Different Domains Cooperate to Target the Human Ribosomal L7a Protein to the Nucleus and to the Nucleoli*

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The human ribosomal protein L7a is a component of the major ribosomal subunit. We transiently expressed in HeLa cells L7a-β-galactosidase fusion proteins and studied their subcellular localization by indirect immunofluorescence staining with anti- β -galactosidase antibodies. We have identified three distinct domains responsible for the nuclear targeting of the protein: domain I, amino acids 23-51; domain II, amino acids 52-100; domain III, amino acids 101-220, each of which contains at least one nuclear localization signal (NLS). Through subcellular localization analysis of deletion mutants of L7a-B-galactosidase chimeras, we demonstrate that domain II plays a special role because it is necessary, although not sufficient, to target the chimeric β -galactosidase to the nucleoli. In fact, we demonstrate that the nucleolar targeting process requires the presence of domain II plus an additional basic domain that can be represented by an NLS or a basic stretch of amino acids without NLS activity. Thus, when multiple NLS are present, each NLS exerts distinct functions. Domain II drives nucleolar accumulation of a reporter protein with the cooperative action of a short basic amino acid sequence, suggesting a mechanism requiring protein-protein or protein-nucleic acid interactions.

The biogenesis of eukaryotic ribosomes is a complex process that takes place in the cell nucleus. Soon after synthesis in the cytoplasm, ribosomal proteins (r-proteins)¹ are transported to the nucleus and subsequently accumulated in the nucleoli where they are associated with the precursor-rRNAs (pre-rR-NAs), which are concomitantly processed into mature rRNA molecules (1). The assembled ribosomal subunits are eventually exported to the cytoplasm to function in protein biosynthesis. Thus, the biogenesis of eukaryotic ribosomes entails an intensive traffic of molecules across the nuclear membrane and, therefore, r-proteins are a good model with which to study the mechanism of nuclear transport and nucleolar accumulation of proteins. The nuclear transport of proteins depends upon the presence of one or more nuclear localization signals (NLS). These sequences have been found throughout the

polypeptide chain (2) and, in most cases, consist of either short basic amino acid sequences like the NLS of the SV40 large T-antigen (126PKKKRKV132) (3, 4) or longer bipartite sequences consisting of two stretches of basic amino acids separated by about 10 amino acids (5). NLS are both necessary and sufficient to target a cytoplasmic protein to the nucleus (4). Much less is known about the mechanism of nucleolar targeting of proteins.

Studies on the nucleolar accumulation of viral proteins have suggested that, like nuclear transport, nucleolar transfer is mediated by short amino acid sequences, namely nucleolar localization signals (NOS) (6-9). A NOS motif, however, is not present in the cellular nucleolar proteins that have been identified so far, e.g. NO38 (10), nucleolin (11, 12), NSR1 (13), GAR1 (14). Studies on the targeting mechanism of the nucleolar protein NO38 have revealed that a domain of 24 amino acids at the carboxyl terminus, with no similarity to the viral nucleolar targeting signal, is essential for its nucleolar accumulation (10). However, when this domain is fused to a reporter protein it is unable to target the hybrid protein to the nucleolus, indicating that the cooperative action of different domains is required for the nucleolar accumulation of NO38. The nucleolar targeting of nucleolin, the major nucleolar protein in vertebrate cells (15, 16), requires both the glycine/arginine-rich (GAR) domain (11, 12) and the RNA binding domains. Nevertheless, the fusion of each of these domains to a reporter protein does not result in directing the chimeric protein to the nucleolus. A similar analysis of NSR1, a yeast nucleolar protein related to mammalian nucleolin, has shown that its nucleolar accumulation is mediated either by different combinations of regions in the NH₂ terminus that contain NLS binding motifs or by the RNA binding domains (13). Because no membrane envelope is involved in selecting nucleolar molecules (17), these results have suggested that nucleolar accumulation of proteins is not due to one or more general nucleolar targeting signals but rather to functional interactions between one or more domains of the protein with other macromolecules residing in the nucleolus. A similar mechanism drives the subnuclear localization of a variety of nuclear components, e.g. the nuclear lamin proteins that specifically assemble to the nuclear membrane (18), and the splicing factors $su(w^a)$ and tra (19).

We describe the three domains of the human r-protein L7a responsible for its nuclear targeting and nucleolar accumulation.

EXPERIMENTAL PROCEDURES

Plasmids—All constructs were prepared in pSVEX-βgal, a derivative of pDEX.² Briefly, an expression cassette including the β -globin promoter followed by the dihydrofolate reductase cDNA was removed from pDEX. Then, an EcoRI-XbaI DNA fragment containing the coding se-

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[§] To whom correspondence should be addressed. ¹ The abbreviations used are: r-protein, ribosomal protein; NLS, nuclear localization signal(s); NOS, nucleolar localization signal(s); PBS, phosphate-buffered saline.

² G. Russo, A. Gast, E. Schlaeger, A. Angiolillo, and C. Pietropaolo, manuscript in preparation.



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1MPKGKKAKGKKVAPAPAVVKKQ22

23EAKKVVNPLFEKRPKNFGIGQDIQPKRDL51	domain	I
52TRFVKWPRYIRLQRQRAILY <u>KRLK</u> VPPAINQFTQALDRQTATQLLKLAH100	domain	II
101KYRPETKQEKKQRLLARAEKKAAGKGDVPTKRPPVLRAGVNTVTTLVENKKAQLVVIA HDDPIELVVFLPALCRKMGVPYCIIKGKARLGRLVHRKTCTTVAFTQVNSEDKGALAKLVE220	domain	111

221AIRTNYNDRYDEIRRHWGGNVLGPKSVARIAKLEKAKAKELATKLG266

FIG. 1. Panel A, restriction map of the pLacZ vector. bp, base pairs. Panel B, nucleotide sequence of the HindIII-PstI linker coding for the 5'-untranslated region and start codon (in boldface) of L7a, and the spurious eight NH_2 -terminal amino acids of β -galactosidase; Val is the first authentic β -galactosidase amino acid. Panel C, amino acid sequence of human r-protein L7a (GenBank accession number X52138), showing the nuclear localization-competent domains.

quence, starting at the eighth amino acid codon, of *Escherichia coli* LacZ was excised from the vector pC4 β gal (20) and cloned in the *Eco*RI-XbaI sites of the pDEX vector polylinker. The resulting vector is indicated as pSVEX- β gal. Two complementary oligonucleotides were synthesized (5'-AATTGAAGCTTCTCTCTCTCTCCCGCCGCCCAGA<u>AT</u>_<u>G</u>GCTGCAGG3-' and 5'-AATTCCTGCAGC<u>CAT</u>CTTGGGCGGGGGAGGAGAGAGAGAGCTTC-3'), coding for the 5'-untranslated region and the start codon of L7a (shown underlined), flanked by *Hind*III and *Pst*I recognition sites at the 5' and 3' termini to be used, eventually, to obtain in-frame fusions to the eighth codon of the β -galactosidase. The linkers terminated with *Eco*RI site staggered ends and were inserted in the *Eco*RI site of pSVEX- β gal to generate the vector indicated as pLacZ

in Fig. 1A. As a result, the pLacZ vector contained a chimeric cDNA coding for the 5'-untranslated region of L7a and followed by a coding sequence of a β -galactosidase in which the first eight amino acids at the NH₂ terminus were different from the amino acids of the natural protein (see Fig. 1B). In DNA transfection experiments, pLacZ produced a protein localized in the cytoplasm. To produce a protein targeted to the cell nucleus, we constructed the pLacZ-NLS vector by inserting in the *Hin*dIII-*PstI* sites of pLacZ a synthetic double strand DNA sequence coding for the NLS, ¹²⁶PKKKRKVE¹³³, which is responsible for the nuclear targeting of the SV40 T-antigen.

To create fusion constructs containing the entire L7a cDNA (pL7a) or cDNA fragments encoding different domains of the L7a protein, we



FIG. 2. Diagrams of fusion constructs containing the L7a domain (black bar) fused to β -galactosidase cDNA, and subcellular localization of the chimeric protein. \diamond and \P indicate constructs for which immunofluorescence data, obtained with a Zeiss Axiovert 10 (\diamond) or with a Zeiss laser scan (\P) microscope are shown. Similar immunofluorescence data were obtained with the other constructs (data not shown). *Panel A*, constructs containing domains of the NH₂ moiety of L7a. *Panel B*, constructs containing domains of the COOH moiety of L7a.

synthesized appropriate primers to use in polymerase chain reaction amplification of L7a cDNA templates. Primers used for the amplification of cDNA coding for domains in the NH₂ moiety of L7a (pL7a1–220, pL7a1–100, pL7a1–51, pL7a1–40, pL7a1–22, pL7a1–17; see Fig. 2) were designed to carry at the 5' and 3' termini the recognition sites for *Hind*III and *Pst*I, respectively, to allow cloning in the *Hind*III-*Pst*I sites of the pLacZ vector. Primers for the amplification of cDNA coding for the domains in the COOH moiety of L7a (pL7a221–266, pL7a139–266, pL7a101–266, pL7a52–266, see Fig. 2) or the domain II of L7a (pL7a52–100, see Fig. 5) were designed to allow cloning in the *PstI-EcoRI* sites of the pLacZ vector.

The vectors pL7a52–100-NLS and pL7a101–266-NLS were obtained by cloning complementary synthetic linkers coding for the NLS sequence of the SV40 T-antigen in the *Hin*dIII-*Pst*I sites of pL7a52–100 and pL7a101–266. The mutated forms of NLS from SV40 T-antigen (NLS* vectors in Fig. 5) were obtained using the same procedure as for the vectors containing the wild type NLS sequence (NLS vectors in Fig. 5) and synthetic oligonucleotide linkers carrying the required mutation. For vector pL7a52–100-b a *Hin*dIII-*Pst*I fragment from pLa7a1–17 was cloned in the *Hin*dIII-*Pst*I sites of pL7a52–100. Vector pL7a52–100-h was constructed by inserting *Hin*dIII-*Pst*I linkers encoding amino acids 12–19 of L7a in the *Hin*dIII-*Pst*I sites of pL7a52–100.

The accuracy of all fusion constructs was verified by nucleotide sequencing, using the dideoxy chain termination procedure (21) as indicated in the Sequenase 2.0 sequencing kit (U. S. Biochemical Corp.).

Cell Culture and DNA Transfection—HeLa cells (ATCC CCL-2.2) were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 5 mM L-glutamine, 50 μ g/ml streptomycin, and 50 units/ml penicillin. Cells were seeded on coverslips in 60-mm dishes; 18 h after plating, DNA transfection was carried out using 10 μ g of plasmid DNA and the calcium phosphate precipitation method (22). 48 h after transfection, the subcellular localization of the β -galactosidase was revealed by indirect immunofluorescence staining.

Immunofluorescence Microscopy—Cells grown on coverslips were fixed 48 h after DNA transfection with 3.7% formaldehyde in phosphate-buffered saline (PBS), kept for 20 min at room temperature and then permeabilized in 0.1% Triton X-100 in PBS for 5 min. The coverslips were incubated for 15 min at room temperature with a mouse polyclonal antiserum anti- β -galactosidase (Sigma Immunochemicals, Germany). The antiserum was used at a 1:600 dilution in a PBS, 0.2% gelatin solution. Coverslips were washed with PBS, 0.2% gelatin solution and incubated for 15 min at room temperature with fluorochromeconjugated secondary antibodies. As secondary antibodies, an antimouse IgG (Fc-specific) fluorescein isothiocyanate conjugate (Sigma Immunochemicals) was used at a 1:600 dilution in PBS, 0.2% gelatin. The coverslips were washed several times with PBS, 0.2% gelatin and then mounted with Mowiol (Hoechst, Germany) on microscope slides. Cells were analyzed on a Zeiss Axiovert 10 photomicroscope using a $63 \times$ Plan-Apochromat lens, or a Zeiss laser scan microscope (LSM 410), using fluorescein filters (450–490, FT510-LP520).

RESULTS

L7a Protein, as Well as the NLS from SV40 T-antigen, Direct β-Galactosidase to the Nucleus-Human r-protein L7a (23) contains 266 amino acid residues, for a molecular mass of 30 kDa (see Fig. 1C). To identify region(s) of the protein responsible for its nuclear and nucleolar localization we have constructed and expressed several chimeras by fusing portions of the L7a cDNA with the 5' terminus of the coding sequence of the E. $coli \ lacZ$ gene (20). The *lacZ* gene product, β -galactosidase, has been used extensively as a reporter protein to study nuclear translocation of a variety of viral (24), yeast (4, 13, 25), Xenopus (10, 12, 26), and human (27-29) proteins, exploiting both the immunodetection of the protein (4, 10, 12, 13, 24-26, 28) and the β -galactosidase activity that is retained in hybrid proteins (27, 29). In our case, when introduced into mammalian cells, each construct led to a chimeric L7a- β -galactosidase protein. The subcellular distribution of the L7a- β -galactosidase chimeric proteins was examined 48 h after transfection by indirect immunofluorescence using anti- β -galactosidase antibodies. As a control, we used the pLacZ construct (Fig. 1A); the β -galactosidase produced by this vector appeared in the cell cytoplasm together with some nuclear staining (Figs. 2A and 3). This nuclear staining could be due to an intrinsic ability of β -galactosidase to enter the nucleus or to technical artifact where strong cytoplasmic fluorescence at the bottom of the cell makes it appear as a nuclear fluorescence. To address this issue, we examined at a confocal microscope cells transfected with pLacZ vector. Fig. 4 shows a confocal multisection analysis of β -galactosidase-positive cells (pLacZ in Fig. 4). The nucleus appears to be free of β -galactosidase, thus confirming that β -galactosidase is an appropriate reporter for nuclear transport studies.



FIG. 3. Immunofluorescence localization of L7a- β -galactosidase fusion proteins. Cells grown on coverslips were transiently transfected with plasmids encoding the constructs shown in Fig. 2. Cells were fixed with formaldehyde 48 h after transfection and processed for immunofluorescence microscopy, using anti- β -galactosidase polyclonal antibodies as primary antibodies, followed by fluorescein isothiocyanateantimouse IgG.

The β -galactosidase was translocated to the nucleus upon the addition of the T-antigen NLS sequence, as shown by the pLacZ-NLS construct (Figs. 2A and 3). A L7a- β -galactosidase fusion protein, carrying the entire L7a coding sequence (pL7a in Fig. 2), was imported in the nucleus and accumulated in the nucleoli, as expected (Figs. 3 and 4).

L7a Protein Contains Multiple NLS-We designed a series of deletion mutants lacking regions at the COOH or NH₂ terminus of L7a in an attempt to identify the domain(s) responsible for the nuclear import of L7a. The mutant cDNAs were obtained by using the polymerase chain reaction technique and were inserted into the pLacZ vector in-frame with β -galactosidase cDNA (Fig. 1). The whole L7a protein and pL7a1-220, coding for residues 1-220 of L7a, produced a protein that entered the nucleus and accumulated in the nucleoli (Fig. 2A). Deletion of 120 amino acids, as in construct pL7a1-100 (Fig. 2A), led to a protein still targeted to the nucleus and which accumulated in the nucleoli (Fig. 3). However, in all experiments we noted a light immunostaining of the nucleus which was never observed in experiments with the entire L7a. Consequently we conducted a confocal analvsis, which demonstrated that nucleoli were strongly stained in both samples, but nuclei retained a diffuse staining in cells transfected with the pL7a1-100 construct (Fig. 4). A further deletion of 50 amino acids, as in construct pL7a1-51, produced a chimeric protein that entered the nucleus (Figs. 2A and 3) but did not accumulate in the nucleoli. Thus, in the

latter protein, the processes of entry into the nucleus and nucleolar accumulation were dissociated. The deletion of another 10 amino acids resulted in a protein distributed in the nucleus and the cytoplasm (pL7a1-40 in Fig. 4). The transfection of construct pL7a1-22, coding for residues 1-22 of L7a, or construct pL7a1-17, coding for residues 1-17 of L7a, completely abolished the nuclear and nucleolar staining in the transfected cells; in fact, the β -galactosidase produced by these vectors resides exclusively in the cytoplasm (Fig. 2A). These results demonstrate that a nuclear targeting signal for the L7a protein is present in the NH₂-terminal domain, more specifically defined by amino acid residues 22–51, whereas residues 52–100 might play a role in the nucleolar accumulation of a L7a- β -galactosidase fusion protein.

We also dissected the COOH-terminal moiety of the L7a protein to look for NLS. We constructed vectors expressing fusion proteins lacking portions of the NH₂-terminal region of L7a (Fig. 2B). Vector pL7a52–266 coding for residues 52–266 of L7a gave, like the entire L7a, nucleolar staining of transfected cells (Figs. 2B and 3). pL7a101–266 produced a protein that entered the nucleus but did not accumulate in the nucleoli (Figs. 2B and 3). A further deletion of 38 amino acids, as in the pL7a139–266 construct, led to a β -galactosidase chimera distributed between the cytoplasm and the nucleus (Figs. 2B and 3). The COOH-terminal region (pL7a221–266) of L7a did not direct the reporter protein to the nucleus (Fig. 2B). Thus, it seems reasonable to conclude that at least one other NLS is present in the region defined by amino acid residues 101–220 of L7a.

Subnuclear Localization of L7a-β-Galactosidase Chimaeric *Proteins*—We concluded from the data reported above that both the NH₂-terminal and the COOH-terminal moieties of the L7a protein contain at least one NLS. In fact, the 23-51 amino acid region is able, as well as the 101-220 amino acid region, to direct a reporter protein to the cell nucleus. However, only chimeric proteins containing a domain defined by the 52-100 amino acid region of L7a accumulate in the nucleoli. When amino acids 52-100 are deleted (constructs pL7a1-51 in Fig. 2A and pL7a101-266 in Fig. 2B), the resulting chimeric protein does not accumulate in the nucleoli, although it is targeted to the nucleus. This indicates that the failure of the truncated protein to accumulate in the nucleoli is not simply due to the inability to enter the nucleus. To investigate the role of the domain defined by amino acid residues 52-100 in the nucleolar accumulation of L7a, other fusion proteins were produced, and their cellular localization was tested by anti- β -galactosidase antibodies and visualized through immunofluorescence staining. The cDNA segment coding for the 52-100 amino acid region of L7a was first fused to the NH_2 terminus of β -galactosidase cDNA, and the chimeric DNA construct pL7a52-100 was transfected into HeLa cells (Figs. 5 and 6). The reporter protein did not accumulate in the nucleolus; however, it entered the nucleus. Therefore, there is another functional NLS in the L7a region defined by amino acids 52-100. Interestingly, a protein containing both the NLS from SV40 T-antigen and the newly defined nuclear localization-competent domain of L7a entered the nucleus and accumulated in the nucleoli (Figs. 5 and 6).

Cooperation among Protein Domains Is Required to Direct a Chimeric Protein to the Nucleoli—To understand whether the nucleolar accumulation of the chimeric β -galactosidase produced by construct pL7a52–100-NLS was correlated with a redundancy of NLS, we added the SV40 T-antigen NLS to the chimeric protein containing the domain defined by amino acid residues 101–266 of L7a, which enables targeting of a reporter protein to the nucleus (construct pL7a101–266-NLS FIG. 4. Confocal analysis of β -galactosidase localization directed by L7a or by deletion mutants. Cells grown on coverslips were transiently transfected with plasmids encoding the indicated constructs. For immunofluorescence staining, cells were processed as described under "Experimental Procedures" and in the legend to Fig. 3. The serial optical sections were obtained by scanning from the adherent base of the cell to the apical surface in 0.5- μ m steps.





FIG. 5. Diagrams of fusion constructs and subcellular localization of the chimeric protein. In each construct, the *black* \hat{bar} represents an L7a domain fused to the β -galactosidase. PKKKRKVE represents the NLS of the SV40 T-antigen; the NLS* constructs contain mutated forms of NLS. represents the 1-17 amino acid domain of L7a (MP-KGKKAKGKKVAPAPA), \Box represents the 12-19 stretch of hydrophobic amino acids of L7a protein, VAPAPAVV. ◊ indicates constructs for which immunofluorescence data are shown. Similar immunofluorescence data were obtained with other constructs (data not shown).

in Figs. 5 and 6). The β -galactosidase produced by this construct was predominantly nuclear. On the other hand, single point mutations in SV40 T-antigen NLS, which abolished its nuclear targeting activity (constructs NLS* in Fig. 5), did not affect its ability to cooperate in the nucleolar accumulation of the chimeric β -galactosidase (Fig. 6). These results indicate that the 52-100 region of L7a plays a fundamental role in the nucleolar accumulation of L7a, but some helper function is required, which can be surrogated by an NLS domain, although independent from its nuclear targeting activity. A constant feature of nuclear targeting signals is a cluster of basic amino acids; amino acids 1-17 at the NH₂ terminus of L7a represent a positively charged stretch of amino acids which cannot direct a protein to the nucleus (construct pL7a1-17 in Fig. 2A). A cDNA construct in which the cDNA coding for amino acids 1-17 of L7a was fused to the cDNA coding for the 52-100 amino acid region (construct pL7a52100-b) was transfected in HeLa cells and produced a β -galactosidase that was targeted to the nucleoli (see Figs. 5 and 6). On the other hand, we had already demonstrated that a mutated SV40 T-antigen NLS, which had lost its ability to target a protein to the nucleus, was able to cooperate with the 52-100 region of L7a to target a protein to the nucleoli (constructs pL7a52-100-NLS* in Figs. 5 and 6). Thus, taken together, these results indicate that a basic domain added to the 52-100 amino acid region restores the latter's ability to target a reporter protein to the nucleolus. Support for this finding came from an experiment in which we fused the amino acid region 12-19 of L7a to the 52-100 region (construct pL7a52-100-h in Fig. 5). The 12-19 amino acid region of L7a does not contain any functional NLS or positively charged amino acid. The β -galactosidase produced by this construct was found exclusively in the nucleus of the cell (Fig. 6).



FIG. 6. Immunofluorescence localization of L7a- β -galactosidase fusion proteins produced by constructs shown in Fig. 5. For immunofluorescence staining, cells were processed as described under "Experimental Procedures" and in the legend to Fig. 3.

DISCUSSION

Nuclear Targeting of the L7a Protein—By using a series of in-frame lacZ-L7a cDNA fusions we have identified within the human r-protein L7a three distinct nuclear localization-competent domains defined by: amino acid residues 23–51 (domain I), amino acid residues 52–100 (domain II), and amino acid residues 101–220 (domain III). The addition of each of these domains at the NH₂ terminus of β -galactosidase resulted in nuclear localization of the reporter protein in cells transfected with a vector carrying the fusion cDNA. Besides having unique features, these three domains share a high content of basic amino acids.

Domain I contains peptide ³⁴KRPKNFGIGQDIQPKR⁴⁹ (boldface in Fig. 1C), which could represent a bipartite NLS consensus sequence similar to the NLS of nucleoplasmin (30); however, a chimeric L7a- β -galactosidase protein in which the putative bipartite NLS has been disrupted (see construct pL7a1-40 in Fig. 2A), still enters the nucleus albeit less efficiently (pL7a1-40 in Fig. 4). When peptide ³⁴KRPKNF-GIGQDIQPKR⁴⁹ is removed from the natural L7a context, as in the fusion protein where the peptide is located at the NH₂ terminus of β -galactosidase (not shown), it is unable to direct the fusion protein to the nucleus. Thus, even though endowed with NLS activity, the ³⁴KRPKNFGIGQDIQPKR⁴⁹ peptide is not comparable to the SV40 large T-antigen NLS. It is sufficient that the T-antigen NLS prototype be exposed on the protein surface (2) to interact with the receptor responsible for triggering the nuclear import (31), whereas, in addition to correct positioning, a weak NLS seems to require additional sequences in the natural protein to be fully efficient, as reported for the nsp2 protein (24) and the NLS of polyoma Tantigen (32). A tetrapeptide consensus (KR/KXR/K) has been detected in a significant number of nuclear localization signals (33); the tetrapeptide 34 KRPK 37 (*underlined* in Fig. 1C) fitting the consensus could be involved in the partial nuclear localization of the chimeric protein containing a disrupted putative bipartite NLS (pL7a1-40 in Fig. 4).

Domain II, comprising amino acid residues 52–100, contains a single cluster of amino acids, ⁷²KRLK⁷⁵ (*boldface* and *underlined* in Fig. 1C), which fits the NLS consensus tetrapeptide proposed by Chelsky *et al.* (33). The region is positively charged, containing 11 positively charged residues *versus* one aspartate residue. A chimeric β -galactosidase carrying domain II of L7a at the NH₂ terminus translocates to the nucleus. However, a R73N mutation did not affect the NLS activity of this region (not shown), indicating that such a strict clustering of positively charged amino acids is not a prerequisite for nuclear targeting. Again, the nuclear targeting of a reporter protein cannot be ascribed simply to the presence of the short peptide sequence because it occurred only when a contribution in terms of sequence or folding was supplied by the natural protein context.

The third nuclear localization-competent domain, domain III, spans through residues 101-220. In fact, whereas the 101-266 amino acid region of L7a directed the reporter protein β -galactosidase to the nucleus (see Fig. 2B), the COOH-terminal region defined by amino acid residues 221-266 did not (see Fig. 2B). In the 101–220 amino acid region two peptides, ¹¹⁰KKQRLLARAEKK¹²¹ and ¹²⁰KKAAGKGDVPTKR¹³², could represent partially overlapping bipartite NLS (boldface in Fig. 1C). Construct pL7a101-266, which includes the putative multiple NLS region, produced a β -galactosidase targeted to the nucleus (Figs. 2B and 3); deletion of the region resulted in a reporter protein that had lost almost completely the ability to translocate to the nucleus (Figs. 2B and 3). However, fusion of the peptide ¹¹⁰KKQRLLARAEKK¹²¹ to the NH₂ terminus of β -galactosidase did not result in nuclear targeting. It is feasible that a cumulative effect, which includes a protein context contribution, can overcome the low efficiency of a single NLS.

The efficiency of nuclear translocation promoted by the three NLS-containing domains is different: domains II and III are more efficient than domain I (see Figs. 2-6).

Nucleolar Accumulation of the L7a Protein-Domain II, comprising amino acid residues 52-100, plays a special role in nucleolar accumulation of L7a: the chimeric proteins carrying a deletion of this region (see construct pL7a1-51 in Fig. 2A and pL7a101-266 in Fig. 2B) did not accumulate in the nucleoli, although they all translocated to the nucleus with the efficiency determined by the corresponding NLS. On the other hand, the presence of domain II alone did not result in the nucleolar accumulation of the chimeric β -galactosidase (construct pL7a52-100 in Figs. 5 and 6). A complete process, i.e. nuclear targeting and nucleolar accumulation of a reporter protein, is restored when a positively charged region is added to the chimeric protein containing domain II. In fact, this cooperative effect is exerted by: (a) a functional NLS from the SV40 Tantigen (see construct pL7a52-100-NLS in Figs. 5 and 6); (b) a mutated, nonfunctional NLS from the SV40 T-antigen (see constructs pL7a52-100-NLS* in Figs. 5 and 6); (c) the NH₂terminal stretch of 17 amino acids from L7a which is rich in positive charges, but unable, alone, to target a reporter protein to the nucleus (compare construct pL7a1-17 in Fig. 2 and construct pL7a52–100-b in Figs. 5 and 6). When this positively charged sequence is replaced by a sequence lacking positively charged amino acids (i.e. amino acid residues 12-19 from L7a protein; see construct pL7a52-100-h in Figs. 5 and 6) the chimeric protein does not accumulate in the nucleoli.

Functional Domains Cooperate in Nucleolar Targeting and Accumulation—Because no membrane or physical barriers confine nucleoli to the cell nucleus (17), it appears that targeting of proteins to the nucleoli occurs by means of functional domains rather than through linear sequences acting as nucleolar localization signals (10, 13, 14, 24, 29). Once in the nucleus, the nucleolar protein could accumulate in the nucleolus through the interaction of functional domains with RNA or proteins residing in the nucleolus. RNA binding domains are involved in the nucleolar accumulation of nucleolin, in vertebrates (11), and of the yeast NSR1 (13). The target sequences of nucleolin on pre-ribosomal RNA have recently been identified (34). In both nucleolin and NSR1 the cooperation among RNA binding domains and other domains facilitates the association of the protein with the nucleoli. The nucleolar accumulation of rproteins could be mediated by RNA-protein interactions, protein-protein interactions, or even by both kinds of interaction. A new RNA binding motif has been proposed in L7a based on the secondary structure rather than on primary sequence homology with identified RNA binding domain in r-proteins (35). The putative RNA binding motif includes amino acid residues 130-161, which is a region distinct from the domain involved in the nucleolar targeting of L7a (amino acid region 52-100).

Acidic nucleolar proteins have been implicated in ribosome biogenesis (36) because of their structural features and because they recognize NLS motifs in vitro (37-40). This finding led to the speculation that the acidic proteins residing in the nucleoli establish electrostatic interactions with the basic amino acid residues, including the basic amino acids in the nuclear localization sequence, thus favoring the association of r-proteins with the ribosomal RNA. Our results are consistent with this model. In fact, the nucleolar accumulation competence of domain II was restored by the addition of a basic domain. We do not know the mechanism by which this domain can drive the nucleolar accumulation of a reporter protein; however, our results are consistent with a model whereby nucleolar targeting is not simply mediated by a linear sequence of amino acids, and more complex interactions involving two or more regions are needed for a nucleolar protein to accumulate in the nucleoli.

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