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Nuclear domains of the RNA subunit of RNase P

Marty R. Jacobson[1], Long-Guang Cao[1,*], Krishan Taneja[2], Robert H. Singer[2,†], Yu-li Wang[1] and Thoru Pederson[1,‡]

[1]Cell Biology Group, Worcester Foundation for Biomedical Research, Shrewsbury, MA 01545, USA
[2]Department of Cell Biology, University of Massachusetts Medical Center, Worcester, MA 01655, USA

*Present address: Department of Molecular Genetics and Microbiology, University of Florida, PO Box 100266, Gainesville, FL 32610, USA
†Present address: Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY 10461-1975, USA
‡Author for correspondence (e-mail: werme@sci.wfbr.edu)

SUMMARY

The ribonucleoprotein enzyme RNase P catalyzes the 5′ processing of pre-transfer RNA, and has also recently been implicated in pre-ribosomal RNA processing. In the present investigation, in situ hybridization revealed that RNase P RNA is present throughout the nucleus of mammalian cells. However, rhodamine-labeled human RNase P RNA microinjected into the nucleus of rat kidney (NRK) epithelial cells or human (HeLa) cells initially localized in nucleoli, and subsequently became more evenly distributed throughout the nucleus, similar to the steady-state distribution of endogenous RNase P RNA. Parallel microinjection and immunocytochemical experiments revealed that initially nucleus-microinjected RNase P RNA localized specifically in the dense fibrillar component of the nucleolus, the site of pre-rRNA processing. A mutant RNase P RNA lacking the To antigen binding domain (nucleotides 25-75) did not localize in nucleoli after nuclear microinjection. In contrast, a truncated RNase P RNA containing the To binding domain but lacking nucleotides 89-341 became rapidly localized in nucleoli following nuclear microinjection. However, unlike the full-length RNase P RNA, this 3′ truncated RNA remained stably associated with the nucleoli and did not translocate to the nucleoplasm. These results suggest a nucleolar phase in the maturation, ribonucleoprotein assembly or function of RNase P RNA, mediated at least in part by the nucleolar To antigen. These and other recent findings raise the intriguing possibility of a bifunctional role of RNase P in the nucleus: catalyzing pre-ribosomal RNA processing in the nucleolus and pre-transfer RNA processing in the nucleoplasm.

Key words: RNase P, Nuclear RNA localization, RNA traffic

INTRODUCTION

Ribonuclease P (RNase P) is a phyletically ubiquitous ribonucleoprotein enzyme responsible for the endonucleolytic cleavage of transfer RNA (tRNA) precursor molecules, generating the 5′ termini of mature tRNAs (Altman et al., 1988; Baer et al., 1989; Pace and Smith, 1990; Altman, 1990; Darr et al., 1992), a process which takes place predominantly in the nucleus of eukaryotic cells (Melton et al., 1980). Although it has not yet been purified as an intact enzyme, mammalian RNase P contains a ~340 nt RNA molecule and its buoyant density suggests that it may be greater than 50% protein (Bartkiewcz et al., 1989). The RNA subunits of several mammalian RNase P enzymes have been identified and cloned (Altman et al., 1993), and human RNase P RNA was shown to be transcribed from a single copy gene by RNA polymerase III (Baer et al., 1990; Hannon et al., 1991). In contrast to the RNA subunit of prokaryotic RNase P, mammalian RNase P RNA is not catalytically active in vitro in the absence of the RNase P protein component(s) (for a review see Darr et al., 1992).

A nucleolar ribonucleoprotein enzyme closely related to RNase P is RNase MRP, an endoribonuclease that participates in pre-ribosomal RNA processing (Schmitt and Clayton, 1993; Chu et al., 1994; Lygerou et al., 1994). Although the RNA components of human RNase P and RNase MRP have only limited sequence homology (Gold et al., 1989), they can be folded into similar theoretical caged pseudoknot structures (Foster and Altman, 1990; Schmitt et al., 1993). Human RNase P and RNase MRP share at least two protein components, the 40 kDa To (or Th) antigen (Reddy et al., 1983; Liu et al., 1994) and the ~100 kDa Pop1 protein (Lygerou et al., 1996). Both enzymes cleave substrate RNAs to generate 5′ phosphates and 3′ hydroxyl termini in a divalent cation-dependent reaction, and both are capable of cleaving the same substrate RNA in vitro (Potuschak et al., 1993). These findings and other considerations suggest a close evolutionary relatedness of these two ribonucleoprotein enzymes (Morrissey and Tollervey, 1995; Lygerou et al., 1996). Candidate protein components of mammalian RNase P have recently been described (Eder et al., 1997).

In bacteria, which have only the RNase P enzyme, a transfer RNA element is located in the pre-ribosomal RNA spacer region between the 16 S and 23 S rRNA sequences which can act as a substrate for RNase P. Significantly, this region corresponds to ITS1 in eukaryotes, which contains the RNase MRP cleavage site A3 (reviewed by Morrissey and Tollervey, 1995).

Recently, Chamberlain et al. (1996) have shown that mutation of conserved positions of the *Saccharomyces cerevisiae* RNase P RNA subunit ($T_{315}\Delta T_{307}$) negatively affects pre-ribosomal RNA processing. In addition to this recent finding, there have been several clues that RNase P, in addition to its role in pre-transfer RNA processing, might also play a role in eukaryotic pre-ribosomal RNA processing (reviewed by Clayton, 1994).

Little is known about the nuclear sites of RNase P ribonucleoprotein assembly or function. In the present investigation we have employed in situ hybridization and fluorescent RNA cytochemistry techniques to examine the intranuclear localization of RNase P RNA. Our results suggest that the RNA subunit of RNase P has a nucleolar association in its RNA processing, ribonucleoprotein assembly or function, in accord with recent findings suggesting an involvement of RNase P in pre-ribosomal RNA processing, but that RNase P RNA also exists throughout the nucleoplasm as well, where it presumably is involved in pre-transfer RNA processing. The concept emerges of a bifunctional role of RNase P in the nucleus.

MATERIALS AND METHODS

Plasmids, in vitro transcription and fluorescent labeling of RNA

The methods used were essentially as described previously (Wang et al., 1991; Jacobson et al., 1995) with only minor modifications. Human RNase P RNA was transcribed from *Dra*I digested plasmid pGEM1/H1 (Bartkiewicz et al., 1989) using T7 RNA polymerase (Gibco/BRL, Bethesda, MD). A RNase P To domain RNA, consisting of only nucleotides 1-88 of human RNase P RNA was transcribed from *Bfa*I digested plasmid pGEM1/H1 using T7 RNA polymerase. A mutant RNase P RNA lacking the To antigen binding domain was transcribed using T3 RNA polymerase (Gibco/BRL) from *Ecl*136II digested plasmid pMH1-1. Plasmid pMH1-1 was constructed by deleting the *Kpn*I-*Xba*I fragment (vector multiple cloning site sequences) from a mouse RNase P RNA clone originally constructed by Kathleen Collins and Carol Greider (Cold Spring Harbor Laboratory) and kindly given to us by Sidney Altman's laboratory (Yale University). This clone consists of nucleotides 61-297 of the mouse RNase P RNA sequence (Altman et al., 1993; corresponding to nucleotides 71-329 of human RNase P RNA) cloned by PCR and ligated into the *Not*I site of Bluescript II KS(+) (Stratagene). Human pre-$tRNA^{Ser}$ was transcribed from *Ava*I digested plasmid pUC19pSer (kindly provided by Cecilia Guerrier-Takada and Sidney Altman, Yale University) using T7 RNA polymerase. Human U6 small nuclear RNA was transcribed from *Dra*I cut pHU6-1 (Mayrand et al., 1996) using T7 RNA polymerase. Human U3 RNA was transcribed from *Hinc*II digested plasmid pHU3.1 using T7 RNA polymerase. Plasmid pHU3.1 was constructed by PCR amplification of a human U3 RNA gene from plasmid pBU3-SP6 (Maser and Calvet, 1989) using two oligodeoxynucleotide primers, 5′-TAATACGACTCACTATAGGGA-AGACTATACTTTCAGGGATC-3′ (having the T7 RNA polymerase promoter and U3 RNA 5′end sequences) and 5′-CTCGAAA-TTAACCCTCACTAAAGGGTCGACCACTCAGACCGCGTTCGC-3′ (having the U3 RNA 3′ end sequence, immediately followed by a *Sal*I/*Hinc*II site and T3 RNA polymerase promoter sequences). The PCR product was made blunt using Klenow DNA polymerase as described by the suppliers (Gibco/BRL), and ligated into the *Hinc*II site of the vector pUC118 (Viera and Messing, 1987), thus eliminating the vector *Hinc*II site.

All RNAs were transcribed from their appropriately digested plasmid DNAs in the presence of 1 mM ATP, CTP, GTP, UTP (Pharmacia, Piscataway, NJ) and 0.1 mM 5-(3-aminoallyl)-UTP (Sigma, St Louis, MO). When desired, 2 μM [α-^{32}P]CTP (New England Nuclear Research Products, Boston, MA) was added to the transcription reaction as a tracer. The cap analog 7-mG(5′)ppp(5′)G (1 mM; New England BioLabs, Beverly, MA) or γ-monomethyl-guanosine-5′-triphosphate (1 mM; Singh and Reddy, 1989) was also included in the U3 RNA and U6 RNA transcription reactions, respectively. The RNA was recovered by ethanol precipitation and coupled to tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes, Eugene, OR) as previously described (Langer et al., 1981; Agrawal et al., 1986; Wang et al., 1991). After coupling, the fluorochrome-labeled RNA was purified by gel filtration on a 5 ml Bio-Gel P-60 (Bio-Rad, Hercules, CA) column. RNA from the peak column fractions was twice ethanol precipitated and then resuspended in sterile 5 mM Tris-acetate buffer (pH 7.0) at a concentration of approximately 50 μg/ml and centrifuged in a Beckman 42.2 Ti rotor at 25,000 rpm for 30 minutes to remove any residual precipitate which might complicate microinjection. The integrity of the fluorescently labeled RNA was examined by denaturing polyacrylamide gel electrophoresis (Wang et al., 1991; Jacobson et al., 1995). All RNAs were heat denatured at 75°C and rapidly cooled to room temperature just prior to microinjection. Additional details of fluorescent RNA preparation are described by Jacobson and Pederson (1997) and Jacobson et al. (1997).

RNP assembly and immunoprecipitation

In vitro transcribed RNase P RNA (15 ng) was incubated for 30 minutes at 30°C followed by an additional 30 minutes at 0°C in a 50 μl reaction containing 60% (v/v) micrococcal nuclease treated nuclear extract (50 μg; Temsamani et al., 1991), 1 mM ATP, 20 μg yeast tRNA, 75 mM KCl, 2.2 mM $MgCl_2$, 0.25 mM dithiothreitol, and 20 mM Hepes (pH 8.0) buffer. Antibody selection from the reaction mixture was performed as previously described (Patton et al., 1989; Jacobson et al., 1995) using either a human autoimmune serum, L122 (kindly provided by Walter van Venrooij, University of Nijmegen, The Netherlands), which is a To/Th class serum containing antibodies against both the RNase P and RNase MRP ribonucleoprotein particles, or an antibody specific for the human Pop1 protein that is a component of both RNase P and RNase MRP (Lygerou et al., 1996), kindly provided by Bertrand Seraphin, European Molecular Biology Laboratory, Heidelberg, Germany. RNA was extracted from the bound fraction after elution and analyzed by denaturing polyacrylamide gel electrophoresis.

Cell culture, microinjection and immunocytochemistry

A subclone of normal rat kidney epithelial cells (NRK-52E; American Type Culture Collection, Rockville, MD) was cultured in F-12K medium (JRH Bioscience, Lenexa, KS) containing 10% fetal bovine serum (JRH Bioscience), 1 mM L-glutamine, 50 μg/ml streptomycin, and 50 μg/ml penicillin. A line of HeLa cells selected for flat attachment in monolayer culture was similarly cultured in F-12K medium. Human diploid fibroblast, Detroit 551 (D551) cells were grown either in F-12K medium or DME medium (Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum, 1 mM L-glutamine, 50 μg/ml streptomycin, and 50 μg/ml penicillin. The methods used for microinjection and immunocytochemistry have been previously described in detail (Wang et al., 1991; Fishkind and Wang, 1993; Jacobson et al., 1995).

Fluorescent images were obtained using a Zeiss Axiovert-10 inverted microscope equipped for epifluorescence (Jacobson et al., 1995). When appropriate, images were digitally processed using a previously described nearest neighbor algorithm (Castleman, 1979; Agard, 1984; Shaw and Rawlings, 1991) and point spread function obtained with our optical system (Fishkind and Wang, 1993; Jacobson et al., 1995).

In situ hybridization

The methods used were essentially those described previously (Taneja et al., 1995) with only minor modification. Oligodeoxynucleotides

containing amino-modified thymidine (see Table 1) were labeled with either the red dye CY3 (Biological Detection Systems, Pittsburgh, PA) or fluorescein-5-isothiocyanate (FITC; Molecular Probes Inc., Eugene, OR) by incubation in 0.1 M $NaHCO_3$/Na_2CO_3 (pH 9.0) overnight at room temperature in the dark (Langer et al., 1981; Agrawal et al., 1986), and purified as previously described (Taneja et al., 1995).

Fluorescence in situ hybridization images were obtained using a Zeiss IM-35 microscope (Carl Zeiss Inc., Thornwood, NY) equipped for epifluorescence and digitally processed using exhaustive photon restoration, a constrained iterative algorithm which restores light to its original point source, as described previously in detail (Taneja et al., 1992; Carrington, et al., 1995). The calculated *z*-axis resolution for the deconvolved optical sections derived from this imaging system was 0.25 μm. Laser scanning confocal images were acquired on a Nikon Diaphot equipped for epifluorescence using a Bio-Rad MRC-1024 Laser Scanning Confocal Imaging System. The *z*-axis resolution of the confocal microscope was 0.4 μm.

RESULTS

Intranuclear localization of endogenous RNase P RNA

Human RNase P is a ribonucleoprotein complex consisting of an ~340 nt RNA (Bartkiewicz et al, 1989; Fig. 1) and at least two protein components, the ~40 kDa To/Th antigen (Hashimoto and Steitz, 1983; Reddy et al., 1983; Reimer et al., 1988; Gold et al., 1988, 1989) and the ~100 kDa hPop1 protein (Lygerou et al., 1996). Antibodies to hPop1 efficiently precipitated >95% of the RNase P RNA from HeLa cell nuclear extracts and these anti-hPop1 immunoprecipitates possess RNase P enzymatic activity, demonstrating that hPop1 is intimately associated with the active pool of nuclear RNase P (Lygerou et al., 1996). Both the To/Th antigen and hPop1 protein localize predominantly in nucleoli; however, some nucleoplasmic staining has also been observed by immunocytochemistry (Reimer et al., 1988; Jacoboson et al., 1995; Lygerou et al., 1996).

To investigate the steady-state spatial distribution of endogenous RNase P, we examined the intranuclear localization of RNase P RNA by in situ hybridization, using fluorescently-labeled complementary oligodeoxynucleotide probes (Table 1). As shown in Fig. 2A, endogenous nuclear RNase P RNA was diffusely localized throughout the nucleus of rat kidney (NRK) epithelial cells. To examine more closely the subnuclear localization of endogenous RNase P RNA, cells were optically sectioned and the out-of-focus light was removed from each section using an iterative algorithm which restores light to its original point source (Materials and Methods; Taneja et al., 1992; Carrington et al., 1995). The restored optical sections confirmed the conclusion from conventional fluorescent microscopy (Fig. 2A) that endogenous RNase P RNA is diffusely distributed throughout the nucleus of NRK cells, human diploid fibroblasts, and HeLa cells (data not shown).

The RNA component of the endoribonuclease RNase MRP is evolutionarily related to RNase P RNA (as reviewed by Morrissy and Tollervey, 1995) and is predominantly localized in nucleoli (Reddy et al., 1981; Reimer et al., 1988; Li et al., 1994). Endogenous MRP RNA was therefore used as a marker for nucleoli in a dual MRP RNA-RNase P RNA in situ hybridization experiment (Fig. 2E-H). Using laser scanning confocal microscopy, the majority of nuclear RNase MRP RNA was found to be localized in nucleoli in both NRK cells (Fig. 2F) and HeLa cells (Fig. 2H). In contrast, RNase P RNA in the same cells was diffusely localized throughout the nucleus (Fig. 2E and G, respectively). Similar results were observed using fluorescence microscopy coupled with digital image processing as described by Taneja et al. (1992) (data not shown). These results demonstrate that although they are evolutionarily closely related, RNase P RNA and RNase MRP RNA display different patterns of steady-state intranuclear localization.

In vitro assembly of fluorescently labeled RNase P RNA into a specific ribonucleoprotein complex

Some patients with autoimmune disease produce antibodies against a 40 kDa nucleolar protein, designated the To or Th antigen, which immunoprecipitate human RNase P RNA and RNase MRP RNA (Hashimoto and Steitz, 1983; Reddy et al., 1983; Reimer et al., 1988; Gold et al., 1988, 1989). This predominantly nucleolar To/Th antigen (Reimer et al., 1988) is antigenically related to the only protein component of *Escherichia coli* RNase P, the C5 protein (Mamula et al., 1989), and readily associates with human RNase P RNA (Fig. 1) in vitro (Liu et al., 1994). As shown in Fig. 3A, rhodamine-coupled, ^{32}P-labeled RNase P RNA assembled into a ribonucleoprotein complex immunoselectable by a To/Th monospecific autoimmune serum. This demonstrates that the

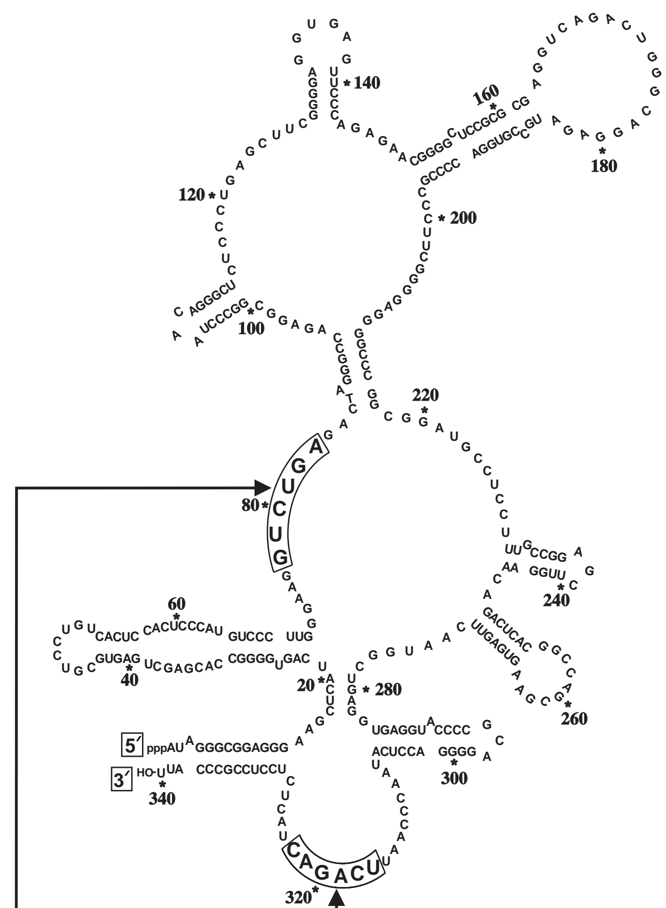

Fig. 1. Human RNase P RNA. The proposed secondary structure is from Altman et al. (1993). Boxed bold type indicates nucleotides involved in a base-pairing interaction (indicated by the arrowheads and connecting line) which forms a theoretical pseudoknot.

Table 1. Oligodeoxynucleotide in situ hybridization probes*

Probe	Sequence	Position
Rat RNase P RNA probes:		
MJ-8	5′-T^{5}CAGGGGCCGAGAGAGT^{5}GACGAGCACT^{5}CAGCGCGTGT^{5}T-3′	30-71†
MJ-9	5′-T^{5}AACTCACCT^{5}CCCCACT^{5}CGGGGAGAGCCCGGTT^{5}A-3′	97-130
MJ-10	5′-T^{5}CCGGGGGCTT^{5}CTCCTGCCCAGT^{5}CTGCCCACGCGT^{5}T-3′	160-194
Human RNase P RNA probes:		
MJ-1	5′-AT^{5}GGGAGTGGAGT^{5}GACAGGACGCACT^{5}CAGCTCGT^{5}G-3′	30-64
MJ-2	5′-T^{5}CCACGGCATCT^{5}CCTGCCCAGT^{5}CTGACCT^{5}CGCGC-3′	160-192
MJ-3	5′-TT^{5}AGGGCCGCCTCT^{5}GGCCCTAGTCT^{5}CAGACCTT^{5}CC-3′	73-107
MJ-25	5′-CACT^{5}TCGCTGGCCGT^{5}GAGTCTGTCT^{5}C	242-267
MJ-26	5′-TT^{5}GAACTCACTT^{5}CGCTGGCCGT^{5}GAGTCTGTT^{5}CC-3′	242-274
Rat RNase MRP RNA probes:		
MJ-5	5′-T^{5}CGAAAGGAT^{5}AAGGAACAAGGT^{5}CCCCCGTACGT^{5}A-3′	30-62
MJ-6	5′-T^{5}GCCTGCGTCACT^{5}AGGAGAACT^{5}GACGGAT^{5}GACAC-3′	192-225
MJ-7	5′-T^{5}CGCCCCGGAGGGGT^{5}CGCTTCTT^{5}GGCGGGCT^{5}AACA-3′	137-171
Human RNase MRP RNA probes:		
MJ-11	5′-T^{5}CGGAAAGGGGAGGAACAGAGT^{5}CCTCAGTGTGT^{5}A-3′	30-62
MJ-12	5′-T^{5}GCCTGCGTAACT^{5}AGAGGGAGCT^{5}GACGGATGACGCT^{5}T-3′	192-225
MJ-13	5′-T^{5}CTCAGCGGGAT^{5}ACGCTTCTT^{5}GGCGGACTTTGGAGT^{5}G-3′	137-171
Control oligonucleotide probes‡:		
MJ-1R	5′-GT^{5}GCTCGACT^{5}CACGCAGGACAGT^{5}GAGGTGAGGGT^{5}A-3′	
MJ-2R	5′-CGCGCT^{5}CCAGTCT^{5}GACCCGTCCT^{5}CTACGGCACCT^{5}T-3′	
MJ-8R	5′-TT^{5}GTGCGCGACT^{5}CACGAGCAGT^{5}GAGAGAGCCGGGGACT^{5}T-3′	
MJ-9R	5′-AT^{5}TGGCCGGAGAGGGGCT^{5}CACCCCT^{5}CCACTCAAT^{5}T-3′	

*T^5 in the oligonucleotide sequences = 5-amino-thymidine.
†Numbers indicate the nucleotide sequence within the RNA molecule to which the probes are complementary.
‡The nucleotide sequences of control oligonucleotides MJ-1R, MJ-2R, MJ-8R and MJ-9R are the reverse sequences (3′-to-5′) of oligonucleotide probes MJ-1, MJ-2, MJ-8 and MJ-9, respectively. As such, these control oligonucleotides are essentially random probes having the same nucleotide composition as their corresponding test probe counterparts.

presence of the fluorochrome on RNase P RNA (5-8 rhodamine groups per RNA molecule) does not impair its assembly into a ribonucleoprotein complex with the To/Th antigen, a known component of the human RNase P enzyme. We also carried out the same type of experiment with an antibody to the human Pop1 protein, also a component of human RNase P (Lygerou et al., 1996). Rhodamine-substituted and non-substituted RNase P RNAs were equivalently selected by this antibody following their ribonucleoprotein assembly in nuclear extracts (Fig. 3B), again demonstrating by this second criterion that the rhodamine-labeled RNase P RNA is unimpaired in its capacity to correctly assemble with the appropriate proteins.

Nuclear localization of microinjected RNase P RNA

Fluorescent RNA cytochemistry is a method we have developed and used previously (Wang et al., 1991; Jacobson et al., 1995) to examine the movement and localization of RNAs in living cells. Here we have used fluorescent RNA cytochemistry to examine the distribution of rhodamine-labeled RNase P RNA after microinjection into the interphase nucleus of living normal rat kidney (NRK) cells. As shown in Fig. 4A, the majority of RNase P RNA became localized in nucleoli within only 3 minutes after nuclear microinjection. However, at 21 minutes and 90 minutes after nuclear microinjection the rhodamine-labeled RNase P RNA became increasingly more evenly distributed throughout the nucleus (see Fig. 4B,C, respectively), resembling the pattern observed for endogenous RNase P RNA by in situ hybridization (Fig. 2). Similar results were obtained in HeLa cells (data not shown). Rapid nucleolar localization was also observed upon nucleus-microinjection of U3 small nuclear RNA (Fig. 4E), and RNase MRP RNA (Jacobson et al., 1995), two nucleolar RNAs known to be involved in ribosomal RNA processing (Baserga and Steitz, 1993; Clayton, 1994). As shown in Fig. 4F and H, the nucleolar localization of RNase P RNA observed shortly after nuclear microinjection (Fig. 4A) was not altered by fixation. In contrast to the initial nucleolar localization of RNase P RNA (Fig. 4F), fluorescein-labeled dextran co-microinjected with rhodamine-labeled RNase P RNA remained in the nucleoplasm, and was in fact excluded from the nucleoli (Fig. 4G; see also overlay in Fig. 4H).

The rapidity of the nucleolar localization of introduced RNase P RNA is striking. Although the earliest timepoint shown in Fig. 4 was 3 minutes after microinjection, similar results were obtained as early as 30 seconds after nuclear microinjection (data not shown). This rapid nucleolar localization of RNase P RNA is similar to the extremely fast nucleolar localization we previously observed for the RNA component of RNase MRP (Jacobson et al., 1995), and contrasts with the more gradual movements of microinjected pre-mRNAs to nucleoplasmic sites of accumulation, which take approximately 30-60 minutes (Wang et al., 1991).

In contrast to the rapid nucleolar localization of RNase P RNA, when pre-tRNA (also an RNA polymerase III transcript) was microinjected into the nucleus no evidence of nucleolar localization was observed (data not shown). We also injected into nuclei rhodamine-labeled human U6 small nuclear RNA, another RNA polymerase III transcript (Kunkel et al., 1986). Initially the U6 RNA was observed diffusely throughout the nucleus, and was partially excluded from nucleoli (Fig. 4I). No nucleolar localization of nucleus-microinjected U6 RNA was observed even after extended periods of observation. However, approximately 30 minutes after nuclear microinjection the U6

RNA was observed in a pattern of multiple nucleoplasmic loci (Fig. 4K) reminiscent of the 'speckle' domains of pre-mRNA splicing factors (Fu and Maniatis, 1990; Spector, 1990; Wang et al., 1991). This subnuclear localization of U6 RNA was observed for both γ-monomethyl phosphate capped and uncapped RNAs (data not shown), and is therefore not dependent on the presence of the γ-monomethyl phosphate cap on the 5′-end of endogeneous human U6 RNA (Singh and Reddy, 1989). These results for pre-tRNA and U6 small nuclear RNA thus establish that the nucleolar localization of RNase P RNA is not a general property of RNA polymerase III transcripts.

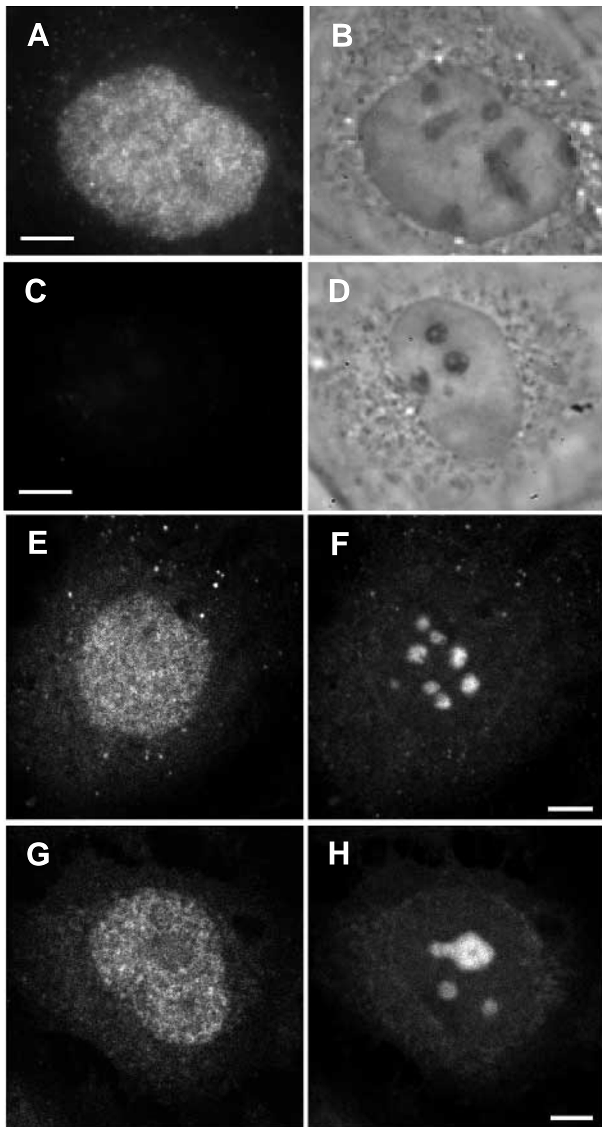

Fig. 2. Localization of endogenous RNase P RNA by in situ hybridization. Cells were cultured, fixed and subjected to in situ hybridization as described in Materials and Methods. (A) A representative NRK cell probed with a mixture of two Cy3-labeled probes (MJ-8 and MJ-9; see Table 1) complementary to different domains of rat RNase P RNA. (C) A representative NRK cell probed with a mixture of two Cy3-labeled random negative control probes (MJ-8R and MJ-9R; see Table 1). For orientation, the corresponding phase contrast images (B and D) are also shown. (E-H) Localization of endogenous RNase P RNA and RNase MRP RNA in the same cell, visualized by confocal laser scanning microscopy (calculated *z*-axis resolution = 0.5 μm) as described in Materials and Methods. NRK cells were hybridized with a probe mixture consisting of Cy3-labeled rat RNase P RNA specific oligonucleotides (E; MJ-8, MJ-9 and MJ-10) and FITC-labeled rat RNase MRP RNA specific oligonucleotides (F; MJ-5, MJ-6 and MJ-7). HeLa cells were hybridized with a probe mixture consisting of Cy3-labeled human RNase P RNA specific oligonucleotides (G; MJ-1, MJ-2 and MJ-3) and FITC-labeled human RNase MRP RNA specific oligonucleotides (H; MJ-11, MJ-12 and MJ-13). Bars, 5 μm.

Identification of the nucleolar sites of rapid RNase P RNA localization

The 'lobular' appearance of nucleolus-localized RNase P RNA (Fig. 4) is reminiscent of the dense fibrillar component of the nucleolus, known to be the site of the early steps in pre-rRNA processing and ribosomal subunit assembly (Fakan and Bernhard, 1971; Hadjiolov, 1985; Thiry and Thiry-Blaise, 1989, 1991; Puvion-Dutilleul et al., 1991). To determine if the nucleolar sites of RNase P RNA localization correspond to the dense fibrillar component, we used fluorescent RNA cytochemistry combined with subsequent immunocytochemistry to compare the localization of nucleus-microinjected RNase P RNA with that of fibrillarin, a dense fibrillar component protein (Ochs et al., 1985; Reimer et al., 1987; Scheer and Benavente, 1990) associated with several small nucleolar ribonucleoprotein particles involved in pre-rRNA processing (Tyc and Steitz, 1989). As shown in Fig. 5, RNase P RNA microinjected into the nucleus of NRK cells became localized in nucleoli (Fig. 5A; red) in a pattern virtually

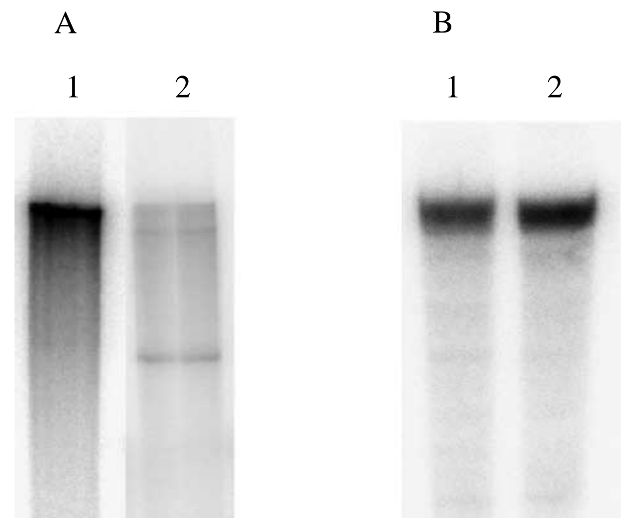

Fig. 3. Ribonucleoprotein assembly of rhodamine-labeled RNase P RNA. In vitro transcribed ^{32}P-labeled and ^{32}P+rhodamine-labeled human RNase P RNAs were purified, incubated with a micrococcal nuclease treated HeLa cell nuclear extract, immunoselected with antiserum prebound to Protein A-Sepharose and the bound and unbound RNA fractions separated by elution and analyzed by denaturing polyacrylamide gel electrophoresis as described in Materials and Methods. (A) Bound (lane 1) and unbound (lane 2) ^{32}P+rhodamine-labeled RNase P RNA immunoselected with human serum L122 (a To/Th class human autoimmune serum; see Materials and Methods). (B) Bound ^{32}P-labeled (lane 1) and ^{32}P+rhodamine-labeled (lane 2) RNase P RNA immunoselected with anti-hPop1 serum (Lygerou et al., 1996). Equal amounts of ^{32}P-labeled and ^{32}P+rhodamine-labeled RNase P RNAs (15 ng) were incubated in the assembly reactions and equivalent amounts of each were immunoselected using the anti-hPop1 serum.

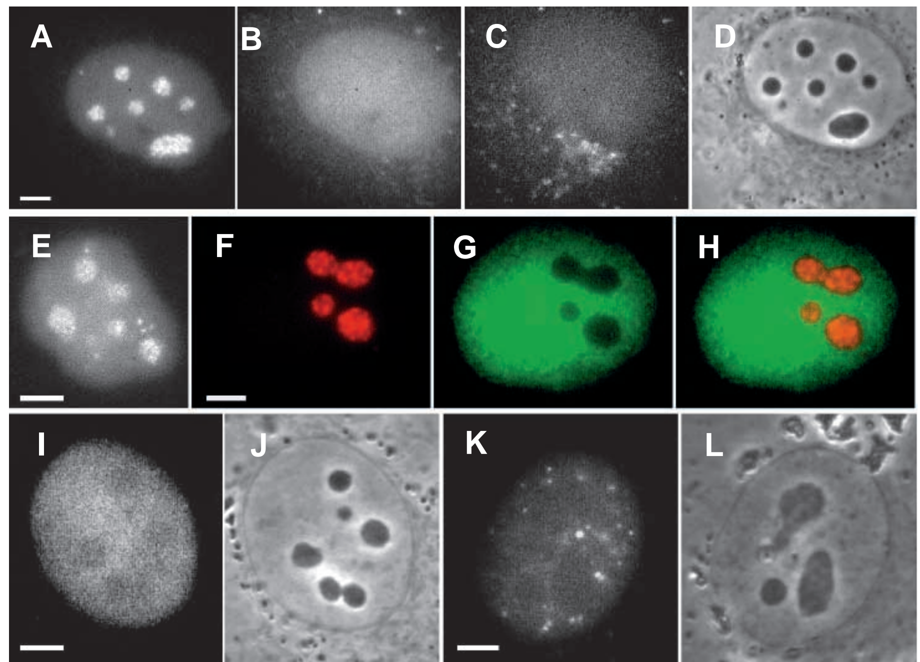

Fig. 4. Localization of human RNase P RNA after microinjection into the nucleus of NRK cells. (A-D) RNase P RNA was transcribed in vitro, labeled with rhodamine, purified and microinjected into the nucleus of NRK cells as described in Materials and Methods. The subsequent localization of RNase P RNA was visualized in the same living cell 3 minutes (A), 21 minutes (B), and 90 minutes (C) after nuclear microinjection. (D) Phase contrast image 90 minutes after nuclear microinjection. (E) Localization of rhodamine-labeled human U3 snRNA in a living cell 3 minutes after nuclear microinjection. (F-H) Localization of rhodamine-labeled RNase P RNA (F) and fluorescein-labeled dextran (G) co-injected into the nucleus of a NRK cell. The cell was fixed 4 minutes after microinjection and visualized as described in Materials and Methods. (H) Overlay of the images in F and G. (I-L) Localization of rhodamine-labeled U6 small nuclear RNA in living NRK cells 6 minutes (I) or 36 minutes (K) after nucleus microinjection. (J) Phase contrast image of the cell in I. (L) Phase contrast micrograph of the cell in K. Bars, 5 µm.

identical to that of endogenous fibrillarin (Fig. 5B; green). Superimposition of these two images revealed a precise co-localization, as indicated by the yellow signal in Fig. 5C. Three-dimensional stereo pairs of the cell shown in Fig. 5, generated by digital optical sectioning microscopy (Fishkind and Wang, 1993; Jacobson et al., 1995) demonstrated that the nucleolar localized RNase P RNA was present throughout the nucleoli in a pattern very similar to that observed for fibrillarin (data not shown). This pattern consists of an interlinking network of lobular foci connected by thinner strands which is the classical organization of the dense fibrillar component as defined by electron microscopy (Fakan and Bernhard, 1971; Hadjiolov, 1985; Thiry and Thiry-Blaise, 1989, 1991; Puvion-Dutilleul et al., 1991).

Identification of the RNase P RNA nucleolar targeting domain

Nucleotides 20-75 (but not nucleotides 76-342) of human RNase P RNA are readily bound by the To/Th antigen (Liu et al., 1994), a 40 kDa predominantly nucleolar protein (Reimer et al., 1988). As shown in Fig. 6A, an RNA consisting of only nucleotides 1-88 of RNase P RNA became rapidly localized in nucleoli after nuclear microinjection, similar to the behavior of full length RNase P RNA (Fig. 4A). However, unlike full-length RNase P RNA, this 3′ truncated RNase P RNA (nucleotides 1-88) remained in nucleoli throughout the observation period (Fig. 6B; 105 minutes), with no change in the ratio of nucleolar-to-nucleoplasmic signal (compare Fig. 6A with B). Thus, nucleotides 1-88 of RNase P RNA, which contain the To binding domain (nucleotides 20-75), are sufficient for nucleolar localization of RNase P RNA. We also microinjected a mutant RNA which corresponds to nucleotides 71-329 of human RNase P RNA (see Fig. 1) that lacks the To antigen binding site. This RNA failed to localize in nucleoli (Fig. 6D,E), indicating that this domain, like the comparable To-binding element in RNase MRP RNA (Jacobson et al., 1995) is required for nucleolar localization of RNase P RNA.

DISCUSSION

The fluorescent RNA cytochemistry method we have developed (Wang et al., 1991; Jacobson et al., 1995, 1997; Jacobson and Pederson, 1977) affords the study of RNA trafficking in living

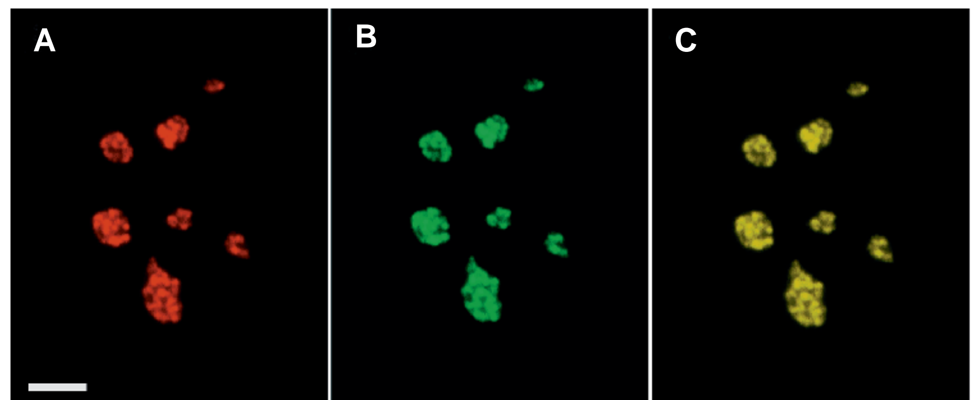

Fig. 5. Nucleus-injected RNase P RNA initially localizes to the dense fibrillar component of nucleoli. Rhodamine-labeled RNase P RNA was microinjected into the nucleus of a NRK cell and the cell was fixed 1 minute after microinjection, permeabilized and stained for fibrillarin as described in Materials and Methods. Antibody-antigen complexes were detected with fluorescein-conjugated anti-mouse IgG. (A) Rhodamine-labeled RNase P RNA (red); (B) endogenous fibrillarin (green); (C) superimposition of A and B. Bar, 5 µm.

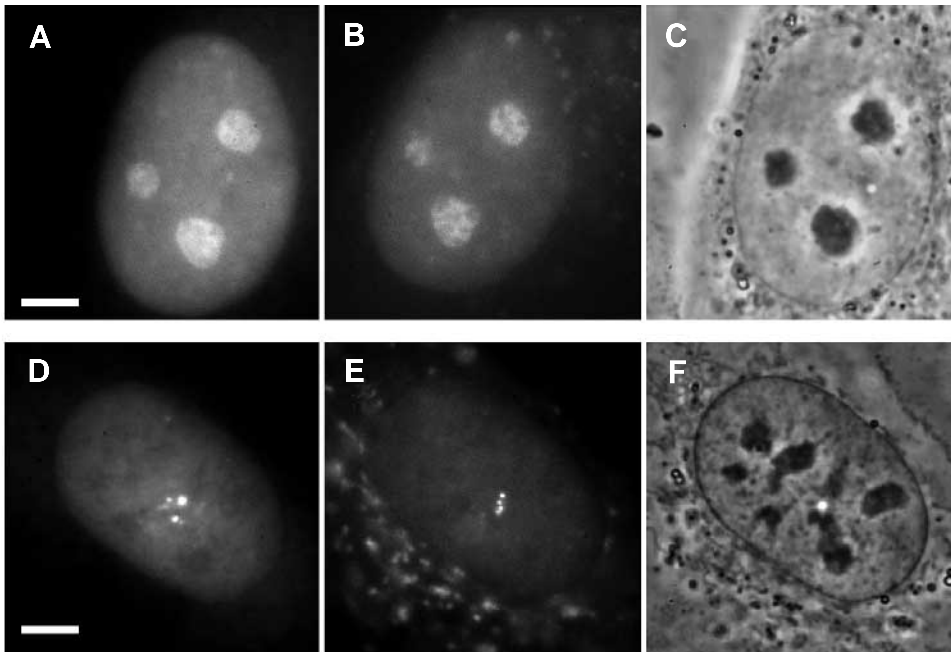

Fig. 6. Nucleolar localization of RNase P RNA requires the To antigen binding domain. An RNA consisting of nucleotides 1-88 of human RNase P RNA containing the To antigen binding domain and 5′ end sequences was transcribed, rhodamine labeled and microinjected into the nucleus of NRK cells as described in Materials and Methods. The localization of the RNase P To domain RNA was visualized in a living cell 3 minutes (A) and 105 minutes (B) after nuclear microinjection. (C) Phase contrast image of cell in A and B. A rhodamine-labeled mutant RNase P RNA (corresponding to nucleotides 71-329 of the human RNase P RNA sequence) lacking the To antigen binding domain was similarly nucleus-microinjected and visualized in a living NRK cell 1 minutes (D) and 20 minutes (E) after microinjection. (F) Phase contrast image of cell in D and E. Bar, 5 μm.

cells. We have previously demonstrated that nucleus-microinjected pre-mRNAs localize in nucleoplasmic ‘speckles’ (Wang et al., 1991), sites known to be rich in snRNP antigens and the pre-mRNA splicing factor SC35 (Spector, 1990; Fu and Maniatis, 1990; Wang et al., 1991). In addition, we have shown that the RNA subunit of RNase MRP, a known nucleolar ribonucleoprotein (Reddy et al., 1981), rapidly accumulates in nucleoli after nucleus-microinjection (Jacobson et al., 1995). Moreover, fluorescent U3 RNA, a small nucleolar RNA, rapidly localizes in the nucleolus (Fig. 4E) while fluorescent U6 RNA, one of the spliceosomal snRNAs, localizes in a nucleoplasmic pattern resembling the distribution of snRNP-rich speckles (Fig. 4K). Thus, several microinjected RNAs (pre-mRNA, MRP RNA, U3 RNA and U6 RNA) localize at the specific nuclear sites at which their endogenous counterparts are present.

In the present investigation we have examined the intranuclear localization of the RNA subunit of the ribonucleoprotein enzyme RNase P. We have demonstrated that microinjected RNase P RNA rapidly localizes in nucleoli, then gradually redistributes throughout the nucleus (Fig. 4). The resulting uniform intranuclear redistribution of microinjected RNase P RNA is very similar to the steady-state distribution of endogenous RNase P RNA observed by in situ hybridization (Fig. 2). The rapid but transient nucleolar localization of nucleus-microinjected RNase P RNA observed in this investigation contrasts with our recently described pattern of intranuclear localization of the RNA subunit of RNase MRP (Jacobson et al., 1995). This RNA, which like RNase P RNA is a RNA polymerase III transcript (Chang and Clayton, 1989), also rapidly localizes in the nucleoli after nucleus-microinjection, but a substantial portion remains there throughout the period of observation (Jacobson et al., 1995). In addition, the localization of endogenous MRP RNA, determined by in situ hybridization, is mostly nucleolar (see Fig. 2F and H). Taken together, these results suggest a transient nucleolar phase in the maturation, ribonucleoprotein assembly or function of RNase P RNA in mammalian cells. In this respect it is of considerable interest to note that there have been increasing hints that, in addition to its established role in pre-transfer RNA processing, RNase P might also function in pre-ribosomal RNA processing (reviewed by Clayton, 1994). This idea has reached considerable momentum with the recent discovery by Chamberlain et al. (1996) that a mutation in the RNA subunit of *S. cerevisiae* RNase P affects pre-ribosomal RNA processing. The hypothesis that emerges is that both RNase MRP and RNase P have nucleolar functions, i.e. pre-ribosomal RNA processing, but that RNase P is more transiently associated with the nucleolus and eventually is mobilized into the nucleoplasm.

Our experiments with mutant RNAs (Fig. 6) revealed that nucleotides 1-88 of RNase P RNA are both necessary and sufficient for nucleolar localization, possibly implicating the To antigen binding domain (nucleotides 20-74; Liu et al., 1994) as the RNase P RNA nucleolar targeting signal, similar to that observed for RNase MRP RNA (Jacobson et al., 1995). Moreoever, our results add another important element to the picture: sequences 3′-ward of nucleotide 88 of RNase P RNA are apparently required for its subsequent transit from the nucleolus to the nucleoplasm. We noted that the To antigen non-binding mutant RNase P RNA (Fig. 6D-F) generates an overall weaker signal in the nucleoplasm and also appears in discrete cytoplasmic foci. It is possible that this RNA, incapable of becoming stably integrated into the nucleolus, either localizes to other sites within the cell, as do for example RNase MRP RNA mutants (Li et al., 1994) mediated by sequences 3′-ward of the To binding domain, or becomes subject to pathways of RNA turnover operating in the nucleus. The binding of microinjected (wild type) RNAs to nuclear structures (e.g. Wang et al., 1991; Jacobson et al., 1995; Figs 4 and 5 of this study) may thus be regarded not only as a report of RNA localization but also as a kind of in vivo nuclease protection experiment. As regards the cytoplasmic sites of localization observed when the RNase P RNA mutant lacking the To antigen binding domain was microinjected into the nucleus (Fig. 6E), these sites may be mitochondria (M. R. Jacobson and T. Pederson, unpublished results).

An important additional clue to the possible nucleolar role of RNase P RNA is conveyed by the specific sub-nucleolar

region in which it becomes localized, the dense fibrillar component (Fig. 5). This nucleolar element is the site of pre-ribosomal RNA processing and nascent ribosomal subunit assembly (Spector, 1993; Hozak, 1995), again adding further weight to the hypothesis that RNase P may, in addition to pre-tRNA processing, also function in pre-rRNA processing. Such a dual catalytic role might, depending upon the kcat's of the respective RNase P cleavages of pre-rRNA and pre-tRNA, and the nucleolar residence time of RNase P, contribute to a balanced rate of rRNA and tRNA biosynthesis in the cell.

In an in situ hybridization study on the Ro small RNAs, endogenous RNase P RNA was found to have an unusual intranuclear localization in a variable percentage (~70-90%) of HeLa cells, consisting of a relatively homogeneous distribution throughout (or on the surface of) the nucleoli plus concentrated, discrete loci of in situ hybridization reactivity at the edges of the nucleoli, termed perinucleolar compartments, or PNCs (Matera et al., 1995). Similar results were also observed with in situ hybridization probes for MRP RNA. In contrast to the situation in HeLa cells, far fewer (but still some) PNCs were observed in non-transformed cell lines (Matera et al., 1995). Our RNase MRP RNA in situ hybridization results (Fig. 2) revealed a predominantly nucleolar localization pattern, which in many ways is similar to that previously observed (Matera et al., 1995; Lee et al., 1996), but we did not observe any PNC-like structures. Moreover, using either the in situ hybridization procedures detailed here (Fig. 2) or the protocols of Matera et al. (1995) (data not shown), we observed RNase P RNA to be diffusely localized throughout the nucleus with no obvious indication of PNC-like structures, similar to recent findings by Lee et al. (1996) using DNA probes complementary to RNase P RNA. We obtained similar results with non-transformed human diploid fibroblasts (Detroit 551 cells) and normal rat kidney epithelial cells. It is possible that differences in oligonucleotide probes (DNA oligos in our experiments, 2-O′-Me RNA in the other studies), cell lines or cell culture conditions could account for the differences observed.

Viewing the present results and related recent work in the broader context of nuclear structure and gene readout domains, how can the transient nature of the nucleolar localization of fluorescent RNase P RNA be reconciled with its subsequent redistribution throughout the nucleus and the uniform intranuclear pattern of endogenous RNase P RNA? A model consistent with all these observations is that, once released from the transcription machinery, RNase P RNA is rapidly translocated to the nucleolus where it undergoes further modification or additional ribonucleoprotein assembly. Ample precedents exist for such stepwise, multi-site pathways of ribonucleoprotein maturation and assembly in the cell, for example, the cytoplasmic assembly of mammalian spliceosomal snRNPs followed by nuclear import (Zieve and Sauterer, 1990). In the case of RNase P, its envisioned duality of function in pre-ribosomal and pre-transfer RNA processing would require its presence in both the nucleolus and nucleoplasm, and its possible sequential passage between these nuclear domains. The fluorescent RNA cytochemistry approach we have developed (Wang et al., 1991; Jacobson et al., 1995, 1997; Jacobson and Pederson, 1977), together with antibody probes specific for unique protein component(s) of RNase P that are becoming available (Eder et al., 1997), sets the stage for addressing these intriguing aspects of the cell biology of RNase P biosynthesis and function.

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