Localization in the Nucleolus and Coiled Bodies of Protein Subunits of the Ribonucleoprotein Ribonuclease P

Nayef Jarrous, Joseph S. Wolenski, Donna Wesolowski, Christopher Lee, and Sidney Altman

Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520

Abstract. The precise location of the tRNA-processing ribonucleoprotein ribonuclease P (RNase P) and the mechanism of its intranuclear distribution have not been completely delineated. We show that three protein subunits of human RNase P (Rpp), Rpp14, Rpp29 and Rpp38, are found in the nucleolus and that each can localize a reporter protein to nucleoli of cells in tissue culture. In contrast to Rpp38, which is uniformly distributed in nucleoli, Rpp14 and Rpp29 are confined to the dense fibrillar component. Rpp29 and Rpp38 possess functional, yet distinct domains required for subnucleolar localization. The subunit Rpp14 lacks such a domain and appears to be dependent on a piggyback process to reach the nucleolus. Biochemical analysis suggests that catalytically active RNase P exists in the nucleolus. We also provide evidence that Rpp29 and Rpp38 reside in coiled bodies, organelles that are implicated in the biogenesis of several other small nuclear ribonucleoproteins required for processing of precursor mRNA. Because some protein subunits of RNase P are shared by the ribosomal RNA-processing ribonucleoprotein RNP, these two evolutionarily related holoenzymes may share common intranuclear localization and assembly pathways to coordinate the processing of tRNA and rRNA precursors.

Key words: coiled body • nucleolus • RNase mitochondrial RNA processing • ribonuclease P • tRNA

Many processes of fundamental importance to the usage of genetic information in eukaryotes take place, or have their catalytic components assembled, in the nucleolus (for review see Melese and Xue, 1995; Shaw and Jordan, 1995; Lamond and Earnshaw, 1998; Pederson, 1998). Events related to processes critical for the cell cycle, life span, and apoptosis also occur in the nucleoli in some eukaryotes (for review see Guarente, 1997; Bacht and Eledge, 1999). Ribonucleoprotein complexes with catalytic roles in RNA processing and modification are major constituents of nucleoli (Pederson et al., 1998; Yu et al., 1999). These ribonucleoproteins have to find their way to the nucleolus or even to certain compartments within these dynamic structures and to the nearby coiled bodies. Specific sequences in certain proteins have already been identified that function, alone or in concert with sequences in other proteins or nucleic acids, to achieve nucleolar localization. However, to date there is no apparent consensus sequence in proteins that determines nucleolar localization, probably because a variety of different protein–protein and protein–nucleic acid interactions are used in the targeting process. In this report, we describe distinct subnucleolar localization domains found in two protein subunits of the human tRNA-processing ribonucleoprotein ribonuclease P (RNase P).

The precise locations of RNase P in eukaryotic cells have not been completely delineated (Matera et al., 1995; Lee et al., 1996; Jacobson et al., 1997; Pederson, 1998; for review see Wolin and Matera, 1999), although it is well established that processing of the 5′ termini of some precursors to tRNA occurs in the nucleolus (Melton and Cortese, 1979) or nucleolar event (Bertrand et al., 1998). The RNA subunit of human RNase P has been identified in the cytoplasm, nucleoplasm, the perinucleolar compartment, as well as the nucleolus (Matera et al., 1995; Lee et al., 1996; Jacobson et al., 1997), but the majority is nucleoplasmic. Several proteins have been characterized as subunits of human RNase P (Lygerou et al., 1996; Eder et al., 1997; Jarrous et al., 1998, 1999). Extensive sharing of protein components of the yeast nuclear RNase P and the rRNA processing enzyme RNase MRP, have now been established by genetic and some biochemical means (Chamber...
lai et al., 1998). A though the protein composition of human RNase MR P remains to be verified by extensive biochemical purification analysis, several RNase P protein subunits are shared by RNase MR P (L ygerou et al., 1996; E der et al., 1997; J arrous et al., 1999; P lu et al., 1999). The specific functions of these protein subunits in RNase P and RNase MR P assembly and intracellular localization, however, remain unknown.

We show here that several protein subunits of human RNase P are primarily localized in the nucleolus of mammalian cells, as determined by confocal immunofluorescence microscopy. Two RNase P protein (R pp) subunits, R pp14 and R pp29, are localized in the dense fibrillar component, whereas the other subunit, R pp38, is more uniformly distributed in the nucleolus. R pp29 and R pp38 possess functional sequences required for nucleolar localization. R pp14 appears to enter the nucleolus through a piggyback process. R pp29 and R pp38 are also found in coiled bodies, nucleoplastic structures that participate in the transport and sorting of several small nuclear and nucleolar ribonucleoprotein components involved in the processing of m rNA and r rNA precursors as well as in the 3' end formation of histone m rNA precursors (G all et al., 1995; L amond and E arnshaw, 1998).

Materials and Methods

Cell Cultures and Transfection

Mouse Swiss 3T3 fibroblasts, HeLa cells, and human embryonic kidney 293 cells were grown in high glucose DM E (L ife Technologies, Inc.) supplemented with L-glutamine, 25 mM Hepes buffer, pyridoxine hydrochloride, 10% F BS, streptomycin (100 μg/ml), and penicillin (100 U/ml). Cells were incubated in 5% CO2 at 37°C in 75-cm2 flasks. For transient transfections, 1×105 cells grown in 60 × 15 mm style petri dishes containing glass coverslips were transfected with plasmid DNA (2–5 μg) using the SuperFect reagent (Q iagen) following the manufacturer's instructions.

The Journal of Cell Biology, Volume 146, 1999 560

Fig. 1 A) were also prepared as described for pEGFP-Rpp38(260-283). pEGFP-Rpp38(1-245) was constructed by cleaving a PstI-HindIII Rpp38 cDNA (E der et al., 1997) fragment subcloned in pBluescript into the unique KpnI site and subcloned in-frame in pEGFP-C1 digested first with XhoI and HindIII. The entire RNase P coding domain was amplified from G418-resistant 293 HEK cells that constitutively express RNase P from G418-resistant 293 HEK cells that constitutively express RNase P (E der et al., 1997). The PCR product was digested with EcoR1, located in the designed primer sequences, and subcloned in-frame in pEGFP-C1 first cleaved with EcoR1. pEGFP-Rpp38(260-283) was constructed by digestion of a PCR DNA product containing the sequence that codes for amino acids 52-85 of Rpp38 (see Fig. 1 B), with HindIII and BamHI located in the designed primers, and followed by subcloning in pEGFP-C1 cleaved first with the same two restriction enzymes. pEGFP-Rpp29(63-85) was generated by subcloning a BamHI-HindIII deoxyoligonucleotide that codes for the 23 amino acids encompassing positions 63-85 of Rpp29 in pEGFP-C1 digested with BamHI and HindIII. pNS20N N4 and pNS20K N4 were constructed as pEGFP-Rpp29(63-85), but the R QRR or K KKKK residues were substituted with four asparagines, respectively. pEGFP-Rpp14 was generated by subcloning a XhoI-HindIII digested PCR DNA product containing the entire Rpp14 open reading frame (J arrous et al., 1999) into pEGFP-C1 cleaved first with XhoI and HindIII.

pPK-Rpp38 was generated by inserting a KpnI-digested PCR R pp38 cDNA containing the entire open reading frame into the K p nI unique site of the myc-tagged chicken pyruvate kinase in pCDNA 3-PK plasmid (S iomi and D reyfuss, 1995), provided to us by Dr. G ideon D reyfuss (U niversity of Pennsylvania, Philadelphia, PA). All DNA constructs described above were verified by sequencing to ensure in-frame subcloning of the desired inserts with the reporter gene.

Indirect Immunofluorescence

Cells (20% confluent) were grown overnight on coverslips (22×22 mm) before fixation with 2% paraformaldehyde (E lectron Microscopy S ciences) diluted in 1× PBS for 30 min. Cells were treated with 0.5% Triton X-100 for 5–30 min, washed twice with 1× PBS (0.5 liter each), and then blocked with 3% B SA/PBS for 20 min. Rabbit polyclonal antibodies against R pp subunits (J arrous et al., 1999), pBSH (A ndrade et al., 1993), or N opp140 peptide (M eer and B lobel, 1992), diluted 1:50–400 in 3% B SA/PBS, were added to the fixed cells for 1 h, and then washed twice with PBS before incubation for 20 min with 1:50 diluted secondary antib ody, A lexa 568 goat anti-rabbit I G antibody conjugate (M olecular Probes Inc.). When the monoclonal antifibrillarin and anti-my c (9E 10) mouse IgG antibodies or polyclonal anti-B23 goat IgG antibodies (S anta C rit Biotechnology Inc., C A) were included, A lexa 488 goat anti-mouse I G antibody or A lexa 594 donkey anti-goat I G antibody conjugates (M olecular Probes Inc.) were used. Cells were washed twice with PBS and mounted on glass slides using boat sealer (E rnest F ullam).

Microscopy and Imaging

Confocal fluorescence microscopy of living or fixed cells was performed at 22°C (±2°C) using a B io-R ad MR C-1024 laser scanner mounted on a 2FL reflector scanner on a Z eiss A xiovert equipped with differential interference contrast (D I C) optics (P lanaPo 100× 1.4 N A oil immersion objective; C arl Z eiss). Fluorescent images were acquired using Texas red and FITC filters, and then processed using LaserS harp software (B io-R ad L aboratories). Bleedthrough was completely eliminated between fluorophore channels in colocalization studies. Nuclei of living cells were also visualized by DNA staining with 4',6-diamidino-2-phenylindole. Digital processing and color adjustment of images were done using M etaM orph Image acquisition and processing software (U niversal I maging Corp.) and A dobe Photoshop (A dobe S ystems, Inc.).

Purification and Analysis of Human RNase P

RNase P from G418-resistant 293 H EK cells that constitutively express GFP-R pp38 fusion protein was purified as previously described (E der et al., 1997). In brief, 109 cells were pelleted, disrupted, and the cell homogenate was centrifuged at 7,000 rpm followed by another centrifugation at 42,000 rpm in a Beckman Ti50 rotor to obtain 5100 crude extract. This 5100 extract was loaded on a D EAE-Sephose anion exchange chromatography column and RNase P was eluted from the column using a 100–500-mM KCl gradient. The flowthrough, wash, and the eluted fractions were assayed for RNase P activity, and then kept in 25% glycerol in –20°C for further analysis. Cleavage of the 5' leader of the yeast suppressor pre-c RNA (SuP51) by human RNase P was performed as described.
Results

Rpp38 Localizes a Green Fluorescent Protein (GFP) to the Cell Nucleolus and Coiled Bodies

Mouse Swiss 3T3 fibroblasts were transiently transfected with pEGFP-Rpp38, a derivative of the expression vector pEGFP-C1, which contains the Rpp38 open reading frame fused in-frame to the carboxy terminus of a GFP (Fig. 1 A). Expression of the GFP-Rpp38 fusion protein in transfected cells was monitored by confocal fluorescence microscopy (see Materials and Methods). 48 h after transfection, the fluorescence signal of GFP-Rpp38 was seen in the nucleoplasm but was most visible in the nucleoli (Fig. 2, A and B, and see below). Only background fluorescence was observed in the cytoplasm. By contrast, GFP alone was distributed diffusely throughout the cytoplasm and the nucleoplasm, but was completely excluded from nucleoli.

That endogenous Rpp38 in 3T3 fibroblasts is also a nucleolar protein was confirmed by using affinity-purified, polyclonal anti-Rpp38 antibodies (Jarrous et al., 1998) in indirect immunofluorescence analysis (Fig. 2, I–K). As with GFP-Rpp38 (Fig. 2 B), endogenous Rpp38 was uniformly distributed in the nucleolus. A weak signal around the nucleus that is typical of mitochondrial staining was also observed (Fig. 2, I–K), but further work is required to confirm localization of Rpp38 in these cytoplasmic organelles.

We also tested the ability of Rpp38 to target another reporter protein, the cytoplasmic chicken pyruvate kinase (Siomi and Dreyfuss, 1995). A fusion protein of Rpp38 with a myc-tagged pyruvate kinase accumulated in the nucleus of Rpp38 transfected cells (Fig. 2, C and D). Since GFP-Rpp38 was not seen in the cytoplasm, as was GFP alone, this fusion protein must have been retained in the nucleoplasm and nucleoli.

Localization of GFP-Rpp38 in the nucleolus of transfected 3T3 fibroblasts was verified by the colocalization of this fusion protein with the nucleolar protein B23 (Biggiogera et al., 1990) using indirect immunofluorescence analysis (Fig. 2, E–H). B23 is a nuclear localization sequence (NLS)–binding phosphoprotein that is found in the dense fibrillar component and the granular component of the nucleolus (Biggiogera et al., 1990). GFP-Rpp38 is more uniformly distributed than B23 in the nucleolar compartments (Fig. 2, E–H).

Figure 1. Schematic representation of gene constructs. (A) Rpp38 or portions of this protein, Rpp38(1-245) or Rpp38(246-283), was fused in-frame to GFP in pEGFP-C1 expression vector (see Materials and Methods). Numbers indicate positions of residues in the 283-amino acid Rpp38 polypeptide. NLS1 and NLS2 indicate putative NLSs. NS38 represents the amino acid sequence from position 260–283 of Rpp38. Single and multiple substitutions or deletions are shown. ATDP has substitution of arginine and lysine with alanine and threonine, respectively, and the two consecutive proline residues were deleted from NS38.

B) Full-length Rpp29 or the amino acids encompassing positions 52–85 of Rpp29 were fused in-frame with GFP in pEGFP-C1. Full-length Rpp29 or the amino acids encompassing positions 52–85 or 63–85 of Rpp29 were fused in-frame with GFP in pEGFP-C1.
cleoplasm of transfected 3T3 cells and a weak signal was seen in nucleoli (data not shown). This may suggest that this fusion protein is too large (~100 kD) to be efficiently translocated and/or retained in the nucleolus, when compared with GFP-Rpp38. Therefore, GFP was used as the reporter protein throughout this study.

In transfected HeLa cells, GFP-Rpp38 compartmentalized in nucleoli as well as in discrete, intranuclear organelles immunostained with an antibody against p80-coilin (Fig. 3, A–D). These organelles represent coiled bodies as defined by the presence of p80-coilin (Andrade et al., 1993; Bauer et al., 1994). Diffuse immunostaining of p80-coilin was seen in the nucleoplasm and the nucleolus as well (Fig. 3, A–D; Lamond and Earnshaw, 1998). The site of Rpp38 was further identified by the colocalization of GFP-Rpp38 with the nucleolar shuttling protein Nopp140 (Meier and Blobel, 1992; Isaac et al., 1998) in nucleoli and coiled bodies (Fig. 3, E–H). Nopp140 is confined to the dense fibrillar component of the nucleolus (Meier and Blobel, 1992). Clearly, GFP-Rpp38 is more widely distributed in nucleoli than Nopp140. Similar results regarding coiled bodies were obtained with transfected 3T3 fibroblasts (data not shown). All these findings, taken together, demonstrate that the Rpp38 subunit of RNase P is localized in the nucleolus and in coiled bodies of cultured mammalian cells.
The Domain for Nucleolar Localization Resides in the Carboxy Terminus of Rpp38

Examination of the amino acid sequence of Rpp38 shows that it may possess three NLSs, located at positions 63–66, 241–244, and 262–281 (Fig. 1 A; Jarrous et al., 1998). Two DNA constructs, pEGFP-Rpp38(1-245), which contains amino acids 1–245 of Rpp38 and pEGFP-Rpp38(246-283), which possesses the remaining 37 amino acids of the polypeptide (see Fig. 1 A), were separately transfected into 3T3 fibroblasts and the localization of these truncated fusion proteins was determined. GFP-Rpp38(1-245) was concentrated in the nucleoplasm but not in the nucleoli (Fig. 4, A–C), an observation that was also confirmed in a double label experiment in transfected cells immunostained for the nucleolar B23 protein (Fig. 4, G–J). By contrast, GFP-Rpp38(246-283) was targeted to the nucleoli and nucleoplasm of cells with no significant signal seen in the cytoplasm (Fig. 4, D–F and K–N, and Table I). Similar nuclear and nucleolar localization patterns were obtained in 293 HEK cells transfected with the two constructs described above (not shown). Thus, the sequence required for nucleolar localization of Rpp38 exists between positions 246–283.

Defining the Nucleolar Localization Domain of Rpp38

The carboxy terminal 24 amino acids of Rpp38 fused to GFP in pEGFP-Rpp38(260-283) (Fig. 1 A) are capable of introducing the reporter protein into the nucleoli of 3T3 fibroblasts (Table I and data not shown). Nucleolar staining with GFP-Rpp38 and GFP-Rpp38(246-283) was as intense as with GFP-Rpp38 and GFP-Rpp38(246-283) (compare relative intensities in Table I), an indication that the last 24 amino acid sequence of Rpp38, designated NS38, are sufficient for nucleolar localization of the reporter protein.

We investigated whether the lysine residues in NS38 were important for its function by amino acid substitution analysis. The intracellular distributions (cytoplasm, nucleoplasm, and nucleoli) as reflected by the fluorescence signals of several mutants shown in Fig. 1 A were also summarized in Table I. Thus, pNS38K N, in which all the nine lysines in the NS38 sequence were substituted by similar, positively charged asparagine (N) residues, was introduced into 3T3 fibroblasts. The resultant fusion protein failed to enter the nucleoli and the fluorescent signal was detected in the cytoplasm as well as the nucleoplasm (Table I). When cells were transfected with pNS38K N23, in which lysines 2 and 3 in NS38 sequence were substituted, or with pNS38K N45, in which lysines 4 and 5 were substituted, a marked decrease in the nucleolar fluorescence was measured (Table I). These latter two fusion proteins were also distributed evenly throughout the nucleus, when compared with the prominent concentration of GFP-Rpp38(260-283) in the nucleoli (Table I). More-
over, as in the case of GFP-Rpp38(260-283), NS38KN23 and NS38KN45 accumulated in the nucleoplasm (Table I), an indication that their nuclear retention was not completely abolished. Similar results were obtained with NS38KN59 in which lysines 5 and 9 were substituted (Table I). However, the nucleolar localization capability of NS38 was completely abolished when the double mutant NS38KN78, in which lysines at position 7 and 8 in NS38 were replaced by asparagines (Fig. 1 A), was introduced into cells (Table I). NS38KN78 was concentrated in the nucleoplasm rather than the cytoplasm when compared with NS38KN (Table I).

Next, we substituted alanine separately for each of the arginine (R13A), serine (S18A), threonine (T22A), or proline (P23A) residues in the NS38 sequence (Fig. 1 A) and tested the ability of these mutants to localize GFP to the nucleolus. The single mutants, R13A, S18A, and T22A, were found to have no profound effect on the nucleolar localization capability of NS38 (Table I). Therefore, phosphorylation of serine or threonine appears not

Figure 4. A nucleolar localization signal exists in the carboxy terminus of Rpp38. Swiss 3T3 fibroblasts were transfected with pEGFP-Rpp38(1-245) (A–C and G–J) or with pEGFP-Rpp38(246-283) (D–F and K–N). Construct maps are shown in Fig. 1 A. 48 h after transfection, living cells were examined (A–F) using confocal microscopy, or first fixed and subjected to indirect immunofluorescence using antibody against nucleolar protein B23 (G–J and K–N). DIC (A, D, G, and K), GFP (B, E, I, and M, green), B23 (H and L, red), and overlays (C, F, J, and N) are shown. Arrows point to nucleoli. Bars: C and F, 5 μm; J and N, 2.5 μm.
Table I. Relative Intensities of Fluorescence in Three Different Compartments of 3T3 Fibroblasts Transfected with the GFP Fusion Constructs

| Construct | Cytoplasm | Nucleoplasm | Nucleoli |
|-----------|-----------|-------------|----------|
| pEGFP     | + +       | + +         | -        |
| pEGFP-Rpp38 | -        | + +         | + +      |
| pEGFP-Rpp38(1-245) | -        | + +         | -        |
| pEGFP-Rpp38(246-283) | -        | + +         | + +      |
| pNS38     | + +       | + +         | + +      |
| pNS38KN   | + +       | + +         | + +      |
| pNS38KN23 | + +       | + +         | + +      |
| pNS38KN45 | + +       | + +         | + +      |
| pNS38KN78 | + +       | + +         | -        |
| pNS38KN59 | + +       | + +         | + +      |
| pNS38R13A | + +       | + +         | + +      |
| pNS38S18A | + +       | + +         | + +      |
| pNS38T22A | + +       | + +         | + +      |
| pNS38P23A | + +       | + +         | + +      |
| pNS38ATDPP | +        | + +         | + +      |
| pEGFP-Rpp29 | -        | + +         | + +      |
| pEGFP-Rpp29(52-85) | -        | + +         | + +      |
| pEGFP-Rpp29(63-85) | -        | + +         | + +      |
| pNS29RN4  | + +       | + +         | + +      |
| pNS29KN4  | + +       | + +         | + +      |
| pNS29HN   | + +       | + +         | + +      |
| pNS29RHKK | + +       | + +         | + +      |

Constructs indicated above are shown in Fig. 1 A and their construction is described in Materials and Methods. The relative fluorescence intensities distributed in different cell compartments, cytoplasm, nucleoplasm, and nucleus, are presented from + to ++ ranking, and are based on 3–7 transfection experiments with each GFP fusion construct using the same set of parameters for measuring fluorescence intensity by confocal microscopy. Background autofluorescence of 3T3 fibroblasts is indicated by +. The frequency of transfection, as measured by cells exhibiting fluorescence, was ~2–10% with all constructs except for the pEGFP-Rpp38. This latter construct yielded transfectants at about one tenth the frequency of the other plasmids, presumably because RNase P incorporating this fusion protein is less active than in other cases. We note that for each construct tested, all transfected cells (i.e., cells exhibiting fluorescence) yielded the same phenotype as shown in Table I.

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The findings described above show that lysine residues throughout the NS38 sequence are required for its nucleoplasmic retention and nucleolar localization, with the lysines at position 7 and 8 being most critical for its entry to the nucleolus.

GFP-Rpp38 Is Associated with Catalytically Active RNase P Complexes

We obtained evidence that the GFP-Rpp38 fusion protein actually resides in a catalytically active RNase P complex. pEGFP-Rpp38, which expresses the neomycin resistance gene (G418 resistance), was used to establish stably transfected human embryonic kidney (HEK) 293 cells in culture. G418-resistant cell populations obtained in this manner exhibited fluorescent signals in the nucleolus and nucleoplasm, as judged by confocal microscopy (data not shown). To determine if GFP-Rpp38 expressed in these cells can be found in RNase P, S100 crude extracts from these stably transfected cells were fractionated on a DEAE-Sepharose anion exchange column. As determined by processing of a yeast tRNA 5′-Ser precursor, RNase P activity was eluted at 280–340 mM KCl (Fig. 5 A), a salt concentration shown previously to elute active RNase P from untransfected human cells from DEAE-columns (Eder et al., 1997). When fractions across the peak of enzymatic activity were subjected to Western blot analysis using anti-GFP antibodies (see Materials and Methods), a protein of ~75 kD that to be an obligatory modification for NS38 function. The proline substitution to alanine (P23A), however, seemed rather to increase the ratio of the nucleolar to the nucleoplasmic staining when compared with the ratio obtained with the NS38 parental construct (Table I). The two prolines in the RKPP sequence of NS38, by contrast, had no critical role in nucleolar localization as corroborated by the ATDPP construct in which the arginine and lysine (at position 5) were replaced with alanine and threonine, respectively, and the two consecutive proline residues were deleted from NS38 (Table I).

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GFP-Rpp38 Is Associated with Catalytically Active RNase P Complexes

We obtained evidence that the GFP-Rpp38 fusion protein actually resides in a catalytically active RNase P complex. pEGFP-Rpp38, which expresses the neomycin resistance gene (G418 resistance), was used to establish stably transfected human embryonic kidney (HEK) 293 cells in culture. G418-resistant cell populations obtained in this manner exhibited fluorescent signals in the nucleolus and nucleoplasm, as judged by confocal microscopy (data not shown). To determine if GFP-Rpp38 expressed in these cells can be found in RNase P, S100 crude extracts from these stably transfected cells were fractionated on a DEAE-Sepharose anion exchange column. As determined by processing of a yeast tRNA 5′-Ser precursor, RNase P activity was eluted at 280–340 mM KCl (Fig. 5 A), a salt concentration shown previously to elute active RNase P from untransfected human cells from DEAE-columns (Eder et al., 1997). When fractions across the peak of enzymatic activity were subjected to Western blot analysis using anti-GFP antibodies (see Materials and Methods), a protein of ~75 kD that
copurified with enzymatic activity was detected (Fig. 5 B). This protein corresponds to the GFP-R pp38 fusion protein and apparently has an anomalous migration in SDS-PAGE, a property that is shared by several R pp proteins including R pp38 (Jarrous et al., 1998). When polyclonal anti-R pp38 rabbit antibodies were used in Western blotting, the same 75-kD protein was detected (Fig. 5 C). A protein of ~65 kD visible in the blot may be a truncated fragment of GFP-R pp38. Neither flowthrough nor wash fractions from the column contained GFP-R pp38 or endogenous R pp38 protein (Fig. 5 C), an indication that both polypeptides were tightly bound to the column and eluted only with RNase P. At least as demonstrated in vitro, the expression of GFP-R pp38 in human cells does not abolish RNase P function in tRNA processing, although constitutive expression of GFP-R pp38 resulted in cell death after 10–15 passages in culture (data not shown).

R pp29 Localizes a Reporter Protein to the Dense Fibrillar Component and Coiled Bodies

As with R pp38, sequences of contiguous basic residues that may function in nuclear localization are found in R pp29, another protein subunit of RNase P (Jarrous et al., 1999). 3T3 mouse fibroblasts were transfected with pEGFP-R pp29, in which the open reading frame of the R pp29 cDNA was fused in-frame with GFP (see Fig. 1 B). GFP-R pp29 was localized in the nucleoplasm and exhibited very intense staining of nucleoli (Fig. 6, A–C). However, GFP-R pp29 was not evenly distributed in the nucleoli but was concentrated in subregions inside these structures (Fig. 6, A–C). The smaller punctate stainings seen in the nucleoplasm may represent discrete structures other than nucleoli such as coiled bodies (see below). Indirect immunofluorescence analyses revealed that GFP-R pp29 localized in nucleoli with B23 (Fig. 7, A–D). Moreover, endogenous R pp29 colocalized with fibrillarin in untransfected fibroblasts (Fig. 7, E–G). The immunostains of R pp29 and fibrillarin seen in nucleoli are strikingly similar (Fig. 7, E–G), suggesting that R pp29 resides in the dense fibrillar component as fibrillarin.

As shown in Fig. 6, D–F, the sequence responsible for the nucleolar localization of R pp29 is located between positions 52 and 85 of this protein, as demonstrated by the use of pEGFP-R pp29(52-85) (Fig. 1 B) in transfected fibroblasts. This domain was able to localize GFP to subnucleolar regions, but in a less distinct manner than the full-length R pp29 protein. GFP-R pp29(52-85) was almost
exclusively retained in the nucleus and only background fluorescence was seen in the cytoplasm. Amino acids 63–85 of Rpp29 were still sufficient for nucleolar localization (Table I; construct pE GFP-Rpp29(63-85)). This sequence of 23-amino acid of Rpp29 is now designated NS29 (Fig. 1B). Mutational analysis of NS29, summarized in Fig. 1B and Table I, showed that the KKKK residues in NS29 were required for efficient nucleolar localization but were not crucial for function, as determined through the use of NS29K N4 mutant. All the other multiple and single mutants tested, including the substitution of the RQRR residues to asparagines, had no dramatic effect on NS29 function (Table I). In fact, the RHKRK motif is sufficient for nucleolar entry (Table I). We concluded that NS29 and NS38 represent distinct domains required for nucleolar localization.

In HeLa cells, coiled bodies immunostained for p80-coilin contained GFP-Rpp29 (Fig. 8, A–D). In some transfected cells, however, coilin-immunostained structures that were on the periphery of nucleoli exhibited no intense signal of GFP-Rpp29 (Fig. 8, A–D, inserts). In contrast to GFP-Rpp29 and GFP-Rpp38, no prominent signal of GFP-Rpp14 fusion protein was seen in the coiled bodies of HeLa cells transiently transfected for 48 h with pE GFP-Rpp14 construct (Fig. 8, E–H). Whether Rpp14 requires a longer time to localize in coiled bodies, remains unknown. A detailed kinetic study, however, is required to determine whether the several GFP-Rpp fusion proteins presented in this study enter the nucleoli first on their way to coiled bodies, as has been shown with Nopp140 (Isaac et al., 1998).

Nucleolar Localization of Rpp14, an RNase P Subunit, May Be Facilitated by a Piggyback Process

Indirect immunofluorescent analysis using affinity-purified, polyclonal antibodies against Rpp14 (Jarrous et al., 1999) showed localization of this RNase P subunit in the nucleolus of 3T3 fibroblasts (Fig. 9, A–C). Yet, Rpp14 has no sequences of basic residues typical of NLSs, in contrast to Rpp29 and Rpp38 (Jarrous et al., 1999). Nevertheless, Rpp14 fused to GFP was directed to subnucleolar regions in 3T3 fibroblasts transfected for 48 h with pE GFP-Rpp14 (Fig. 9, D–F). No prominent punctate staining was seen in the nucleoplasm, as was the case with Rpp29 and Rpp38. However, we found that GFP-Rpp14 was seen exclusively in the nucleolus only in cells producing low levels of this fusion protein, as reflected in the relatively weak fluorescent signals observed in transfected cells (Fig. 9, E and F, note cell background). In transfected cells that showed more intense signals, comparable in their intensity to those reported here for Rpp38 and Rpp29, most of the GFP-Rpp14 synthesized was not transported to the nucleoli, but...
rather remained in the cytoplasm (Fig. 9, E and F; two cells with stronger signals are indicated). Moreover, when cells were tested at earlier times (<24 h) after transfection, most of the GFP-Rpp14 stain was visible in the cytoplasm (data not shown). These observations suggest that, when compared with the rapid and efficient entry of Rpp38 and Rpp29 into the nucleolus, the localization process of Rpp14 under the same conditions is inefficient, and thus may require a limiting, endogenous factor. As demonstrated by co-localization analysis, GFP-Rpp14 was compartmentalized into subnucleolar regions occupied also by endogenous Rpp29 in transfected cells (Fig. 9, G–I), an indication that these two subunits colocalized in the dense fibrillar component.

Discussion

This study shows that the nucleolus of cultured mammalian cells serves as the major site of localization of several protein subunits of human RNase P. In contrast to the dispersed distribution of Rpp38 in nucleoli, the Rpp14 and Rpp29 subunits are localized in the dense fibrillar component. The differential pattern of nucleolar localization was also observed for these subunits when fused to GFP and expressed in living cells. These RNase P subunits, thus, define different sites that, in turn, may reflect distinct biological functions. This conclusion is supported further by our findings that at least two subunits, Rpp29 and Rpp38, are also found in functionally distinct organelles, the coiled bodies. The nucleolus and the coiled bodies appear to be involved in the biogenesis of the ribonucleoprotein RNase P and, therefore, in the process of maturation of tRNA precursors.

Molecular Aspects of Localization Domains in RNase P

Nucleolar localization of proteins usually involves multiple domains in targeting sequences that can interact with ribonucleic acids or with other proteins (Peculis and Gall, 1992; Creancier et al., 1993; Yano and Mielese, 1993; Mears et al., 1995; Michael and Dreyfuss, 1996; Antoine et al., 1997; Li, 1997; Russo et al., 1997; Zirwes et al., 1997a,b). Nucleolar localization domains of some proteins, such as nucleolin, p120 nucleolar protein, and ribosomal proteins L5 and L7a are not functional by themselves when transferred to a reporter protein; they require additional, noncontiguous domains for function (Schmidt-Zachmann and Nigg, 1993; Valdez et al., 1994; Michael and Dreyfuss, 1996; Russo et al., 1997). On the other hand, NS38 and NS29 are functional and transferable. However, these two domains seem not to be sufficient for targeting a reporter protein to the coiled bodies as well.

NS38 has no arginine- or arginine/glycine–rich motifs (Burd and Dreyfuss, 1994), as has been found in domains in nucleolin (C23) and in the human immunodeficiency virus Tat protein that may facilitate RNA-binding and/or protein–protein interactions (Dang and Lee, 1989;
Schmidt-Zachmann and Nigg, 1993; Mears et al., 1995; Bouvet et al., 1998). The single arginine residue found in NS38 has no essential role either in the nucleoplasmic retention or in the nucleolar localization capability of this domain. Lysine residues at different positions throughout the NS38 sequence, instead, are required for efficient nucleolar localization. Adjacent lysines at positions 7 and 8, but not at positions 2 and 3, have a critical role in NS38 function. Numerous K.K.K repeats are found in several protein subunits of yeast nuclear RNase P (Chamberlain et al., 1998), but as in many other cases of nucleolar proteins such repeats were proved nonessential for nucleolar targeting (Gautier et al., 1997). NS38 shows no identity at the primary amino acid sequence to NS29. It is thus likely that structural features and the placement in space of side chains of both hydrophobic and charged amino acids (lysines) determine the function of these sequences.

Both NS29 and NS38 act early and efficiently to introduce a reporter protein to the nucleoli of mouse and human cultured cells. Similar conclusions were made for the full-length proteins, Rpp29 and Rpp38. In contrast, Rpp14 entry to the nucleolus seems slow and limited. Rpp14, which lacks any basic residues typical of nuclear or nucleolar targeting domains, may require other proteins that oc-

Figure 9. Subnucleolar localization patterns of Rpp14. 3T3 fibroblasts were subjected to indirect immunofluorescent analysis using anti-Rpp14 antibodies (A–C). C is an overlay of A and B. Fibroblasts transfected for 48 h with pEGFP-Rpp14 were examined under confocal microscope (D–F) before fixation and immunofluorescence analysis with anti-Rpp29 antibodies (G–I). F is an overlay of DIC (not shown) and E; two cells with high fluorescent signal are indicated by arrowheads. I is an overlay of G and H. Intense yellow color is seen in nucleoli. Bars: C and D, 3.3 μm; F and I, 10 μm.
cur in limited amounts in the cell for its nucleolar transport.

Furthermore, we were able to show that the nucleolar localization processes of Rpp subunits are dependent on ongoing transcription in functional, intact nucleoli. Thus, selective inhibition of rRNA transcription by a low concentration of actinomycin D (0.2 μg/ml; Pombo et al., 1999) leads to disintegration of the nucleoli and to dispersed nucleoplasmic staining by Rpp29, Rpp38, or their nucleolar localization domains fused to GFP (data not shown). Inhibition of protein synthesis by cycloheximide, by contrast, seems to have no effect on the nucleolar localization properties of these subunits.

**Localization Sites of RNase P and Biological Functions**

As judged by rRNA hybridization analysis in situ, most of the RNA subunit of the yeast nuclear RNase P is localized in the nucleolus with some unprocessed tRNA precursors that contain 5' leader sequences (Bertrand et al., 1998). In contrast, the majority of the human RNase P RNA is concentrated in the nucleolus rather than the nucleoplasm (Matera et al., 1995; Lee et al., 1996; Jacobson et al., 1997; Wolin and Matera, 1999). Moreover, H1 RNA that was microinjected to the nucleoplasm only transiently enters the dense fibrillar component of the nucleolus before it is redistributed in the nucleoplasm (Jacobson et al., 1997). Our study now shows, using both indirect immunofluorescence and cell transfection analyses, that several protein subunits of human RNase P reside in the nucleolus. Since the estimated copy numbers of RNase P RNA and RNase subunits of human RNase P are known (Jacobson et al., 1997), the concentration of actinomycin D (0.2 μg/ml) seems to have no effect on the nucleolar localization properties of these subunits.

In contrast, the majority of the human RNase P RNA is concentrated in the nucleolus with some unprocessed tRNA precursors that contain 5' leader sequences (Bertrand et al., 1998). In contrast, the majority of the human RNase P RNA is localized in the nucleolus rather than the nucleoplasm (Matera et al., 1995; Lee et al., 1996; Jacobson et al., 1997; Wolin and Matera, 1999). Moreover, H1 RNA that was microinjected to the nucleoplasm only transiently enters the dense fibrillar component of the nucleolus before it is redistributed in the nucleoplasm (Jacobson et al., 1997).

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**References**

Andrade, L. E. C., E. M. Tan, and E. K. L. Chan. 1993. Immunocytochemical analysis of the ribosome body in the cell cycle and during cell proliferation. Proc. Natl. Acad. Sci. USA. 90:1947–1951.

Antoine, M., K. Reimers, C. Dickson, and P. Kiefer. 1997. Fibroblast growth factor 3, a protein with dual subcellular localization, is targeted to the nucleus and nucleolus by the concerted action of two nuclear localization signals and a nucleolar retention signal. J. Biol. Chem. 272:29475–29481.

Bachant, J. B., and S. J. Elledge. 1999. Mitotic stores in the nucleolus. Nature. 396:757–758.

Bauer, D. W., C. Murphy, Z. Wu, C. H. Wuu, and J. G. Gall. 1994. In vitro assembly of coiled bodies in Xenopus egg extracts. Mol. Biol. Cell. 5:633–644.

Bertrand, E., F. Houser-Scott, A. Kendall, R. H. Singer, and D. R. Engelke. 1998. Nuclear localization of early RNA processing. Genes Dev. 12:2463–2468.

Bignigiera, M., K. Burki, S. H. Kaufmann, J. H. Shaper, N. Gas, F. A. Malric, and S. Fakan. 1990. Nucleolar distribution of proteins B23 and nucleolin in mouse preimplantation embryos as visualized by immunoelectron microscopy. Development. 110:1263–1270.

Bouvet, P., J. J. Diz, K. Kinde,ber, J. J. Madjar, and F. A. Malric. 1998. Nucleolin interacts with several ribosomal proteins through its RGG domain. J. Biol. Chem. 273:19025–19029.

Bur, C. G., and G. Dreyfus. 1994. Conserved structures and diversity of functions of RNA-binding proteins. Science. 263:615–621.

Chamberlain, J. R., Y. Lee, W. S. Lane, D. R. Engelke. 1998. Purification and characterization of the nuclear RNA holoenzyme complex reveals extensive subunit overlap with RNase MRP. Genes Dev. 12:1678–1690.

Cerniacni, L., H. Prats, C. Zanoni, F. A. Malric, and B. Bugler. 1993. Determination of the functional domains involved in nucleolar targeting of nucleolin. Mol. Biol. Cell. 4:1239–1250.

Dang, C. V., and W. M. Lee. 1989. Nuclear and nucleolar targeting sequences of c-erb-A, c-myc, N-unc, p53, HSP70, and HIV tat proteins. J. Biol. Chem. 264:18019–18023.

Edler, P. S., R. Kekuda, V. Stoic, and S. A. Itman. 1997. Characterization of two scleroderma autoimmune antigens that copurify with the ribonucleoprotein ribonucleoprotein P. Proc. Natl. Acad. Sci. USA. 91:1101–1106.

Forster, A. C., and S. A. Itman. 1990. Similar cage-shaped structures for the RNA components of all ribonuclease P and ribonucleoside MRP enzymes. Cell. 62:407–409.

Gall, J. G., A. Tsvetkov, Z. Wu, C. Murphy. 1995. Is the sphere organelle/coiled body a universal nuclear component? Dev. Genet. 16:25–35.

Gautier, T., T. Berges, D. Tollervey, and E. Hutt. 1997. Nuclear K K E D repeat proteins Nop56p and Nop35p interact with Nop1p and are required for ribosome biogenesis. Mol. Cell. Biol. 18:7088–7096.

Guarante, L. 1997. Link between aging and the nucleolus. Genes Dev. 11:2449–2455.

Ishii, C., Y. Ang, and U. T. Meier. 1998. Nop140 functions as a molecular link between the nucleus and the coiled bodies. J. Cell Biol. 142:319–329.

Jacobson, M. R., L. G. Cao, Y. L. Wang, and T. Pederzon. 1995. Dynamic localization of RNase MRP RNA in the nucleolus observed by fluorescent RNA cytochemistry in living cells. J. Cell Biol. 121:1649–1658.

Jacobson, M. R., L. G. Cao, K. Tanaka, R. H. Singer, Y. L. Wang, and T. Pederzon. 1997. Nuclear domains of the RNA subunit of RNase P. J. Cell Sci. 110:829–837.

Jarras, N., P. S. Eder, C. Guerrier-Takada, C. Hooq, and S. A. Itman. 1998. A u-
to antigenic properties of some protein subunits of catalytically active complexes of human ribonuclease P. RNA. 4:407–417.

Jarrous, N., P.S. Eder, D. Wesolowski, and S. Altman. 1999. Rpp14 and Rpp29, two protein subunits of human ribonuclease P. RNA. 5:153–157.

Lamond, A.J., and W.C. Earnshaw. 1998. Structure and function in the nucleus. Science. 280:547–553.

Lee, B., A.G. Matera, D. Ward, and J. Craft. 1996. Identification of RNase mitochondrial RNA processing enzyme with ribonuclease P in higher ordered structures in the nucleolus: a possible coordinate role in ribosome biogenesis. Proc. Natl. Acad. Sci. USA. 93:11471–11476.

Li, Y.P. 1997. Protein B23 is an important human factor for the nucleolar localization of the human immunodeficiency virus protein Tat. J. Virol. 71:4098–4102.

Lygerou, Z., H. Pluk, W.J. van Venrooij, and B. Seraphin. 1996. hPop1: an autoantigenic protein subunit shared by the human RNase P and RNase MRP ribonucleoproteins. EMBO (Eur. Mol. Biol. Organ.) J. 15:5936–5948.

Matera, A.G., M.R. Frey, K. Margelot, and S.L. Wolin. 1995. A perinucleolar compartment contains several RNA polymerase III transcripts as well as the polypyrimidine tract–binding protein, hnRNP I. J. Cell Biol. 129:1181–1193.

Mears, W.E., V. Lam, and S.A. Rice. 1995. Identification of nuclear and nucleolar localization signals in the herpes simplex virus regulatory protein ICP27. J. Virol. 69:935–947.

Meier, U.T., and G. Blobel. 1992. Nopp140 shuttles on tracks between nucleolus and cytoplasm. Cell. 70:127–138.

Melese, T., and Z. Xue. 1995. The nucleolus: an organelle formed by the act of building a ribosome. Curr. Opin. Cell Biol. 7:319–324.

Melton, D.A., and R. Cortese. 1979. Transcription of cloned tRNA genes and the nuclear partitioning of a tRNA precursor. Cell. 18:1165–1172.

Michael, W.M., and G. Dreyfuss. 1996. Distinct domains in ribosomal protein L5 mediate S S rRNA binding and nucleolar localization. J. Biol. Chem. 271:11571–11574.

Peculis, B.A., and J.G. Gall. 1992. Localization of the nucleolar protein NO38 in amphibian oocytes. J. Cell Biol. 116:1–14.

Pederson, T. 1998. The plurifunctional nucleolus. Nucleic Acids Res. 26:3871–3877.

Pluk, H., H. van Ensennaam, S.A. Rutjes, G.J. Pruinj, and W.J. van Venrooij. 1999. RNA-protein interactions in the human RNase MRP ribonucleoprotein complex. RNA. 5:512–524.

Pombo, A., D.A. Jackson, M. Hollinshead, Z. Wang, R.G. Roeder, and P.R. Cook. 1999. Regional specialization in human nuclei: visualization of discrete sites of transcription by RNA polymerase III. EMBO (Eur. Mol. Biol. Organ.) J. 18:2241–2253.

Russo, G., G. Ricciardielli, and C. Pietropaolo. 1997. Different domains cooperate to target the human ribosomal L7a protein to the nucleus and to the nucleolus. J. Biol. Chem. 272:5229–5235.

Schmidt-Zachmann, M.S., and E.A. Nigg. 1993. Protein localization to the nucleolus: a search for targeting domains in nucleolin. J. Cell Sci. 105:799–806.

Shaw, P.J., and E.G. Jordan. 1995. The nucleolus. Annu. Rev. Cell Dev. Biol. 11:93–121.

Shaw, P.J., M.J. Highett, A.F. Beven, and E.G. Jordan. 1995. The nucleolar architecture of polymerase I transcription and processing. EMBO (Eur. Mol. Biol. Organ.) J. 14:2896–2906.

Siomi, H., and G. Dreyfuss. 1995. A nuclear localization domain in the hnRNP A1 protein. J. Cell Biol. 129:551–560.

Valder, B.C.L., Perlaky, D. Henning, Y. Sajo, P.K. Chan, and H. Busch. 1994. Identification of the nuclear and nucleolar localization signals of the protein p120. Interaction with translocation protein B23. J. Biol. Chem. 269:23776–23783.

Wolin, S.L., and A.G. Matera. 1999. The trials and travels of tRNA. Genes Dev. 13:1–10.

Yan, C., and T. Melese. 1993. Multiple regions of N5R1 are sufficient for accumulation of a fusion protein within the nucleolus. J. Cell Biol. 123:1081–1091.

Yu, Y.-T., E.C. Schari, C.M. Smith, and J.A. Steitz. 1999. The growing world of small nuclear ribonucleoproteins. In The RNA World. Second edition. Cold Spring Harbor Press, Cold Spring Harbor, New York. 487–524.

Zirwes, R.F., A.P. Kouzmenko, J.M. Peters, W.W. Franke, and M.S. Schmidt-Zachmann. 1997a. Topogenesis of a nucleolar protein: determination of molecular segments directing nucleolar association. Mol. Biol. Cell. 8:231–248.

Zirwes, R.F., M.S. Schmidt-Zachmann, and W.W. Franke. 1997b. Identification of a small, very acidic constitutive nucleolar protein (NO29) as a member of the nucleoplasmin family. Proc. Natl. Acad. Sci. USA. 94:11387–11392.