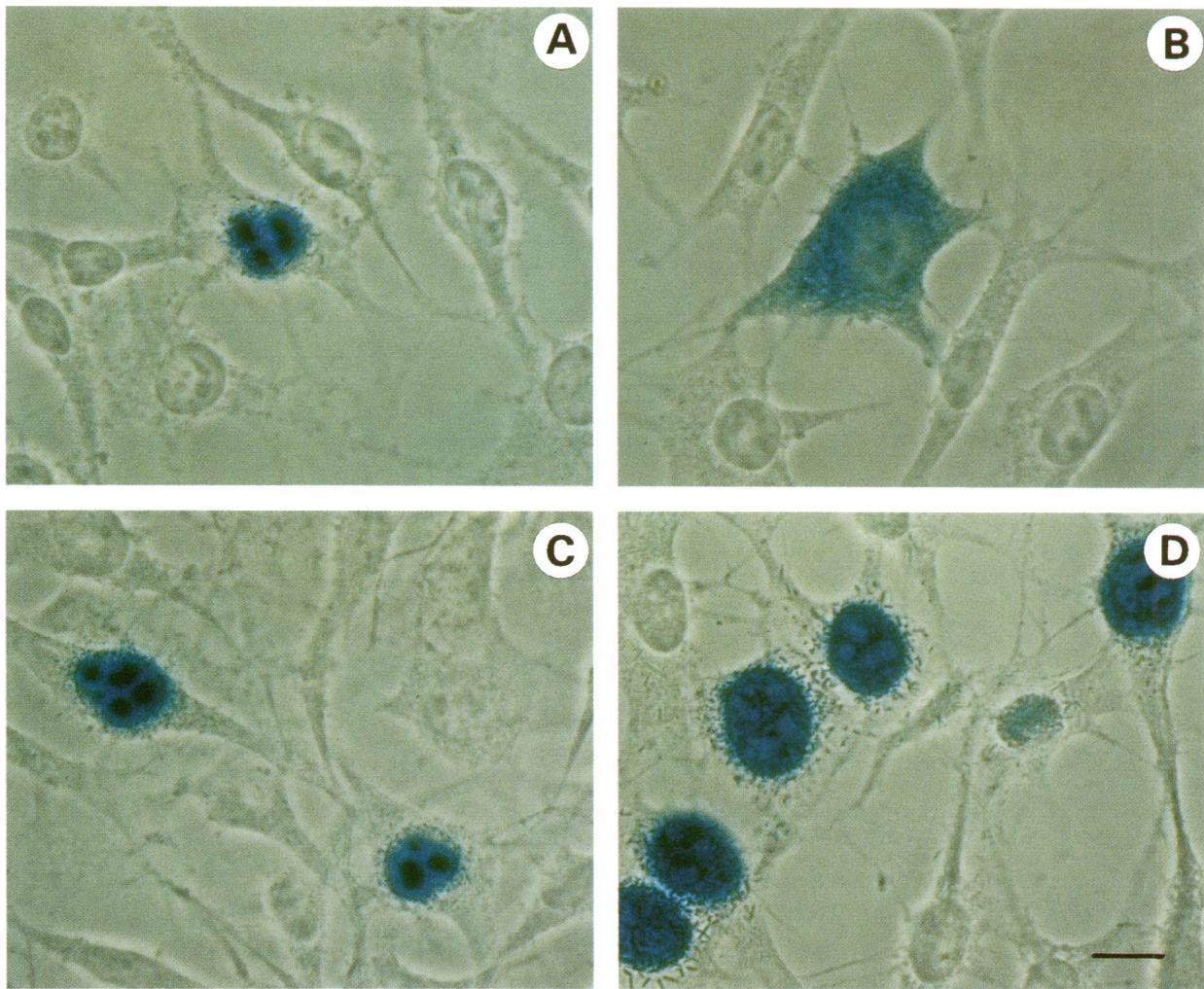


Figure 2. Intracellular localization of S6 β -galactosidase chimera in L cells. The constructs shown in Figure 1B were expressed in L cells and the enzymatic activity of the fusion proteins was localized after enzymatic conversion of X-gal. (A) pPbxS6-1; (B) pPbxS6-2; (C) pPbxS6-3; and (D) pPbxS6-4. Bar, 10 μ m.

rapeptide sequences were chosen from the C-terminal half of the S6 protein. The hydrophilicity plot of S6 (Hopp and Woods, 1981) and the surface probability (Emeni *et al.*, 1985) were taken as additional criteria for the selection of potential NLSs.

Starting from amino acid residue 138 there are six basic tetrapeptides at the C-terminal region (Heinze *et al.*, 1988). Three of these sequences (namely ¹⁶⁷KKPR¹⁷⁰, ¹⁸⁸KRRR¹⁹¹, and ²³⁰KRRR²³³) are compatible with Chelsky's suggestion. Therefore, both sequences, and in addition ²⁰⁰KKNK²⁰³ as a control, were fused to the amino terminus of β -galactosidase, transiently expressed, and intracellularly localized by their β -galactosidase activity (Figure 3A). The amino acid sequences surrounding the chosen tetrapeptides are shown in Figure 3B.

An asparagine in the X position of Chelsky's consensus sequence completely abolishes the nuclear targeting function of the putative signal as predicted. In accordance with Chelsky's suggestion, a proline in the X position leads to a preferential nuclear localization of the fusion protein with a faint cytoplasmic staining in approximately 30% of the cells. The tetrapeptide KRRR that fulfills Chelsky's hypothesis directs β -galactosidase exclusively into the nucleus (Figure 3A). Interestingly, both tetrapeptides that successfully targeted the fusion proteins into the nucleus could not induce a nucleolar accumulation of the chimeras that we observed with the larger S6 fragments (Figure 2, A and C). Thus, the experiments suggest that there are at least three NLSs in the ribosomal protein S6 as indicated in Figure 3A.

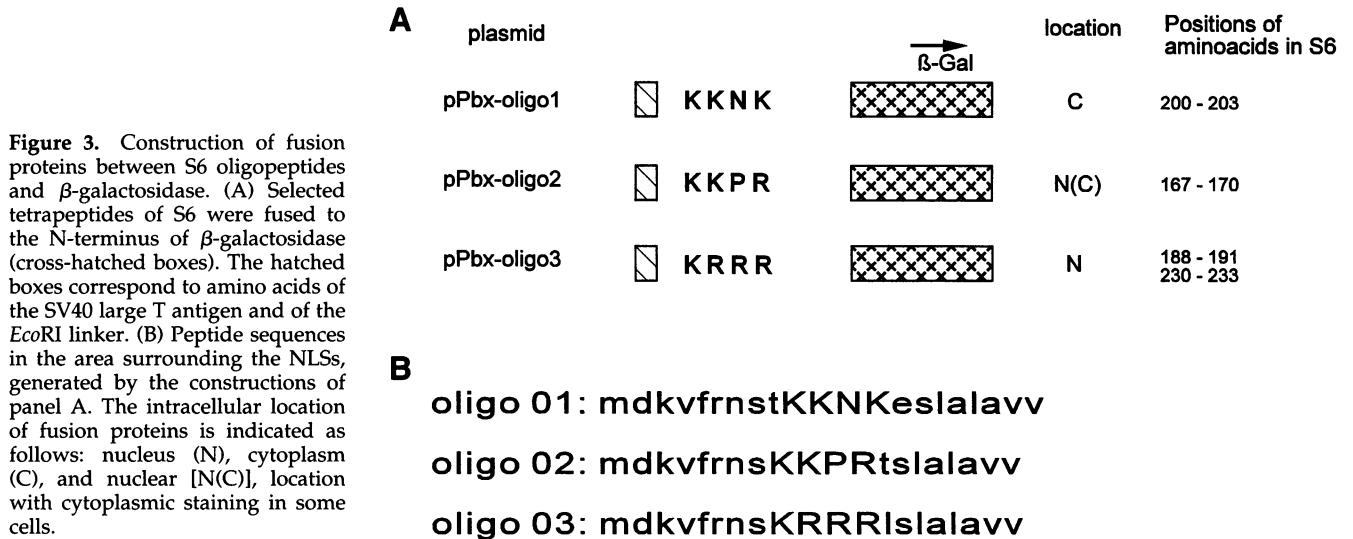


Figure 3. Construction of fusion proteins between S6 oligopeptides and β -galactosidase. (A) Selected tetrapeptides of S6 were fused to the N-terminus of β -galactosidase (cross-hatched boxes). The hatched boxes correspond to amino acids of the SV40 large T antigen and of the *EcoRI* linker. (B) Peptide sequences in the area surrounding the NLSs, generated by the constructions of panel A. The intracellular location of fusion proteins is indicated as follows: nucleus (N), cytoplasm (C), and nuclear [N(C)], location with cytoplasmic staining in some cells.

Targeting Properties of NLS Deletion Mutants of S6

To corroborate the conclusion drawn from the experiments shown in Figure 3, we used site-directed mutagenesis to delete first one, then two, and finally all three NLSs from S6, and transiently expressed the resulting S6- β -galactosidase fusion proteins in L cells (Figure 4 and Table 1).

Deletion of only one NLS (Figure 4) did not interfere with the nuclear import of the corresponding fusion proteins (Table 1). In all single deletion mutants the resulting fusion proteins accumulated in the nucleoli. Deletion of two NLSs (Figure 4) resulted in a nucleolar accumulation of the fusion proteins, although an additional faint staining of the cytoplasm was detectable in two mutants in a few cells (Table 1). Removal of all three NLSs produced a S6- β -galactosidase protein that did not enter the nucleus at all but instead remained in the cytoplasm (Table 1), which appeared evenly blue stained (Figure 6F). Taken together, the characterized constructs of S6 cDNA fragments and oligonucleo-

tides as well as deletion mutants indicate that the S6 protein contains three independent signals for nuclear targeting.

Analysis of Nucleolar Accumulation of S6 Using cDNA Fragments

Most ribosomal proteins have to accumulate in the nucleolus to take part in the assembly of the ribosomal subunits (Hadjiolov, 1985; Warner, 1990; Raué and Planta, 1991). Knowing the nucleolar targeting properties of the construct pPbxS6-3 (Figures 1B and 2C), we decided to further analyze the sequence requirements for nucleolar accumulation of ribosomal protein S6. Initially we expected a short nucleolar signal sequence (Table 2). The 228 nucleotides of S6 in the construct pPbxS6-3 were trimmed at the 3' end, resulting in the S6(182) fragment that encodes an oligopeptide of 61 amino acids that still leads to a nucleolar staining of the corresponding fusion protein

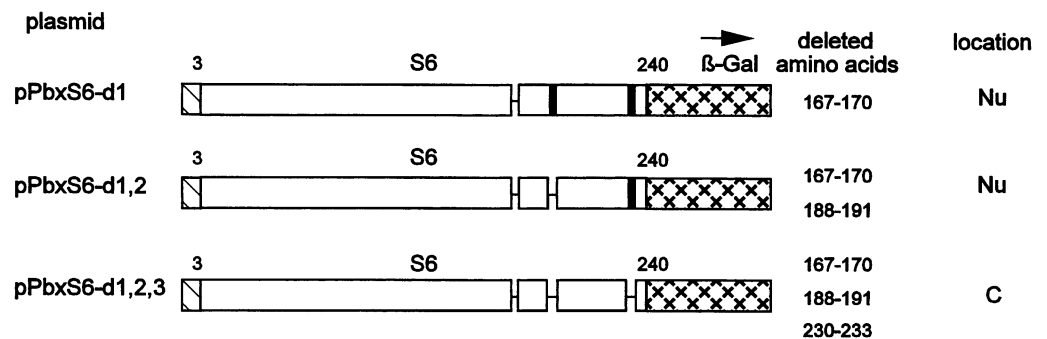


Figure 4. Deletion mutagenesis of pPbxS6.S6 cDNAs (blank boxes) in which NLS coding sequences had been deleted by site-directed mutagenesis (thin lines) were fused to the lacZ gene (cross-hatched boxes). Numbers refer to positions of amino acids in the S6 sequence;

black bars indicate the positions of NLSs; and hatched boxes correspond to an octapeptide sequence derived from amino acids of SV40 large T antigen and the *EcoRI* linker. The intracellular location of fusion proteins is indicated as follows: nucleolus (Nu) and cytoplasm (C).

Table 1. Location of the S6 deletions mutants

Plasmid	Deleted amino acids	Location	
		Nu/N (%)	C (%)
S6-d1	¹⁶⁷ KKPR ¹⁷⁰	100	—
S6-d2	¹⁸⁸ KRRR ¹⁹¹	100	—
S6-d3	²³⁰ KRRR ²³³	100	—
S6-d1,2	167–170; 188–191	100	—
S6-d1,3	167–170; 230–233	90	10
S6-d2,3	188–191; 230–233	90	10
S6-d1,2,3	167–170; 188–191; 230–233	—	100

The distribution of the corresponding β -galactosidase fusion proteins was evaluated in 100 transfected cells. Chimeras with 100% in the column Nu/N showed a preferential staining of the nucleolus whereas an exclusive staining of the cytoplasm was detected in the triple mutant. Transfections with S6-d1,3 and S6-d2,3 showed a strong staining of the nucleolus and the nucleoplasm and in addition a faint staining of the cytoplasm in approximately 10% of the cells; all other cells transfected with these two constructs showed a preferential staining of the nucleolus.

(Figures 5 and 6C). This result suggested that the pentapeptide ¹⁹⁹TKKNK²⁰³ of the putative NuLS (Table 2) was dispensible for nucleolar targeting of the hybrid protein. We separated NLS1 from NLS2 by cleavage of the cDNA sequence with the endonuclease *Mae*III. The division of S6(182) into the 5' fragment S6(125) (Figure 6A) and 3' fragment S6(57) (Figure 6B) destroyed the nucleolar-targeting function observed with the uncleaved S6(182). The inability of the S6(57)- β -galactosidase fusion protein to localize in the nucleolus indicated that residues 180–198 of S6, although containing a sequence that is related to the retroviral nucleolar signals (Table 2), do not function by themselves as NuLSs.

Next, we extended the construct pPbxS6–4 on the 5' end (Figure 1B), which by itself showed nuclear targeting (Figure 2D) by adding fragment S6(57) (Figure 6B). The extended fragment S6(168) was able to direct β -galactosidase into the nucleolus (Figure 6D). Thus, this construct corroborated the importance of the peptide segment 180–198 containing a putative NuLS (Table 2) for nucleolar targeting of S6. The complementary fragment S6(541) coding for the N-terminal

portion of S6 and containing only NLS1 did not lead to nucleolar accumulation of β -galactosidase but instead exhibited nuclear targeting with low efficiency (Figure 6E).

Taken together, these experiments define a minimal sequence for nucleolar targeting of S6 within the segment of amino acids 183–198; however, the construct S6(57), which contains this minimal sequence, cannot direct the fusion protein into the nucleolus. Therefore, a domain that is necessary but not sufficient for nucleolar accumulation has been defined. Additional peptide sequences at the N-terminal and/or C-terminal side of this domain are essential for nucleolar accumulation of S6 (Figure 5).

DISCUSSION

Why Does Ribosomal Protein S6 Carry Three NLSs?

The three tetrapeptide sequences NLS 1 (¹⁶⁷KKPR¹⁷⁰), NLS 2 (¹⁸⁸KRRR¹⁹¹), and NLS 3 (²³⁰KRRR²³³) have been identified in the carboxy terminal region of S6 function as nuclear-targeting sequences because they direct fusion proteins consisting of these amino acid sequences and β -galactosidase into the cell nucleus (Figure 3) and because deletions of all three NLSs alter the corresponding chimeric S6- β -galactosidase into a cytosolic resident protein (Figure 6F and Table 1). Three NLSs in a single protein have also been detected in p53 protein (Shaulsky *et al.*, 1990) whereas one and two NLSs have been observed in numerous proteins (Garcia-Bustos *et al.*, 1991).

The three NLSs of S6 may function by increasing the transport rate of S6 into the nucleus because it has been observed that import of ribosomal protein into the nucleus is not limited under normal circumstances

Table 2. Nucleolar localization signals

Protein	NuLS-Sequence
rex ^a	² PKTRRRPRRSQQRKRPPTP ¹⁹
rev ^b	³⁵ RQARRNRRRRWRERQR ⁵⁰
tat ^c	⁴⁸ GRKKRRQRRAHQ ⁶¹
S6 ^d	¹⁸⁸ KRRRIALKKQRTKKNK ²⁰³
angiogenin ^e	³¹ RRRGL ³⁵

The NuLS were compiled from the following: ^aSiomi *et al.*, 1988; ^bCochrane *et al.*, 1990; ^cDang and Lee, 1989; and ^eMoroianu and Riordan, 1994. ^dThe S6 peptide sequence (Heinze *et al.*, 1988), which is homologous to the NuLS shown, is included for comparison.

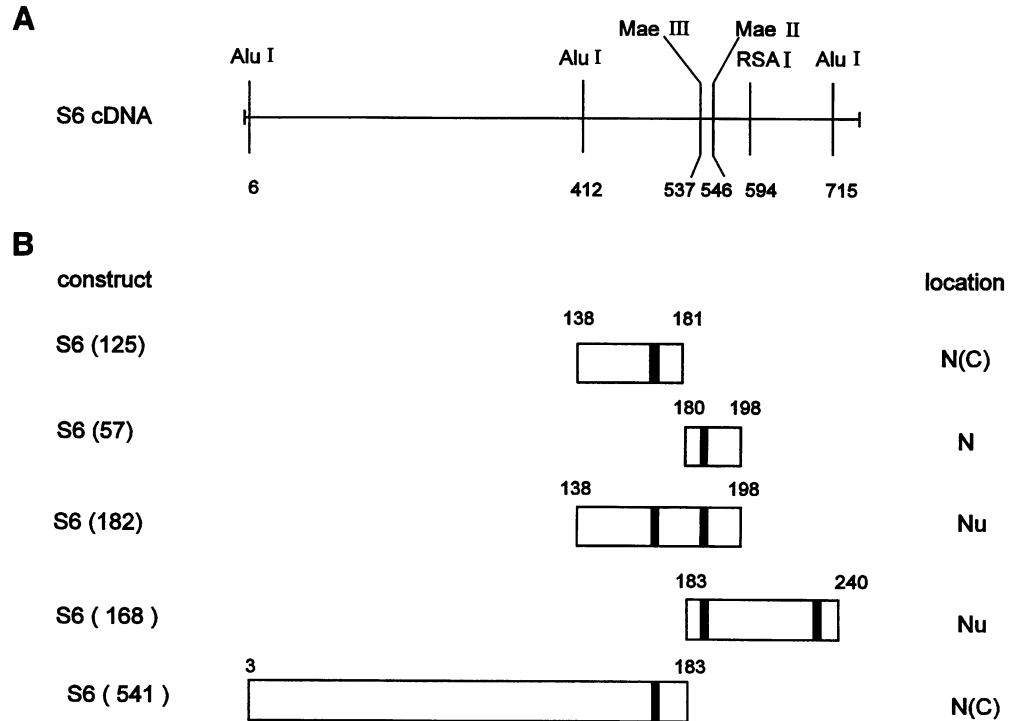


Figure 5. Construction of β -galactosidase fusion proteins for the analysis of nucleolar targeting of S6. (A) Restriction sites of S6 cDNA used in the construction of fusion proteins in panel B. Numbers refer to nucleotide positions in the S6 cDNA; black bars indicate the positions of the NLSs. (B) S6 fragments were fused to the 5' end of the lacZ gene. Numbers refer to the positions of amino acids in the S6 sequence. The intracellular location of the resulting chimeric proteins is indicated as in Figure 3.

(Raué and Planta, 1991). An increased nuclear uptake rate is important because free ribosomal proteins in the cytosol become rapidly degraded (Tsay *et al.*, 1988). Similarly, Lanford *et al.* (1986) and Dworetzky *et al.* (1988) experimentally found a positive correlation between the number of NLS sequences and the import rate of the corresponding proteins into the nucleus. The faster nuclear import could be due to the increased probability of the proteins to interact with the NLS receptor (Adam and Adam, 1994; Görlich *et al.*, 1994), which subsequently catalyses a rapid translocation through the nuclear pores (Melchior *et al.*, 1993; Moore and Blobel, 1994a,b).

How Do the NLSs of S6 Compare with Known NLSs of Yeast Ribosomal Proteins?

This is the first report that describes in detail the nuclear localization signals and nucleolar-targeting properties of a mammalian ribosomal protein. Up to now only NLSs of three ribosomal proteins from the yeast *S. cerevisiae* have been published. Protein L3 has one NLS (¹MSHRLTEAPRHGHLGFLPRKRA²²) (Moreland *et al.*, 1985), protein L25 contains two NLSs (¹MAPSAKATAAKKAVVGK¹⁷ and ¹⁸TNGKKALKVRT²⁸) (Schaap *et al.*, 1991), and protein L29 also contains two NLSs (⁶KTRKHR¹¹ and ²³KHRKHP²⁸) (Underwood and Fried, 1990). Some of these sequences contain histidine in their basic cluster of the targeting signals. This has been very rarely found in

nuclear proteins of higher eukaryotes (Garcia-Bustos *et al.*, 1991). None of these karyophilic signals of yeast is compatible with the minimal consensus sequence proposed by Chelsky *et al.* (1989). It is unknown whether these major differences are species specific or due to the special ribosomal proteins studied. It is hoped that further clarification will result after elucidation of additional NLS signals of ribosomal proteins.

Differential Import Efficiencies of the NLSs of S6

Nuclear targeting sequences consist either of a short basic cluster like the prototype sequence PKKKRKV of SV40 large T antigen (Kalderon *et al.*, 1984) or are constructed of two essential domains of basic amino acids that function in an interdependent manner and a 10-amino acid spacer region that separates the two basic motifs and tolerates mutations (Dingwall and Laskey, 1991; Robbins *et al.*, 1991). The two identical NLSs KRRR of S6 represent a relatively strong signal because when this tetrapeptide is fused to β -galactosidase it directs the hybrid protein exclusively into the nucleus (Figure 3).

Efficiency of nuclear import can be influenced by the protein context of the NLS (Roberts *et al.*, 1987; Nelson and Silver, 1989), by distant sequences (Gao and Knipe, 1992), and by the interdependence of NLSs that have been experimentally demonstrated with p53

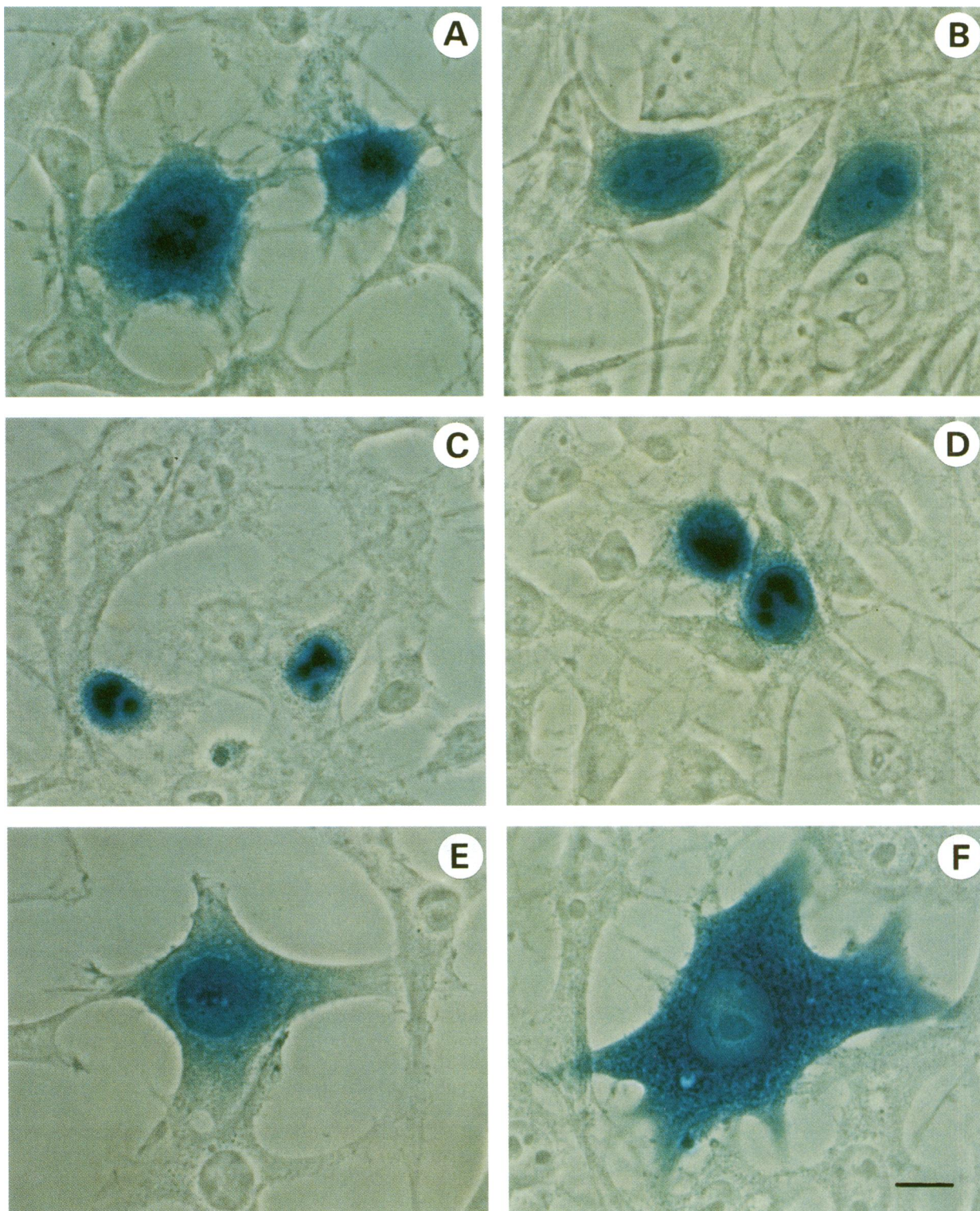


Figure 6. Intracellular distribution of hybrid proteins. The constructs of Figure 5B were transiently expressed in L cells and the intracellular distribution of the resulting fusion proteins was determined by their β -galactosidase activity. (A) S6(125); (B) S5(57); (C) S6(182); (D) S6(168); (E) S6(541); and (F) pPbxS6-d1,2,3. Bar, 10 μ m.

(Shaulsky *et al.*, 1990) and viral proteins (Morin *et al.*, 1989; Nath and Nayak, 1990).

The tetrapeptide KKPR itself (Figure 3) and all chimeras containing only this NLS1 (Figure 6, A and E; Table 1) show a slightly reduced efficiency of nuclear targeting, e.g. in approximately 90% of the cells transfected by S6-d2,3 the corresponding fusion protein accumulated exclusively in the nucleus whereas in the remaining 10% of the cells β -galactosidase activity was detected in the nucleus as well as in the cytosol (Table 1). Similar observations have been reported by Shaulsky *et al.* (1990) who found that none of the individual NLSs of p53 gave rise to an exclusively nuclear localization. Shuttling of nuclear and nucleolar proteins (Schmidt-Zachmann and Nigg, 1993; Schmidt-Zachmann *et al.*, 1993) could also explain our data of lower stringency in nuclear localization, which we observed with chimeras containing NLS1 as the only signal (Figure 6, A and E; Table 1) and with the double mutant S6-d1,3 (Table 1).

Nucleolar Accumulation of S6 in Transfected L Cells

S6, which assembles early into the 40S subunit (Westermann *et al.*, 1981), has to migrate into the nucleolus. S6- β -galactosidase chimeras are presumably targeted into the nucleolus as tetrameric complexes because β -galactosidase activity presupposes tetramer formation. Because each 40S subunit contains only a single S6 molecule we assume that integration of the S6- β -galactosidase fusion protein into preribosomes (if it occurs at all) will inhibit further maturation of ribosomal subunit formation. This assumption would question additional interactions with other surrounding ribosomal proteins under our experimental conditions. Inhibition of 40S biogenesis might be the reason for the dark blue staining of the nucleoli in transfected cells. Nucleolar detection of yeast L25 that was tagged with a myc epitope of 10 amino acids (Evan *et al.*, 1985) proved to be difficult because of its rapid integration into the assembling ribosomal subunits and the subsequently immediate export of the particles. In two other reports on ribosomal proteins of *S. cerevisiae*, fusion proteins with β -galactosidase have been immunologically detected in polysomal fractions (Gritz *et al.*, 1985; Moreland *et al.*, 1985). These previous observations are compatible with the interpretation of our data because in those reports fusion proteins were detected by specific antibodies that may have bound to nascent chains.

S6 does not contain a specific short NuLS similar to the nucleolar localization signals of retroviral regulatory proteins that catalyze migration into the nucleolus by themselves (Table 2). Short nucleolar-targeting signals are not unique to retroviral proteins as the NuLS of angiogenin demonstrates (Table 2). Our analysis of the nucleolar targeting shows that the segment

of amino acids 183–198 is necessary but not sufficient for nucleolar accumulation of S6. Nucleolar localization of S6 requires additional peptide sequences flanking this segment at the amino and carboxy terminal side (Figures 5 and 6). Similarly, in other cellular proteins, nucleolar accumulation is facilitated by cooperation of several functional domains (Milarski and Morimoto, 1989; Peculis and Gall, 1992; Meßmer and Dreyer, 1993; Schmidt-Zachmann and Nigg, 1993; Yan and Mélése, 1993; Mears *et al.*, 1995). The nucleolar localization of S6-d1,3 (Table 1) suggests that the deleted NLS1 and NLS3 are not involved in targeting S6 into the nucleolus.

Because the nucleoli are contiguous with the nucleoplasm and not separated by a lipid membrane, a receptor-mediated uptake mechanism need not be postulated. The peptide sequences that lead to nucleolar accumulation of S6 might be involved in binding to rRNA, because S6 assembles early into the subunits, and/or may be important for interactions with surrounding ribosomal proteins. Our experiments with the deletion mutants can be interpreted by postulating that nuclear translocation through the pores of the nuclear envelope and nucleolar accumulation occur via essentially independent mechanisms, although nuclear import of S6 is a prerequisite for subsequent nucleolar accumulation. For nucleolar localization of S6, a mechanism depending on retention by binding to rRNA molecules may be envisaged. Binding as a model for nucleolar accumulation may explain why no uniform consensus sequence for nucleolar localization has yet emerged. Domains essential for nucleolar anchoring may differ depending on the nature of the nucleolar constituent with which they have to associate.

ACKNOWLEDGMENTS

We thank Ercan Akgün (Heinrich Pette Institut, Hamburg) for the gift of the expression vector pPbxnlsxlacZ. We gratefully acknowledge Adreas Bauche for his expert technical assistance and photography, and Thomas Klokow for designing the figures and printing. We thank Xiaosheng Wu for helpful discussions and critical reading of the manuscript. This work is part of the Ph.D. thesis of C.S. and E.L., and was supported by a grant from the Deutsche Forschungsgemeinschaft to J.K.

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