Nuclear RanGTP is not required for targeting small nucleolar RNAs to the nucleolus

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Summary

The small GTPase Ran is the central regulator of macromolecular transport between the cytoplasm and the nucleus. Recent work has suggested that RanGTP also plays an important role in regulating some intra-nuclear processes. In this study, we have investigated whether RanGTP is required for the intra-nuclear transport of RNAs. Specifically, we directly analyzed the nucleolar localization of Box C/D and Box H/ACA small nucleolar RNAs (snoRNAs) in mammalian tsBN2 cells, Saccharomyces cerevisiae and Xenopus oocytes under conditions that deplete nuclear RanGTP and prevent RNA export to the cytoplasm. Our data suggest that depletion of nuclear RanGTP does not significantly alter the nucleolar localization of U3 snoRNA in tsBN2 cells. Complementary studies in the budding yeast S. cerevisiae using conditional Ran mutants as well as mutants in Ran regulatory proteins also indicate that disruption of the Ran gradient or of Ran itself does not detectably affect the nucleolar localization of snoRNAs. Finally, microinjection into Xenopus oocytes was used to clearly demonstrate that a specific pool of snoRNAs could still be efficiently targeted to the nucleolus even when the RanGTP gradient was disrupted by microinjection of mutant Ran proteins. Taken together, our data from three phylogenetically distinct experimental systems suggest that nuclear RanGTP, which is essential for trafficking of RNAs between the nuclear and cytoplasmic compartments, is not required for nuclear retention or nucleolar localization of snoRNAs.

Key words: Small nucleolar RNA, RanGTP, RNA transport, Nucleolus, tsBN2, Xenopus oocytes, S. cerevisiae

Introduction

In eukaryotic cells, macromolecules must navigate within and between distinct subcellular compartments. Work on a variety of transport processes has demonstrated that a common way of regulating and orchestrating these transport events is through small GTPases that cycle between GTP- and GDP-bound forms. For example, the GTPases Rab and Arf regulate vesicular trafficking (Chavrier and Goud, 1999; Takai et al., 2001) and the Ran GTPase is critical for mediating directional transport between the nucleus and the cytoplasm (Görlich and Kutay, 1999; Izaurralde et al., 1997; Kuersten et al., 2001). The mechanism by which the Ran GTPase imparts directionality on transport between the cytoplasm and the nucleus is understood in some detail (Görlich and Kutay, 1999). Numerous experiments have demonstrated that Ran regulates the assembly and disassembly of both import and export complexes (Görlich and Kutay, 1999). Current models suggest that directionality arises because a RanGTP gradient exists between the nucleus and the cytoplasm. Within the nucleus Ran is primarily in the form of RanGTP, whereas within the cytoplasm it is primarily in the form of RanGDP (Görlich and Kutay, 1999; Izaurralde et al., 1997). This gradient is set up by the compartmentalization of the Ran regulatory proteins; the guanine nucleotide exchange factor, GEF, which catalyzes the conversion of RanGDP to RanGTP (Bischoff and Ponstingl, 1991) is restricted to the nucleus (Nemergut et al., 2001; Ohtsubo et al., 1987) and the Ran GTPase activating protein, RanGAP (Becker et al., 1995; Bischoff et al., 1994; Corbett et al., 1995b), is primarily cytoplasmic (Hopper et al., 1990). Nuclear import complexes form in the cytoplasm where RanGDP is abundant and are then dissociated in the nucleus upon binding to RanGTP (Görlich and Kutay, 1999; Rexach and Blobel, 1995). By contrast, formation of nuclear export complexes in the nucleus requires RanGTP (Görlich and Kutay, 1999). These export complexes are dissociated in the cytoplasm where the RanGTP is converted to RanGDP in the presence of the RanGAP (Becker et al., 1995; Bischoff et al., 1994). Thus, Ran regulates the formation of competent transport complexes for nucleocytoplasmic transport.

In addition to its well characterized role in nucleocytoplasmic transport, there is recent evidence suggesting the direct involvement of Ran in regulating a variety of other important cellular processes including mitotic spindle formation (Gruss et al., 2001; Kahana and Cleveland, 2001; Nachury et al., 2001), nuclear envelope formation (Hetzer et al., 2000; Hughes et al., 1998; Zhang and Clarke, 2001) and mitotic cell cycle regulation (Matsumoto and Beach, 1991; Quimby et al., 2000b; Sazer and Nurse, 1994). Although Ran shuttles between the nucleus and cytoplasm (Görlich and Kutay, 1999), it exhibits a primarily steady-state localization...
within the nucleus (Quimby et al., 2000a), and evidence is accumulating that Ran is important for regulating intra-nuclear processes. Some of the evidence suggesting intra-nuclear roles for the Ran protein stems from analysis of a conditional mutant in the Ran guanine exchange factor, RCC1 (Ohtsubo et al., 1987). RCC1 (Regulator of Chromatin Condensation 1) was originally isolated as the conditional tsBN2 mutant from hamster cells that showed a premature chromatin condensation phenotype (Dasso, 1993; Ohtsubo et al., 1987). Subsequent biochemical analysis demonstrated that RCC1 is tightly bound to chromatin and acts as a guanine nucleotide exchange factor for the Ran GTPase (Bischoff and Poonstingl, 1991; Nemergut et al., 2001). The chromosome condensation phenotype associated with the RCC1 mutant suggests a role for Ran in regulating important intra-nuclear events such as DNA replication and mitosis. Consistent with this idea, several S. pombe mutants in components of the Ran cycle show clear cell cycle defects (Flegl et al., 2000; Sazer and Nurse, 1994). These observations suggest potential functions for Ran in regulating intra-nuclear processes.

One potential function for Ran within the nucleus could be to regulate intra-nuclear transport events. In addition to movement between the nucleus and cytoplasm, there is also macromolecular trafficking that must occur within the nucleus. RNAs must move from their site of transcription to their site of action. For most newly synthesized cellular RNAs (e.g. messenger RNAs, transfer RNAs, ribosomal RNAs) this means that they must be appropriately targeted from nucleoplasmic genes to nuclear pore complexes for delivery to the cytoplasm (Carmo-Fonseca et al., 1999; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998; Nakiely et al., 1997). Many other classes of RNAs will ultimately function within the nucleus (Terns and Terns, 2002; Will and Luhmann, 2001). Although some of these RNA species (e.g. spliceosomal small nuclear RNAs) are transported to the cytoplasm for processing and maturation (Mattaj, 1986; Yang et al., 1992), other RNAs (e.g. small nuclearular RNAs) exist solely within the nucleus and must be targeted from their site of transcription to their intra-nuclear site of action (Terns and Terns, 2002). Conceivably, RanGTP might be required for this intra-nuclear transport.

Indeed there is evidence to suggest that Ran plays a role in regulating the intra-nuclear trafficking of macromolecules. Previous work has shown that the temperature-sensitive RCC1 mutant, tsBN2, shows a change in the intra-nuclear localization of both small ribonucleoproteins (snRNPs) and the general splicing factor SC35 (Cheng et al., 1995; Huang et al., 1997) following a shift to the non-permissive temperature. Furthermore, biochemical experiments have implicated RCC1 in the transport of some but not all RNAs within the nucleus (Cheng et al., 1995). Cheng et al. used pulse/chase experiments to analyze precursor RNAs and to demonstrate that many of the small RNAs involved in splicing, U1, U2, U4 and U5, are not properly exported to the cytoplasm when RCC1 is inactivated in tsBN2 cells (Cheng et al., 1995). However, this analysis also suggested a role for nuclear RanGTP in the retention of U3 snoRNA within the nucleus (Cheng et al., 1995). When tsBN2 cells were shifted to the non-permissive temperature to deplete RCC1, the biochemical fractionation properties of U3 snoRNA were altered and U3, which is normally strictly localized to the nuclear fraction (in nucleoli), could be detected in the cytoplasm. The appearance of U3 snoRNA in the cytoplasmic fraction was attributed to an inability of the RNA to be retained within the nucleus by virtue of its inability to become localized to the nucleolus in the absence of RanGTP (Cheng et al., 1995). Thus, it was proposed that RCC1 (and hence nuclear RanGTP) was essential to localize U3 to the nucleolus.

The goal of the present study was to determine whether Ran is required for intra-nuclear transport of RNAs and, more specifically, to address whether nuclear RanGTP is essential for the nucleolar localization of snoRNAs. SnoRNAs are a large family of cellular RNAs that are synthesized in the nucleoplasm, actively retained within the nucleus during all steps of maturation and function and specifically targeted to the nucleolus where they function in rRNA processing/modification and ribosome biogenesis (Filipowicz et al., 1999; Terns and Terns, 2002; Tollervey and Kiss, 1997; Weinstein and Steitz, 1999). There are two major families of snoRNAs, the box C/D and the box H/ACA families (Balakin et al., 1996; Ganot et al., 1997). Each snoRNA family associates with a specific subset of proteins including fibrillarin, Nop56, Nop58, p15.5kD (Box C/D) or Cbf5p, Gar1, Nhp2, Nop10 (Box H/ACA) (Terns and Terns, 2002). Localization of the snoRNAs to the nucleolus requires the highly conserved box C/D and box H/ACA motif, respectively (Lange et al., 1998; Lange et al., 1999; Narayanan et al., 1999a; Narayanan et al., 1999b; Ruhl et al., 2000; Samarsky et al., 1998). It is hypothesized that proteins that bind to the motifs are instrumental in localizing snoRNAs to the nucleolus (Terns et al., 1995; Verheggen et al., 2001). Although it is clear that nuclear RanGTP is essential for the assembly of some RNP export complexes within the nucleus (Arts et al., 1998; Dahlberg and Lund, 1998; Izaurralde et al., 1995; Kutay et al., 1998), it is not known whether the intra-nuclear assembly of a snoRNP complex and/or nucleolar localization of snoRNPs requires nuclear RanGTP. In this study, we have disrupted the Ran gradient or Ran itself in three different cellular systems and our results indicate that nuclear RanGTP is not required for nuclear retention or nucleolar localization of snoRNA.

Materials and Methods
tsBN2 cell culture, fluorescence in situ hybridization and immunofluorescence

The temperature-sensitive RCC1 mutant cell line, tsBN2 (Ohtsubo et al., 1987), was cultured in Dulbecco’s modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum at 33°C. The localization of U3 snoRNA at both the permissive (33°C) and non-permissive (40°C) temperature was determined by fluorescence in situ hybridization (FISH) as described elsewhere (http://singerlab.aecom.yu.edu/protocols). Briefly, the cells were seeded onto coverslips at approximately 80% confluency and allowed to adhere overnight and then half of these coverslips were shifted to 40°C. Cells were fixed in 4% formaldehyde (Electron Microscopy Science), 10% acetic acid (Sigma), 1×PBS at 2 and 6 hours after the temperature shift. Cells maintained at 33°C in parallel were also fixed at the same time points. After permeabilization with 70% ethanol overnight at 4°C, the cells were rehydrated with 2×SSC, 50% formamide and probed with 30 ng of Cy3-conjugated U3-specific antisense deoxyoligonucleotide probe for 3 hours at 37°C in hybridization buffer (10% dextran sulfate, 2 mM vanadyl-ribonucleoside complex, 0.02% RNase-free BSA, 40 μg E. coli tRNA, 2×SSC, and 50% formamide). The human U3 probe (obtained from Operon
Technologies) that was used is 5′-GTTCTCTCTCCCTQCRACTCC-C
AAQAAGGAGAAGAGGACCATCAGGCGG 3′ where Q indicates
aminomethyl-modified T residues incorporated for Cy3
coupling. Cy3 was obtained from Amersham Pharmacia Biotech
and the coupling was performed following the manufacturer’s rec-
dominations. The modified oligo was purified from unreactive Cy3 dye
using a nucleotide removal kit (Qiagen). Coupled probes were run on
8% nondenaturing polyacrylamide gels and detected by UV illumina-
tion to estimate the efficiency of coupling and recovery after purifica-
tion. Indirect immunofluorescence was performed as described
elsewhere (http://singerlab.aecom.yu.edu/protocols) using a 1:100
dilution of anti-trimethyl guanosine cap (TMG) antibodies (Brinkmann
et al., 1983) and a 1:50 dilution of Cy2-conjugated anti-
rabbit secondary antibodies (Jackson Immunoresearch Laboratories).
Coverslips were mounted on slides with 90% glycerol in 1×PBS
containing 1 mg/ml p-phenylene diamine and 1 μg/ml 4,6-diamino-2-
phenylindole (DAPI).

Yeast strains and media
All yeast strains were grown in yeast extract peptone dextrose (YE PD)
medium under standard conditions (Adams et al., 1997). The relevant
genotype for the wild-type strain, ACY192, is Mata ura3-52, trpl-
Δ63, leu2-Δ1. The following temperature-sensitive mutants were also
used: ACY212, Mata GSP1::HIS3 GSP2::HIS3 ura3-52 leu2-Δ1,
trpl-Δ63 (transformed with a gsp1-1, LEU, AMP, CEN plasmid
(pAC413) or with a gsp1-2, LEU, AMP, CEN plasmid (pAC414))
(Wong et al., 1997); ACY109 Mata prp20-1 trpl leu2-Δ1 ura3
(Amborg et al., 1993); and ACY61, Mata rna1-1 leu2-Δ1 ura3-52 his3
(Corbet et al., 1995a).

Yeast fluorescence in situ hybridization and immunofluorescence
FISH analysis and immunofluorescence in yeast was performed as
described (Amborg et al., 1992; Wong et al., 1997) except that Cy3-
labeled DNA probes were used. The anti-sense deoxyoligonucleotide
probes against yeast U3 snoRNA (5′-ATTCAGTGCTTGTGGAA-
GAGTCAAAGAGGAGCGTATCCTATAGAAAGTA 3′) snR10
snoRNA (5′-CAGACGACAGAAAGACGCTGGTCACCCAGATC-
GATAATTTGTTCCTCCAGTC 3′) and poly(A)+ mRNA (oligo
d(T)50) were synthesized by Operon Technologies with a single Cy3
label at the 5′ end. Briefly, the strains were grown in YEPD at room
temperature and shifted to 37°C for 3 hours. Formaldehyde-fixed cells
were harvested, spheroplasted with 300 μg of Zymolyase 100T (US
Biological), resuspended in P solution (1.2 M sorbitol in 0.1 M
potassium phosphate buffer, pH 6.5) and applied to 14-well Teflon-
faced microscope slides (CellPoint Scientific, Inc.) precoated with
0.1% polylysine. Following permeabilization with 0.5% IGEPA, the
hybridization was performed using 100-150 ng of Cy3-labeled probe
overnight at 37°C. In some experiments, indirect immunofluorescence
was also performed as described previously (Wong et al., 1997) using
a 1:1000 dilution of anti-Nop1p (A66) monoclonal antibodies (Aris
and Blobel, 1988) and a 1:50 dilution of Texas-Red-conjugated anti-
mouse secondary antibodies (Jackson Immunoresearch Laboratories).
Cells were stained with 1 μg/ml 4,6-diamino-2-phenylindole (DAP),
air dried and mounted in 1 mg/p-phenylene diamine/ml 90% glycerol
in 1×PBS.

Xenopus laevis oocyte microinjection and RNA analysis
The nuclear retention and nuclear localization of microinjected
fluorescently labeled U3 snoRNA or U65 snoRNA was assayed as
described previously (Narayanan et al., 1999a; Narayanan et al.,
1999b; Speckmann et al., 1999) in Xenopus oocytes in which the Ran
system was disrupted using Ran T24N. The RNAs for microinjection
were transcribed in vitro with a fluorescein-12-UTP label (for
detection by fluorescence microscopy) and a 32P-GTP label (for
detection by gel electrophoresis and autoradiography). Recombinant
T24N mutant Ran was expressed and purified from E. coli as
described previously (Lounsbery et al., 1996). 32P labeled, U1sm-
snoRNA (and U3 snoRNA in Fig. 5C and D) was coincubated with
the fluorescein-labeled RNAs as controls for RNA nuclear export (or
retention). 40 fmoles of Ran T24N in 10 nl of microinjection buffer
(10 mM Na2PO4, pH 7.2, 70 mM KCl, 1 mM MgCl2 and 10 mg/ml
blue dextran) or 10 nl of microinjection buffer alone were injected
into the nuclei of stage V/VI oocytes. The oocytes were incubated at
18°C for 1 hour before a second nuclear injection containing 1 fmoles
of fluorescein/32P-GTP-labeled U3 or U65. For analysis of intra-
nuclear localization, the oocytes were incubated at 18°C, and the
nuclei were dissected four hours after injection of the RNA. The
contents of each dissected nucleus were transferred to a microscope
slide. The slides were centrifuged, fixed, mounted and subjected to
fluorescence microscopy as described (Narayanan et al., 1999a;
Narayan et al., 1999b). For analysis of nucleocytoplasmic distribution,
RNA was extracted from the nucleus and cytoplasmic fractions from
others of the set of injected oocytes, and the radiolabeled RNAs were detected by 8% denaturing gel electrophoresis and
autoradiography.

Microscopy
Microscopy was performed using a Zeiss Axiovert S 100 inverted
fluorescence microscope equipped with differential interference
contrast optics (Thornwood, NY, USA). Images were acquired using
a cooled charge-coupled device camera (Quantix-Photometrix, AZ,
USA) and IP Lab Spectrum software (Signal Analytics, VA).

Results
Several recent studies have uncovered critical intra-nuclear functions
for the small GTPase, Ran (Azuma and Dasso, 2000; Clarke and Zhang,
2001; Dasso, 2001; Kahana and Cleveland, 1999). To test for a role of Ran in intra-nuclear trafficking of
RNAs, we utilized several complementary approaches and
systems to determine whether an intact RanGTP gradient is
required to target small nucleolar RNAs to the nucleolus.

Mammalian cells
Previous biochemical studies have suggested that a functional
Ran guanine nucleotide exchange factor, RCC1, is required for
microinjection of U3 snoRNA to the nucleolus (Cheng et al., 1995). We
examined the subcellular localization of the U3 snoRNA in
tsBN2 cells, which carry a temperature-sensitive allele of
RCC1 that results in rapid depletion of the RCC1 protein soon
after shift to the non-permissive temperature (Nishijima et al.,
2000; Ohtsubo et al., 1987). The U3 snoRNA was detected by
fluorescence in situ hybridization (FISH) with an anti-sense U3
snoRNA probe, which was previously demonstrated to
selectively detect U3 snoRNA (Michienzi et al., 2000). As
shown in Fig. 1, U3 snoRNA is localized to the nucleolus even
following a 6 hour shift to the non-permissive temperature (40°C). To verify that the cells have undergone the temperature
shift, the localization of the trimethyl guanosine cap (TMG), a
marker for the TMG-capped U snRNAs, was also examined.
As previously reported, U snRNA localization shifts from
widespread nucleoplasmic staining to less numerous foci when
the RanGTP gradient is disrupted (Cheng et al., 1995; Huang
et al., 1997). These data suggest that in contrast to previous
To address whether Ran function or the Ran gradient is required for snoRNA localization in \textit{S. cerevisiae}, we examined the targeting of snoRNAs to the nucleolus in several yeast mutants that are known to perturb the Ran gradient. As a control we also examined the localization of poly(A)+ RNA in these cells since many studies have demonstrated that export of poly(A)+ RNA from the nucleus is blocked when the Ran gradient is disrupted (Saavedra et al., 1996; Wong et al., 1997).

We first examined the localization of U3 and snR10 in \textit{rna1-1} mutant cells. The \textit{RNA1} gene encodes the yeast Ran GTPase-activating protein or RanGAP, which is required for GTP hydrolysis by Ran (Becker et al., 1995), and thus maintenance of the RanGTP gradient. The \textit{rna1-1} mutant is a well characterized temperature-sensitive allele of \textit{RNA1} (Hopper et al., 1990). The \textit{rna1-1} mutant cells were grown at the permissive temperature (25°C), the culture was split, and half the cells were shifted to the non-permissive temperature (37°C) whereas the other half was retained at 25°C. FISH was performed following a 3 hour shift to the non-permissive temperature.

Fig. 3A shows the localization of U3 snoRNA, snR10 snoRNA and poly(A)+ RNA in \textit{rna1-1} cells at the permissive and non-permissive temperatures. The localization of the snoRNAs is somewhat punctate at the non-permissive temperature, which is consistent with previous reports that the nucleolus fragments when the RanGTP gradient is disrupted in \textit{S. cerevisiae} (Saavedra et al., 1996; Wong et al., 1997). This suggests that, despite the nucleolar fragmentation, the snoRNAs are still associated with the nucleolus in the \textit{rna1-1} mutant. As expected when the Ran gradient is disrupted, there is a clear accumulation of poly(A)+ RNA within the nucleus of \textit{rna1-1} cells (Corbett et al., 1995b).

To confirm that the fragmented signal observed for the snoRNAs at the non-permissive temperature is coincident with the nucleolar fragments, we took advantage of previous work, which demonstrated that the nucleolar fragmentation phenomenon is dependent on ongoing RNA polymerase II transcription (Kadowaki et al., 1994). We combined the \textit{rna1-1} mutation with a mutation in RNA polymerase II, \textit{rhp1-1}, which rapidly shuts down poly(A)+ RNA synthesis at 37°C.
Ran-independent snoRNP trafficking was shown (Nonet et al., 1987). Previous work has shown that the nucleolus no longer fragments when mutants that disrupt the Ran gradient are combined with the rbp1-1 mutant (Kadowaki et al., 1994; Nonet et al., 1987). Fig. 3B shows the localization of U3, snR10, and poly(A)+ RNA in the rna1-1 rbp1-1 double mutant cells. In these cells, the nucleolar fragmentation is virtually eliminated and the localization of both snoRNAs to the nucleolus is clear. Consistent with a decrease in poly(A)+ RNA synthesis, very little signal is detected with the oligo dT probe. Taken together, these data strongly suggest that the RanGTP gradient in S. cerevisiae is not required for nucleolar localization of snoRNAs.

To further assess the role of Ran in snoRNA localization, we took advantage of mutations in the yeast Ran protein, Gsp1p. Two different conditional Gsp1p mutants were used for these experiments, gsp1-1 and gsp1-2 (Wong et al., 1997). Results in Fig. 4A show the localization of U3, snR10, and poly(A)+ RNA in gsp1-1 cells. As observed with the rna1-1 mutant, the snoRNAs are localized to punctate intra-nuclear sites, and poly(A)+ RNA accumulates in the nucleus at the non-permissive temperature. To once again confirm that these punctate signals represent fragmentation of the nucleolus, the gsp1-1 rbp1-1 double mutant was constructed. As shown in Fig. 4B, this eliminated the fragmentation of the snoRNA signal. Localization of U3 and snR10 snoRNAs to nucleoli was also unaffected in strains harboring a different temperature-sensitive allele of Ran (gsp1-2) (Wong et al., 1997) or containing a temperature-sensitive allele (prp20-1) (Aebi et al., 1990; Amberg et al., 1993) of the Ran guanine nucleotide exchange factor (data not shown). Taken together, these results strongly suggest that neither the Ran gradient nor the Ran protein itself is required for correct nucleolar localization of snoRNAs.

**Xenopus oocytes**

Although in both the conditional mutant mammalian and yeast systems used above, the temperature shift was clearly sufficient to alter the intracellular localization of an RNA species (U snRNA in the tsBN2 cells and poly(A)+ RNA in the yeast), it is conceivable that mislocalized snoRNAs would not be readily detected in a background of snoRNAs that had achieved correct localization prior to the temperature shift period. Furthermore, although it is possible that the pool of newly synthesized snoRNAs could be reduced in these mutants, it is known that nascent U3 snoRNA levels are not affected in tsBN2 cells during the temperature shift (Cheng et al., 1995). Moreover, our experiments with the yeast and tsBN2 cells indicate that disruption of the Ran system does not significantly affect the retention of the RNA in the nucleolus.

To directly examine the trafficking and nucleolar targeting of a specific pool of snoRNAs, we took advantage of the Xenopus oocyte system, which permits analysis of retention of newly injected snoRNAs within the nucleus as well as their nucleolar localization. To disrupt the RanGTP gradient in the Xenopus oocyte, we used a well characterized dominant-negative Ran mutant, T24N Ran (Lounsbury et al., 1996; Palacios et al., 1996). The T24N Ran is a mutant form of the Ran protein that is locked in the GDP-bound form (Carey et al., 1996; Palacios et al., 1996). The T24N Ran mutant protein also sequesters cellular RCC1 (RanGEF) (Lounsbury et al., 1996). Thus, when T24N Ran is injected into the nucleus, RCC1 becomes unavailable to replenish RanGTP in the nucleus and overall, nuclear RanGTP levels decrease and RanGDP levels increase (Lounsbury et al., 1996).

For this experiment, recombinant T24N Ran protein was microinjected into Xenopus oocyte nuclei and was followed an hour later by injection of fluorescently and 32P-labeled U3 Box C/D snoRNA (Fig. 5A) or U65 Box H/ACA snoRNA (Fig. 5C).

**Fig. 2.** U3 and the snR10 (Box C/D and Box H/ACA snoRNAs, respectively) are readily detected in the yeast nucleolus by fluorescence in situ hybridization (FISH). (A) U3 and snR10 localize to crescent-shaped nucleoli of S. cerevisiae as revealed by hybridization with Cy3-labeled, antisense probes against U3 or snR11 (RNA panel). The merged image shows that the snoRNA signals (detected by FISH) are directly adjacent to the nucleoplasmic DNA detected by DAPI staining of the same cells. (B) Colocalization of snoRNAs with the nucleolar marker protein, Nop1 (fibrillarin). A combination of FISH (to detect U3 snoRNA) and immunofluorescence (to detect Nop1 protein) was performed. The merge image indicates an overlay of U3 snoRNA and Nop1 protein. DIC, differential interference contrast. Arrowheads point to a nucleolus.
RNAs. As a control to confirm that nuclear RanGTP levels were indeed disrupted and U snRNA export was blocked as previously reported (Izaurralde et al., 1997), 32P-labeled U1sm snRNA was co-injected with the snoRNAs. When T24N Ran was pre-injected into the nucleus, export of co-injected U1sm snRNA from the nucleus was completely blocked, similar to results reported earlier (Izaurralde et al., 1997). As expected, export of U1sm was unaffected in control (mock-injected) oocytes (Fig. 5B,D, compare lane 3 with 6). Under nuclear RanGTP-depleted conditions, fluorescently labeled U3 and U65 snoRNAs were transported and localized to nucleoli (Fig. 5A,C, panels U3+T24N and U65+T24N). The nucleolar structure appeared normal when nuclear RanGTP levels were disrupted and was comparable to the morphology of the nucleoli in cells that were not pre-injected with T24N Ran. In addition, the stability and nuclear retention of U3 and U65 remained unaffected by injection of T24N (Fig. 5B.D, compare lanes 2 and 5). Taken together, these results suggest that in Xenopus oocytes RanGTP is not required to retain Box C/D and Box H/ACA snoRNAs in the nucleus or to localize the snoRNAs to nucleoli.

Discussion

Whether destined for export to the cytoplasm or for function within the nucleus, all cellular RNAs transcribed from nuclear genes undergo intra-nuclear transport. The mechanism(s) by which RNAs travel within the nucleus and achieve proper localization to nuclear pore complexes or sites of intra-nuclear function are not well defined. The Ran cycle has been shown to be important for transport of RNAs between the nucleus and the cytoplasm (Dahlberg and Lund, 1998), and it has been proposed that Ran regulates the intra-nuclear transport of RNA as well (Cheng et al., 1995). The prevailing model for Ran function in RNA transport is based on the ability of Ran to couple and uncouple cargo RNPs to the soluble transport receptor (or adaptors) in a compartment-specific fashion (Görlich and Kutay, 1999). Given that Ran appears to use this basic mechanism to function directly in a number of diverse nuclear functions, it was reasonable to consider that Ran might regulate targeting of RNAs within the intra-nuclear environment. In this study, we have demonstrated that the nuclear retention and nucleolar localization of snoRNAs can occur independently of Ran function in phylogenetic diverse...
systems (mammalian cells, yeast and *Xenopus* oocytes). These results suggest that Ran may not be generally required for intranuclear transport of macromolecules.

A previous study indicated that Ran GTP was required for the nuclear retention and perhaps nucleolar localization of U3 snoRNA in tsBN2 cells (Cheng et al., 1995). Cheng et al. reported a significant portion of newly synthesized U3 snoRNA in a cytoplasmic fraction of tsBN2 cells at the non-permissive temperature, whereas essentially all U3 was observed in the nuclear fraction of cells cultured at the permissive temperature. The model proposed by Cheng et al. to explain the appearance of U3 snoRNA in the cytoplasmic fraction of tsBN2 cells at the non-permissive temperature is that loss of RCC1 (and nuclear RanGTP) prevents the nucleolar localization of U3 and results in mislocalization of U3 to the cytoplasm (Cheng et al., 1995). We have examined the subcellular localization of U3 snoRNA by fluorescence in situ hybridization in intact tsBN2 cells. Our studies did not reveal the presence of U3 snoRNA in the cytoplasm (or a noticeable decrease in U3 snoRNA in nucleoli) within the interphase tsBN2 cells (or *S. cerevisiae*) examined at the non-permissive temperature (Figs 1, 3 and 4). This suggests that retention of snoRNAs within nuclei and nucleoli does not depend on nuclear RanGTP. Although we cannot strictly rule out the possibility that the population of U3 snoRNA that was synthesized during the temperature shift was mislocalized and not localized by FISH, our results in *Xenopus* oocytes argue that RanGTP is not required for the nuclear retention or nucleolar localization of a population of newly injected Box C/D or Box H/ACA snoRNAs. The results of Cheng et al. could be explained if depletion of RanGTP affects nuclear structure in a manner that leads to alteration of biochemical fractionation properties of U3 snoRNA. In addition, U3 might appear in a ‘cytoplasmic’ fraction derived from a population of cells that includes increased numbers of mitotic cells resulting from RCC1 depletion in the tsBN2 cells (Nishimoto et al., 1978).

In *S. cerevisiae*, we also observed proper nucleolar localization of snoRNAs following RanGEF depletion in (data not shown). Indeed, the nucleolar localization of snoRNAs went unperturbed in yeast strains carrying a conditional allele

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**Fig. 4.** snoRNAs localize to nucleoli in Ran mutant strains. The subcellular distribution of U3 and snR10 snoRNAs and poly(A)+ RNA were determined by fluorescence in situ hybridization using Cy3-labeled U3, snR10 and oligo d(T) probes as indicated. FISH analysis was performed in a strain containing a temperature-sensitive mutation in Ran (gsp1-1) or a double mutant (gsp1-1 rbp1-1) containing also a temperature-sensitive mutation in the large subunit of RNA polymerase II required for production of poly(A)+ mRNA. Cells were analyzed both at 25°C (permissive temperature) and after a shift to the non-permissive temperature of 37°C for three hours. The merged image shows the position of each RNA relative to the DAPI-stained nucleoplasmic signal. U3 and snR10 nucleolar signals are observed in both strains at both permissive and non-permissive temperatures. As expected, export of poly(A)+ RNA to the cytoplasm is blocked at the non-permissive temperature.
of another key component of the Ran cycle, RanGAP (Fig. 2) as well as Ran itself (Fig. 3). These findings also strongly argue against an essential role for Ran in the nucleolar localization of snoRNAs.

Although the experiments that we have performed in the mammalian and yeast cells show that an intact Ran gradient is not required for the steady-state nucleolar localization of snoRNAs, the *Xenopus* oocyte microinjection experiments extended these observations by enabling the characterization of a single population of snoRNA molecules. Importantly, our findings in the oocyte system show that snoRNA targeting to the nucleolus per se proceeds even when RanGTP is depleted. Both the nuclear retention and nucleolar targeting of snoRNAs remain unaffected under conditions that fully block the export of co-injected control U1 snRNA (Fig. 4). Though it is possible that low levels of nuclear RanGTP are sufficient to localize snoRNAs to nucleoli, our control experiments [reorganization of TMG capped U snRNA (Fig. 1), block of mRNA export in yeast cells (Figs 2 and 3) and block of snoRNA export in *Xenopus* oocytes (Fig. 4)] indicate that severe or total disruption of the RanGTP cycle was achieved.

In the case of nuclear export of most cellular RNAs, nuclear RanGTP has been shown to be essential for the assembly of an export complex that subsequently translocates through nuclear pore complexes at the nuclear envelope (Dahlberg and Lund, 1998). Our data suggest that Ran binding is not involved in the assembly and nucleolar targeting of snoRNPs (complexes of snoRNAs and snoRNA binding proteins). The nucleolus is not a membrane-bound structure and there is no known physical barrier for entry into this organelle. Transport of the assembled snoRNPs to nucleoli may occur by simple diffusion or be facilitated by extrinsic factors other than Ran. Conceivably, appropriate snoRNP assembly may be the most crucial requirement for the transport and localization of snoRNPs to the nucleolus. Interaction of specific components of the snoRNPs with components of the nucleolus may serve to trap and retain the snoRNPs within the nucleolus, the functional destination of most snoRNPs.

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Fig. 5. Microinjected U3 and U65 snoRNAs are retained within the nucleus and localized to nucleoli of *Xenopus* oocytes after injection of T24N Ran. (A,C) Recombinant RanT24N in microinjection buffer (bottom panels) or microinjection buffer alone (top panels) were injected into separate sets of oocytes. One hour later, one fmole of fluorescently and 32P-labeled U3 or U65 snoRNA was injected into the same set of oocytes. Nuclear spreads were made four hours after injection of the RNA, and slides were analyzed by fluorescence microscopy. Several individual nucleoli are shown in each differential interference contrast (DIC) panel. The fluorescence signals (RNA) show that the injected snoRNAs are targeted to a centrally located subregion of the nucleoli in oocytes, which were injected with the T24N Ran (+T24N). (B,D) Nuclear (N) and cytoplasmic (C) fractions were obtained from the same set of injected cells. RNA was extracted from the N and C fractions and subjected to denaturing PAGE followed by autoradiography. The marker lane (M) indicate samples prior to injection.
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