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Distinct Domains in Ribosomal Protein L5 Mediate 5 S rRNA Binding and Nucleolar Localization*

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Ribosomal protein L5, a 34-kDa large ribosomal subunit protein, binds to 5 S rRNA and has been implicated in the intracellular transport of 5 S rRNA. By immunofluorescence microscopy, L5 is detected mostly in the nucleolus with a fainter signal in the nucleoplasm, and it is known to also be a component of large ribosomal subunits in the cytoplasm. 5 S rRNA is transcribed in the nucleoplasm, and L5 is thought to play an important role in delivering 5 S rRNA to the nucleolus. Using RNA-binding assays and transfection experiments, we have delineated the domains within L5 that confer its 5 S rRNA binding activity and that localize it to the nucleolus. We found that the amino-terminal 93 amino acids are necessary and sufficient to bind 5 S rRNA *in vitro*, while the carboxyl-terminal half of the protein, comprising amino acids 151–296, serves to localize the protein to the nucleolus. L5, therefore, has a modular domain structure reminiscent of other RNA transport proteins where one region of the molecule serves to bind RNA while another determines subcellular localization.

Assembly of ribosomal subunits takes place in eukaryotic cell nucleoli and involves the coordination of several events prior to nuclear export of the mature subunit to the cytoplasm. These events include RNA polymerase I transcription and subsequent processing of 18, 28, and 5.8 S rRNAs, which occurs in nucleoli, the nuclear import, and nucleolar concentration of roughly 30 small subunit and 40 large subunit ribosomal proteins, as well as transcription by RNA polymerase III and nucleolar accumulation of 5 S rRNA (reviewed by Franke (1988), Gerbi *et al.* (1990), Warner (1990), Sollner-Webb and Mougey (1991), Scheer and Weisenberger (1994), and Melese and Xue (1995)). The components of nascent ribosomal subunits therefore originate in at least three different cellular compartments: the cytoplasm (ribosomal proteins), the nucleoplasm (5 S rRNA), and the nucleolus (18, 28, and 5.8 S rRNAs). This requires complex intracellular trafficking in order to ensure that all of the subunit components, in the proper stoichiometry, are present in the nucleoli so that efficient ribosome subunit assembly can occur.

One portion of this process, the biogenesis of 5 S rRNA, is becoming better understood at the level of mechanistic detail. In human somatic cells, transcription of 5 S rRNA occurs mostly on genes clustered in repeats on the telomeric region of the long arm of chromosome 1 (q42–q43, Steffensen *et al.*, 1974; Little and Braaten, 1989). Immediately after transcription, 5 S rRNA is transiently associated with the La protein, which functions in transcription termination of all polymerase III transcripts (Gottlieb and Steitz, 1989). After association with La, 5 S rRNA is bound by ribosomal protein L5 to form an RNP[1] that can be recognized by specific autoantibodies (Steitz *et al.*, 1988). Pulse-chase labeling, followed by immunoprecipitation experiments with these autoantibodies, has demonstrated that the L5–5 S RNP forms prior to, and is therefore a likely precursor to, ribosome assembly. An intranuclear trafficking pathway has been proposed whereby the L5–5 S RNP forms in the nucleoplasm and then migrates to the nucleoli to participate in large ribosomal subunit assembly, and a putative function of delivering 5 S rRNA to the nucleolus was therefore assigned to L5 (Steitz *et al.*, 1988). The 5 S rRNA biogenesis pathway has been more extensively studied in *Xenopus* oocytes. In this system, because of the extraordinary demands for ribosome production in the developing egg, the pathway is far more complex. In previtellogenic oocytes, oocyte-type 5 S rRNA is transcribed in large quantities prior to the production of other ribosomal components and therefore is immediately exported to the cytoplasm. While in the cytoplasm, it is complexed in one of two different storage particles: the 7 S particle, which has transcription factor IIIA (TFIIIA) as a protein component; or the 42 S particle, which contains 5 S rRNA, tRNAs and other proteins (reviewed by Tafuri and Wolffe (1993)). After synthesis of ribosomal proteins begins, during vitellogenesis, 5 S rRNA is exchanged from the storage particles onto L5 (Allison *et al.*, 1991, 1993). The L5–5 S RNP migrates back into the nucleus and then to the nucleoli where subunit assembly occurs. Although this additional cytoplasmic phase of the 5 S rRNA biosynthetic pathway is probably unique to oocytes and does not occur in somatic cells (Allison *et al.*, 1995), it is clear from studies in both systems that L5 plays a significant role in the intracellular trafficking of 5 S rRNA.

As a step toward a more detailed understanding of the 5 S rRNA transport pathway, we were interested in the sequences within L5 that mediate its transport properties. In this report we delineate the domain in L5 that confers its ability to bind 5 S rRNA as well as the region that allows the protein to accumulate in the nucleolus, and find that these domains are separable. Additionally, we find that L5 mutants that maintain 5 S rRNA binding activity cannot localize to the nucleolus if they lack the carboxyl-terminal half of the protein, indicating that 5 S rRNA binding is neither necessary nor sufficient for nucleolar targeting. These results therefore strengthen the idea that L5 functions in part to target 5 S rRNA to the nucleolus.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U48270.

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[1] The abbreviation used is: RNP, ribonucleoprotein.

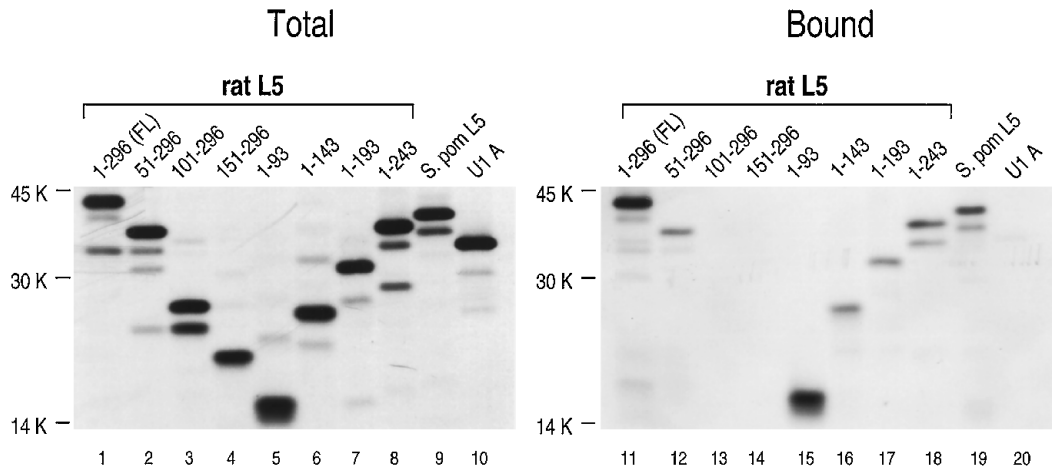

FIG. 1. **Delineation of the 5 S rRNA-binding domain of L5.** *A*, total ^{35}S-labeled translation products of reactions programmed with rat L5 (*lane 1*), rat L5 deletion mutants (*lanes 2–8*, named according to the L5 sequences encoded by that particular plasmid), *S. pombe* L5 (*lane 9*), and human U1-specific small nuclear RNP A protein (*lane 10*). Proteins were fractionated on SDS-PAGE gels, and the gels were subsequently fluorographed to enhance visualization of the proteins. *B*, results of 5 S rRNA-binding reactions with the proteins displayed in *A*.

EXPERIMENTAL PROCEDURES

5 S rRNA-binding Assays—The human 5 S rRNA gene was polymerase chain reaction-amplified using plasmid pH5 SB (Little and Braaten, 1989; kind gift of J. Sylvester) as template and subsequently subcloned as a *Eco*RI-*Hin*dIII fragment into pSP72 (Promega, Madison, WI) to produce plasmid p5TU. Biotinylated 5 S rRNA was produced by transcription *in vitro* as described (Boelens *et al.*, 1993). ^{35}S-Labeled proteins were produced by translation *in vitro* using a TnT kit (Promega, Madison, WI) according to the manufacturer's instructions. Plasmid petg10:A (kind gift of Dr. Carol Lutz) was used to produce U1 A protein. Binding assays and RNP analysis was done exactly as described (Boelens *et al.*, 1993).

Immunological Screening—A *Schizosaccharamyces pombe* cDNA library (Clontech, Palo Alto, CA) was directly screened with antibodies against human RNP proteins according to published procedures (Matunis *et al.*, 1992). A 350-base pair fragment was recovered from the screening and subsequently used to screen by hybridization a *S. pombe* genomic library (kind gift of N. Kaufer), which resulted in the isolation of a 2.2-kilobase pair *Eco*RI fragment containing the entire L5 coding sequences. DNA sequencing was performed using a Sequenase kit (U. S. Biochemical Corp.) according to the manufacturer's instructions.

HeLa Cell Culture, Transfection, and Immunofluorescence—Myc-tagged rat L5 and deletion derivatives were produced by polymerase chain reaction amplification of plasmid pL5–6-4 (Chan *et al.*, 1987; kind gift of I. Wool) to produce a *Eco*RI-*Xho*I fragment, which was then subcloned into plasmid myc-A1 (Michael *et al.*, 1995). HeLa cells were maintained, transfected, and processed for immunofluorescence exactly as described (Michael *et al.*, 1995).

RESULTS

To initiate our studies on the domain structure of L5, we wanted to determine the sequences that contribute to its 5 S rRNA binding activity. To do so, we used a rat L5 cDNA clone (Chan *et al.*, 1987), kindly provided by Dr. Ira Wool, to study binding to human 5 S rRNA using a protein-RNA binding assay that is similar to other published procedures (Boelens *et al.*, 1993; Ashley *et al.*, 1993). Briefly, a human 5 S rRNA gene (a kind gift of Dr. Jim Sylvester) was cloned downstream of a T7 promoter to allow production of the RNA by transcription *in vitro*. Biotin-UTP was included in the transcription reaction to produce biotinylated 5 S rRNA, which was then incubated in binding buffer with nonspecific competitor RNA and ^{35}S-labeled L5 protein derivatives made by *in vitro* translation in reticulocyte lysate. The RNP complexes where then selected by incubation with streptavidin coupled to agarose beads followed by washing and bound proteins were analyzed by SDS-PAGE. Under these assay conditions the full-length rat L5 protein bound tightly to 5 S rRNA (Fig. 1, *lane 11*), whereas another RNA-binding protein, the human small nuclear RNP U1 A protein, had no detectable binding activity (Fig. 1, *lane 20*), demonstrating the specificity of this assay. We next examined the 5 S rRNA-binding capacity of a set of rat L5 deletion mutants and found that deletion mutants that retained the amino-terminal half of the protein maintained binding activity, while mutants lacking these sequences failed to bind. The smallest fragment that retained binding activity corresponds to amino acids 1–93 (Fig. 1, *lane 15*), while another fragment, comprising amino acids 101–296 (Fig. 1, *lane 14*), did not bind, indicating that the first 100 amino acids of the L5 protein are both necessary and sufficient for 5 S rRNA binding activity. Interestingly, the deletion mutant 51–296 (Fig. 1, *lane 12*) also contains binding activity, indicating the L5 RNA-binding domain may be delineated even further within the first 100 amino acids.

As an additional means to gain insight into the particular amino acids within L5 that contribute to 5 S rRNA binding activity, we cloned and sequenced the gene encoding L5 from the lower eukaryote *S. pombe*. An alignment between fission yeast L5 and the rat counterpart is presented in Fig. 2. The two proteins are 46% identical and 70% similar when conservative amino acid substitutions are considered. These numbers are consistent with the level of conservation between the fission yeast protein and the L5 homologues from chicken, frog, budding yeast, and rice (Kenmochi *et al.*, 1991; Wormington, 1989; Tang and Nazar, 1991; Kim and Wu, 1993). When we tested the *S. pombe* L5 protein for binding, we found that, despite being only 46% conserved relative to the rat protein, the fission yeast L5 binds to human 5 S rRNA (Fig. 1, *lane 19*).

In order to understand the relationship between 5 S rRNA binding and the intracellular localization of the rat L5 protein, we transfected HeLa cells with epitope-tagged L5 derivatives and determined their localization by immunofluorescence microscopy. Full-length L5 protein localizes predominantly to the nucleoli and also exhibits a fainter nucleoplasmic staining pattern (Fig. 3). This staining pattern is in good agreement with that observed using an antiserum that recognizes the L5–5 S RNP particle (Steitz *et al.*, 1988), suggesting that uncomplexed L5, if it exists in sufficient quantity to be detected, co-localizes with the RNA-bound form. We next examined two deletion mutants, L5 1–150 and L5 151–296 (Fig. 3). Interestingly, we found that the 1–150 fragment, which binds 5 S rRNA *in vitro*, can enter the nucleus but does not localize to the nucleolus. Conversely, the 151–296 fragment localizes to both the nucleus and nucleolus with wild type efficiency yet cannot bind 5 S

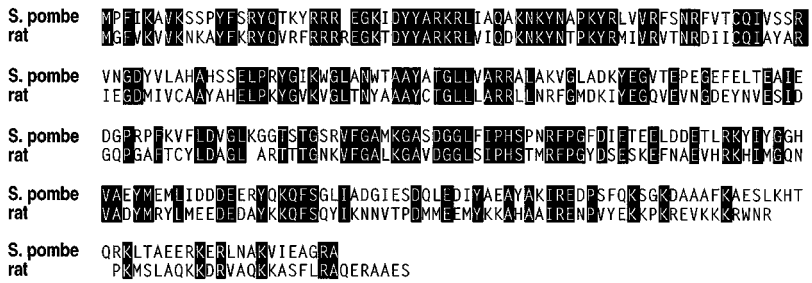

FIG. 2. **Amino acid alignment of the *S. pombe* and rat L5 proteins.** Identical amino acids are highlighted in *black boxes*.

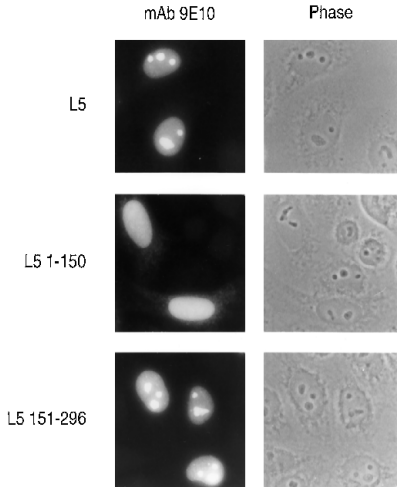

FIG. 3. **Intracellular localization of rat L5 (L5) and deletion mutants comprising amino acids 1–150 (L5 1–150) and 151–296 (L5–151-296).** Plasmids encoding these proteins were transfected into HeLa cells. Forty h post-transfection, the cells were fixed and processed for immunostaining with monoclonal antibody 9E10, which recognizes the Myc tag (panel 9E10). The phase contrast image is depicted in panel (*Phase*).

rRNA. These results demonstrate that the 5 S rRNA-binding domain is not required for the nuclear import or intranuclear transport of the L5 protein and that the signal(s) which mediates nucleolar localization resides in the carboxyl-terminal 150 amino acids. In order to further delineate the sequences within amino acids 151–296 that confer nucleolar localization, we wanted to first transfer the region onto a heterologous, non-nuclear/nucleolar protein and confer nucleolar localization onto that fusion protein. Unfortunately, repeated attempts with either full-length L5 or L5 151–296 fused to a number of different reporters, including the bacterial proteins β-galactosidase and maltose-binding protein as well as chicken pyruvate kinase, resulted in a nuclear, but not nucleolar localization of these proteins (data not shown). The reason for this is unknown, but the inability to transfer a nucleolar localization sequence onto a reporter protein has been previously noted for other nucleolar proteins (Peculis and Gall, 1992; Schmidt-Zachmann and Nigg, 1993). Interestingly, in the case of the β-galactosidase and maltose-binding protein full-length L5 fusion proteins, the ability to bind 5 S rRNA *in vitro* is maintained (data not shown), whereas the nucleolar localization properties are not, which further supports the conclusion that 5 S rRNA binding activity alone is insufficient for nucleolar localization.

DISCUSSION

The L5 5 S rRNA-binding domain—In order to learn more about the domain structure of L5, we carried out RNA-binding assays and found that the first 93 amino acids comprise the minimal domain tested which binds 5 S rRNA. Data base searches with this fragment as well as visual inspection of the sequence did not identify similarities to any other known RNA-binding proteins outside of the L5 family of ribosomal proteins or to any known RNA-binding sequence motifs (reviewed by Burd and Dreyfuss (1994)), suggesting that this domain is unique to this family. Additionally, we found that the fission yeast L5 protein, which is 46% identical to mammalian L5, maintains the capacity to bind human 5 S rRNA. An amino acid alignment of the 5 S rRNA-binding domain from rat, rice, and fission yeast L5, as well as the equivalent region from the highly related, 5 S rRNA-binding archebacteria *Methanococcus vannielii* L18 protein (Jahn *et al.*, 1991) is presented in Fig. 4. The region is very well conserved throughout its length from archebacteria to mammals, suggesting a strong conservation of function. We note the presence of a proline residue at position 57 in the rat sequence, which is absolutely conserved. Proline residues often serve as helix breakers within proteins and can potentially demarcate separate functional domains. If this is the case with L5, then the 5 S rRNA-binding domain can be separated into two parts, as indicated in Fig. 4. Given our result that the 51–296 fragment maintains 5 S rRNA binding activity, it appears possible that the minimal 5 S rRNA-binding domain corresponds to section II in Fig. 4. The RNA-binding assay employed in this work is not suitable for protein fragments of sizes much smaller than 100 residues; therefore, it will be of interest to produce recombinant protein corresponding to amino acids 51–93 of rat L5 and to test it in other RNA-binding assays. Production of this fragment in *E. coli* has, however, so far proven difficult. Nevertheless, the 93-amino acid fragment is sufficiently small as to make possible NMR structural studies, and such experiments are now in progress.

Nucleolar Targeting of L5—The next step toward our understanding of the structure-function relationship of the domains within L5 involved in 5 S rRNA trafficking came from subcellular localization experiments. We found that epitope-tagged L5 localizes primarily in the nucleolus with a fainter signal in the nucleoplasm and virtually no signal in the cytoplasm, which may be due either to absence of the protein from the cytoplasm or to masking of the epitope while buried in the large ribosomal subunit. This pattern is in agreement with earlier localization studies, which looked at endogenous L5–5 S RNPs using a human auto-antibody (Steitz *et al.*, 1988), and is also consistent with the results of *in situ* hybridization experiments, which localized 5 S rRNA in HeLa cells (Carmo-Fonseca *et al.*, 1993). This localization pattern is interesting in light of earlier findings regarding expression levels of the L5–5 S RNP. Knight and Darnell found that only half of the 5 S rRNA molecules in HeLa cells are confined to ribosomes, while the other half is complexed with a protein, now known to be L5 (Knight and Darnell, 1967; Steitz *et al.*, 1988). Additionally, earlier work that examined the relative nucleolar stoichiometry of ribosomal proteins in HeLa cells found that the ratio of L5 in the nucleolus relative to that found in cytoplasmic ribosomes is nearly 10-fold higher than that of any of the other ribosomal protein (Phillips and McConkey, 1976). Taken together with the localization data, these studies indicate that there is a substantial pool of non-ribosomal associated L5–5 S RNP lo-

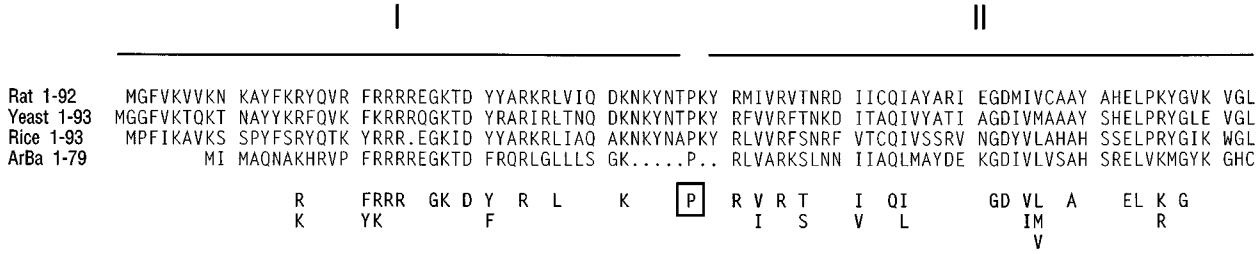

FIG. 4. **Amino acid alignment of the defined 5 S rRNA-binding domain of rat L5 to the corresponding regions in *S. pombe* (*yeast*) and rice (*rice*) L5 proteins and the archebacterial *M. vannielii* L18 protein (*ArBa*).** The residues which are absolutely conserved or highly similar between all four proteins are indicated beneath the alignment in *bold lettering*. The conserved proline at rat L5 position 57 is *boxed* in the consensus, and the regions within the domain flanking the proline are designated *I* and *II*.

cated primarily in the nucleolus at a concentration which greatly exceeds that of assembling ribosomal subunits.

The idea that nucleolar proteins require specific targeting signals to localize to the nucleolus has been challenged recently due to the lack of a clear consensus motif within the several proteins for which this type of signal has been delineated. Rather, it appears plausible that nucleolar localization is a retention-driven process whereby proteins accumulate within nucleoli by virtue of binding to nucleolar components (see Yan and Melese (1993)). For instance, in the case of the abundant nucleolar protein nucleolin, the domains that are necessary for nucleolar accumulation map to the RNA-binding domains of the protein, which indicates that interaction with pre-rRNA is the driving force for nucleolar localization of this protein (Schmidt-Zachmann and Nigg, 1993). Our results with L5 demonstrate that the carboxyl half of the protein contains the sequences required for nucleolar localization, that 5 S rRNA binding activity is neither necessary nor sufficient for L5 nucleolar localization and consequently that 5 S rRNA does not contain the information required to properly localize the L5–5 S RNP to the nucleolus. Therefore, if the retention-driven model is correct, 5 S rRNA does not act as the anchor for L5 nucleolar localization. Additionally, nascent large ribosomal subunits seem to be unlikely candidates as the L5–5 S RNP is present in large excess over nascent subunits within nucleoli (Phillips and McConkey, 1976). One possibility is that the L5–5 S RNP first localizes to the nucleolus by virtue of an interaction between the carboxyl terminus of L5 and some as yet unknown nucleolar component and then serves to nucleate large subunit assembly. This would help explain why the L5–5 S RNP is present in excess over other ribosomal proteins within nucleoli.

5 S rRNA Trafficking—The goal of this study was to gain information about the functional domains within the L5 protein that contribute to the subcellular transport of the L5–5 S RNP. The results presented here substantiate an earlier proposal that L5 functions in part to deliver 5 S rRNA to the nucleolus (Steitz *et al.*, 1988). A model for 5 S rRNA trafficking can be formulated where the L5 amino-terminal domain binds to 5 S rRNA shortly after transcription in the nucleoplasm and the RNP then localizes to the nucleolus by virtue of interactions between a nucleolar component and the carboxyl-terminal domain of L5. After nucleolar localization, the RNP becomes incorporated into large ribosomal subunits and is then exported with them from the nucleus to the cytoplasm. This model is consistent with recent work concerning 5 S rRNA transport in *Xenopus* oocytes, where mutant 5 S rRNA molecules unable to bind either L5 or TFIIIA were shown to be incapable of nucleo-cytoplasmic transport (Guddat *et al.*, 1990). According to our model, one interpretation of this result is that, at least in *Xenopus* oocytes, TFIIIA mediates 5 S rRNA export along an as yet undefined pathway and L5 mediates 5 S rRNA export by virtue of nucleolar localization and subsequent large ribosomal subunit assembly and export. Because the domain within L5 that allows nucleolar localization has now been identified, it becomes possible to search for interacting proteins, which could potentially function in ribosomal subunit assembly and export.

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