RNA: Structure, Metabolism, and Catalysis:

Silencing of RNA Helicase II/Guα Inhibits Mammalian Ribosomal RNA Production

Dale Henning, Rolando B. So, Runyan Jin,
Lester F. Lau and Benigno C. Valdez

J. Biol. Chem. 2003, 278:52307-52314.
doi: 10.1074/jbc.M310846200 originally published online October 13, 2003

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 52, Issue of December 26, pp. 52307–52314, 2003
Printed in U.S.A.

Silencing of RNA Helicase II/Guα Inhibits Mammalian Ribosomal RNA Production*

Received for publication, October 1, 2003, and in revised form, October 13, 2003
Published, JBC Papers in Press, October 13, 2003, DOI 10.1074/jbc.M310846200

Dale Henning, Rolando B. So, Runyan Jin, Lester F. Lau‡, and Benigno C. Valdez§

From the Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030 and the ‡Department of Molecular Genetics, University of Illinois College of Medicine, Chicago, Illinois 60607

The intricate production of ribosomal RNA is well defined in yeast, but its complexity in higher organisms is barely understood. We recently showed that down-regulation of nucleolar protein RNA helicase II/Guα (RH-II/Guα or DDX21) in *Xenopus* oocytes inhibited processing of 20 S rRNA to 18 S and contributed to degradation of 28 S rRNA (Yang, H., Zhou, J., Ochs, R. L., Henning, D., Jin, R., and Valdez, B. C. (2003) *J. Biol. Chem.* 278, 38847–38859). Since no nucleolar RNA helicase has been functionally characterized in mammalian cells, we used short interfering RNA to search for functions for RH-II/Guα and its paralogue RH-II/Guβ in rRNA production. Silencing of RH-II/Guα by more than 80% in HeLa cells resulted in an almost 80% inhibition of 18 and 28 S rRNA production. This inhibition could be reversed by exogenous expression of wild type RH-II/Guα. A helicase-deficient mutant form having ATPase activity was able to rescue the production of 28 S but not 18 S rRNA. A phenotype exhibiting inhibition of 18 S and 28 S rRNA production was also observed when the paralogue RH-II/Guβ was overexpressed. Both down-regulation of RH-II/Guα and overexpression of RH-II/Guβ slowed cell proliferation. The opposite effects of the two paralogues suggest antagonistic functions.

The use of short interfering RNA (siRNA)[1] has revolutionized the way in which the functions and interactions of multiple genes and their encoded proteins are deciphered. Short double-stranded RNA mediates the recognition and degradation of its homologous RNA, leading to the down-regulation of expression of the target gene; the mechanism involves a multiprotein complex (1–3). The application of siRNA facilitates the study of complex cellular processes including the biogenesis of ribosomal RNA (rRNA).

Synthesis and processing of pre-rRNA, maturation of the final products, and formation of ribosomes occur in the nucleolus. The identification of ~350 nucleolar proteins (4, 5) and the use of siRNA to silence each encoding gene provide the genetic and biochemical screenings that are necessary to elucidate the mechanism of rRNA production in mammalian cells, a process that is more defined in yeast (6–8). A number of nucleolar proteins have been functionally associated with mammalian rRNA production mostly based on *in vitro* experiments and indirect *in vivo* observations (9–13). More direct lines of evidence are limited to Bop1 (14), $p19^{arf}$ (15), and the *DKC1* gene product dyskerin (16, 17). Although our laboratory recently showed that antisense-mediated down-regulation of RNA helicase II/Guα in *Xenopus laevis* oocytes resulted in inhibition of production of 18 S rRNA and degradation of 28 S rRNA (18), we have not reported that this enzyme has a similar function in mammalian cells.

RNA helicase II/Guα (RH-II/Guα or DDX21) is a DEAD box nucleolar protein that we previously cloned and characterized (19, 20). The cDNA-derived amino acid sequence shows RNA helicase motifs at the middle region, which is responsible for its 5′ to 3′ RNA unwinding activity, and a functionally distinct domain at its C terminus that is able to introduce secondary structure to single-stranded RNA (21). Its paralogue, RNA helicase II/Guβ, has a marginal RNA helicase activity *in vitro*, and it does not possess an RNA folding activity (22). The high homology in the structures of the two paralogues and close proximity of their genes on human chromosome 10 suggest evolution by gene duplication (23). Their nucleolar localization suggests a functional relationship with the production of rRNA.

To gain more information about the roles of RH-II/Guα and RH-II/Guβ in rRNA biosynthesis, we employed the use of siRNA and overexpression of their wild type or negative mutant forms. Down-regulation of RH-II/Guα slowed the production of 18 and 28 S rRNA. These effects were reversed when wild type RH-II/Guα was overexpressed, whereas inducible expression of a helicase-deficient mutant reversed the inhibitory effects on 28 S rRNA but not on 18 S rRNA production. siRNA-mediated silencing of RH-II/Guβ did not inhibit rRNA biosynthesis, but overexpression of its wild type form inhibited rRNA production. In addition to affecting rRNA production, silencing of RH-II/Guα or overexpression of RH-II/Guβ resulted in inhibition of cell proliferation. The results suggest antagonistic roles of the two paralogues in rRNA production and cell proliferation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulfate. LAP3 cells are genetically modified NIH3T3 cells that are capable of expressing exogenous genes upon IPTG induction as previously described (24). These cells were grown in similar medium containing 10% newborn calf serum.

Cells were transfected with synthetic siRNA oligoribonucleotides (Dharmacon Research, Inc.) using LipofectAMINE 2000 (Invitrogen). The siRNAs used are listed in Table I.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNAs were isolated from monolayer cells using TRIzol reagent (Invitrogen). RT-PCR was done using the OneStep RT-PCR kit (Qiagen). A 20-μl reaction mixture contained 1× buffer, 0.4 mM dNTPs, 50 ng of

* This work was supported by Public Health Service Grants DK52341 from NIDDK, National Institutes of Health (NIH) (to B. C. V.) and CA95627 from NCI, NIH (to L. F. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Pharmacology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-7908; Fax: 713-798-3145; E-mail: bvaldez@bcm.tmc.edu.

[1] The abbreviations used are: siRNA, short interfering RNA; IPTG, isopropyl-β-D-thiogalactopyranoside; RH-II/Gu, RNA helicase II/Gu protein; rRNA, ribosomal RNA; RT, reverse transcription; nt, nucleotides.

TABLE I
siRNAs used in cell transfection

ID	Sequence	Target
si-934	GGGAAAUCACCCUGAAAGGdTdT dTdTCCCUUUAGUGGGACUUUCC	Human and mouse Guα
si-934Scr	GUAACAAUGAGAGCACGGCdTdT dTdTCAUUGUUACUCUCGUGCCG	Scrambled si-934 sequence
si-935	GAAUGGACUCUCCCAACCUdTdT dTdTCUUACCUGAGAGGGUUGGA	Mouse Guα
si-936	CACCAGGUCGUAUCAAAGAdTdT dTdTGUGGUCCAGCAUAGUUUCU	Human Guα

RNA, 0.8 μl of OneStep RT-PCR enzyme mix, 1.5 nM ^{32}P-end-labeled primer 1, and 1.5 nM primer 2. Primers for internal control were also added in the reaction. β-Actin primers were initially used as internal control, but multiple bands were obtained. Better results were obtained using primers for U1 small nuclear RNP-specific C protein mRNA (U1RNPC; GenBank™ accession number X12517). PTC-100 Programmable Thermal Controller (MJ Research, Inc.) was used to do reverse transcription at 50 °C for 30 min followed by 95 °C heating for 15 min to denature reverse transcriptase and activate HotStarTaq DNA polymerase. The succeeding 20 cycles of PCR included denaturation at 94 °C for 0.5 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. A final extension reaction was done at 72 °C for 10 min.

The RT-PCR product was mixed with 2 μl of Bluejuice (Invitrogen), and 6 μl of the mixture was analyzed using 5% polyacrylamide-SDS gel. After electrophoresis at 100 V, the gel was vacuum-dried and exposed to a PhosphorImager screen. All radioactive signals were measured using the Amersham Biosciences Storm 860 PhosphorImager. The primers used for RT-PCR are shown in Table II.

Western Blot—Cells were lysed in Laemmli buffer and boiled for 3 min. Protein extracts were loaded onto 10% polyacrylamide-SDS gels and blotted onto Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). Immunoblot analysis by chemiluminescence was done using the ECL plus Western blotting Detection System (Amersham Biosciences). The characterizations of anti-RH-II/Guα and MS3 anti-C23 antibodies were previously described (20, 25). Anti-B23 monoclonal antibody was obtained from Dr. P. K. Chan (Baylor College of Medicine). Anti-actin (C-11) and anti-FLAG (M2) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Sigma, respectively. After analysis of each membrane with anti-RH-II/Guα, it was stripped and probed with another antibody directed against C23, B23, or actin.

Metabolic Labeling of Cells—Cells were transfected with 10 or 50 nM siRNA for 48 h as described above. Cells were then incubated in phosphate-free medium (Sigma) for 3.5 h. It was replaced with fresh phosphate-free medium containing 40 μCi/ml [^{32}P]orthophosphate (Amersham Biosciences). Incubation at 37 °C with 5% CO_2 was continued for 1.5 h. Cells were then washed twice with a growth medium. The chase experiment was done by incubating the labeled cells in a growth medium containing 500 mM Na_2HPO_4 at 0, 2, and 4 h. Total RNA was isolated using TRIzol Reagent (Invitrogen), and the RNA concentration was determined at 260 nm. Equal weight of RNA was resolved on a formaldehyde-containing 1% agarose gel and blotted onto a Hybond-N nylon membrane (Amersham Biosciences). The membrane was dried and placed in a PhosphorImager cassette for ~4 h or overnight. After analysis, the RNA blot was stained with 0.2% methylene blue to visually check equal loading.

Preparation of LAP3 Stable Clones—All human and mouse RH-II/Gu cDNAs (20, 23, 26) were subcloned into the NheI and BspEI sites of pX12 vector. Both wild type and mutant forms (21) were amplified by PCR using the primers listed in Table III.

Oligonucleotides BV596 and BV597 were used to amplify human RH-II/Guα, and BV598 and BV599 were used for its mouse orthologue. The mouse RH-II/Guβ cDNA was amplified using BV777 (5′-CCACCGCAGGCTAGCATGGACTACAAAGACGATGACGACAAGGGGAAACTCCTCTGGGGAGACATA-3′) and BV778 (5′-AGATTAACTATCTCCGGATCAGTCAAAATTCCGTTTATGGCC-3′). BV596, BV598, and BV777 encode a FLAG epitope. Transfection, selection, and IPTG induction of LAP3 cells were as previously described (27).

Proliferation Assay—HeLa cells, which were transfected two times (24-h interval) with 10 nM siRNA for a total of 48 h, were trypsinized and counted. Equal cell numbers were replated into new 6-well plates for 72 h. Each well contained 50,000 or 100,000 cells after replating. Cells were again trypsinized, and all samples were diluted with equal volumes of medium for replating into 96-well plates, such that sample wells would have a total volume of 100 μl and 10,000 or fewer cells each. A standard curve was generated by serial dilution of untreated HeLa cells, wherein the maximum was 20,000 cells per well and the minimum was 625. Plated cells were allowed to recover and attach for 4 h at 37 °C and 5% CO_2. The CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega) was used to assay for living cells. After an incubation time of 30 min to 1 h at 37 °C, the plates were read on an enzyme-linked immunosorbent assay reader at 490 nm.

RESULTS

siRNA-mediated Down-regulation of RH-II/Guα—This study involved the use of both human and mouse cell lines. We designed siRNA to target both human and mouse RH-II/Guα as well as siRNAs that were species-specific. Down-regulation was examined at the mRNA and protein levels. Preliminary experiments indicated that 20 cycles of RT-PCR correlated linearly with the levels of mRNAs, and the use of U1RNPC as a loading control was better than β-actin.

Treatment of HeLa and LAP3 cells with 0–100 nM si-934 resulted in a decrease in the level of RH-II/Guα mRNA in both cell lines in a dose-dependent manner. When 10 nM si-934 was used, the human homologue decreased by 75%, whereas the mouse homologue decreased by 55% (Fig. 1, *A* and *B*). An siRNA that specifically targets mouse RH-II/Guα, si-935, silenced RH-II/Guα by 75% (Fig. 1*B*) without affecting the human orthologue (Fig. 1*A*). si-936, which was specific to human RH-II/Guα, decreased RH-II/Guα mRNA in HeLa cells by 45% with a minimal effect on the level of mRNA of the mouse enzyme. All three siRNAs did not significantly affect the level of RH-II/Guβ in HeLa and LAP3 cells (Fig. 1, *A* and *B*).

A significant decrease in the protein level of mouse RH-II/Guα was observed using 100 nM si-934 and 100 nM si-935, which resulted in 76 and 89% silencing, respectively (Fig. 1*C*). This decrease in the protein level correlates with the decrease in the mRNA level (Fig. 1*B*). Because of the efficacies of si-934 and si-935, we decided to use these siRNAs in the succeeding experiments.

To determine the kinetics of down-regulation of the human RH-II/Guα at the mRNA and protein levels, samples at different time points were analyzed by RT-PCR and Western blotting. Fig. 2 shows a 50% decrease in the mRNA level within 4 h after transfection without any effect on the protein level. The mRNA level continued to decrease to 10% until 72 h, and a significant decrease in the protein level was not observed until 48 h after transfection (Fig. 2*B*). The delayed effect of si-934 on the protein level of RH-II/Guα is attributed to a longer half-life of the protein. Determination of the time when the protein level decreased was essential in the study of its physiological function as detailed below.

Down-regulation of RH-II/Guα Inhibits Production of rRNA—We hypothesized that down-regulation of RH-II/Guα would result in an aberrant processing of rRNA. After 48 h of siRNA transfection, cells were pulse-labeled with [^{32}P]orthophosphate and then chased with unlabeled phosphate-enriched medium. As a negative control, an siRNA with scrambled si-934 sequence was included (si-934Scr). RT-PCR analysis of total RNAs taken 4 h after the chase began showed a 92%

TABLE II
Primers used for RT-PCR

ID	Sequence	Position
BV993	5′-CAAACTAGATCTCACCAAACTTAA-3′	Human Guα nt 1031–1054 (sense)
BV994	5′-CAGATGCTCCACAGTTATTGCCGT-3′	Human Guα nt 1272–1295 (antisense)
BV998	5′-TCAGATGAATTCTCCAAATCTCATAAG-3′	Human Guβ nt 328–354 (sense)
BV999	5′-TTTCTTTGGAGTCTTTCAATTAAGGGG-3′	Human Guβ nt 663–689 (antisense)
BV459	5′-GAAAAAGGAATTCTTTGAGAAGCAAGCAGACTCTG-3′	Mouse Guα nt 333–367 (sense)
BV985	5′-TCCCTCTTTCTGCTCCACAGGTATTTC-3′	Mouse Guα nt 793–819 (antisense)
BV986	5′-TCAGATGAATTCTCCCCATCTCATAAG-3′	Mouse Guβ nt 329–355 (sense)
BV987	5′-TCCCTTGTTGGAGCCAAAACGAGTACC-3′	Mouse Guβ nt 718–744 (antisense)
U1C5′	5′-GCAACATGCCCAAGTTTTATTGTG-3′	Human U1RNPC nt 11–34 (sense)
U1C3′	5′-TATCCTTATCTGTCTGGTCGAGTC-3′	Human U1RNPC nt 477–500 (antisense)
BV974	5′-AGCATCATGCCCAAGTTTTATTGTGA-3′	Mouse U1RNPC nt 31–56 (sense)
BV976	5′-TTTCTCCCTCCAAAAATATTCAGTTA-3′	Mouse U1RNPC nt 625–650 (antisense)

TABLE III
Primers used for PCR of wild type and mutant RH-II/Guα

ID	Sequence
BV596	5′-AGGGACCAAGCTAGCATGGACTACAAAGACGATGACGACAAGGCGGTTGAGAAGACCGGTCGGCCT-3′
BV597	5′-ATCTTCTATTCCGGATTATTGACCAAATGCTTTACTGAAACT-3′
BV598	5′-AGGGACCAAGCTAGCATGGACTACAAAGACGATGACGACAAGGAAGAGGCCGTTTCCTCCAAAGCA-3′
BV599	5′-CCTTCTGGCTCCGGATCACTGACCAAACGCTTTACTAAAACT-3′

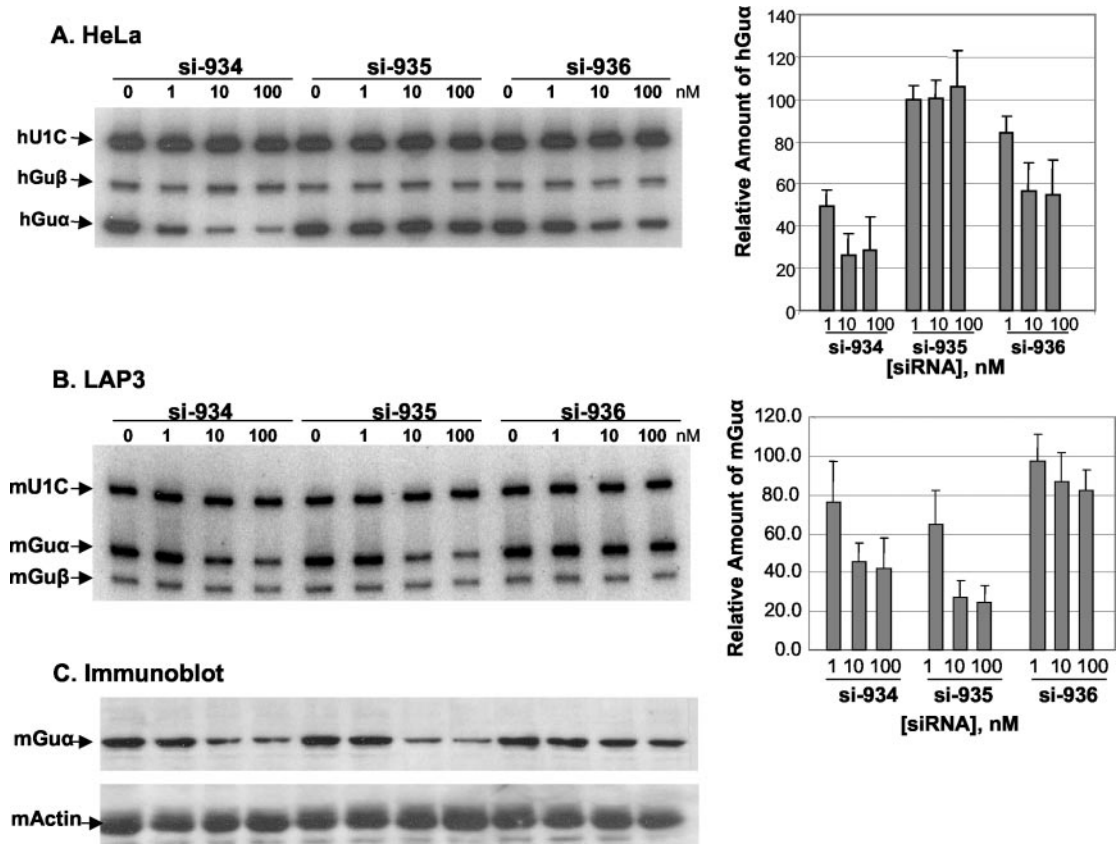

FIG. 1. **Specificity of the siRNA-mediated down-regulation of RH-II/Guα mRNA.** Human HeLa (*A*) and mouse LAP3 (*B*) cells were transfected with different concentrations of siRNAs (0–100 nM). Total RNA was isolated after 48 h and analyzed by RT-PCR. Each reaction contained three pairs of primers to reverse transcribe mRNA and amplify cDNA fragments for U1 small nuclear RNP-specific C protein (*U1C*), RH-II/Guα (*Guα*), and RH-II/Guβ (*Guβ*). One primer in each pair was ^{32}P-end-labeled. BV993, BV994, BV998, BV999, U1C5′, and U1C3′ were used for HeLa RNA (*A*); BV459, BV985, BV986, BV987, BV974, and BV976 were used for LAP3 RNA (*B*). RT-PCR products were resolved on 5% polyacrylamide gels. A quantitative analysis of four independent experiments is shown on the *right*. *C*, a parallel experiment using LAP3 cells was conducted to analyze changes in the protein level of mouse RH-II/Guα after siRNA treatment. *h*, human; *m*, mouse.

decrease in RH-II/Guα in HeLa cells treated with si-934. Mock transfection and si-934Scr did not affect the level of RH-II/Guα mRNA (Fig. 3*A*).

Both mock-transfected and si-934Scr-treated cells showed normal progression in the processing of pre-rRNA within 4 h of chase (Fig. 3*B*). The low ^{32}P activity at 0 h of chase would indicate that little labeled phosphate taken up by the cells had been incorporated into rRNA. Within 2 h, the ^{32}P labeling of RNA increased, and the positions of 47 S/45 S pre-rRNA and 32 S, 28 S, and 18 S rRNA were visualized. Treatment of cells with si-934, which caused a dramatic decrease in RH-II/Guα mRNA (Fig. 3*A*), resulted in an inhibition of rRNA production (Fig. 3*B*). The decrease in the largest visible precursor, 47 S/45 S, was not as marked as the decrease in the final products, 28 and

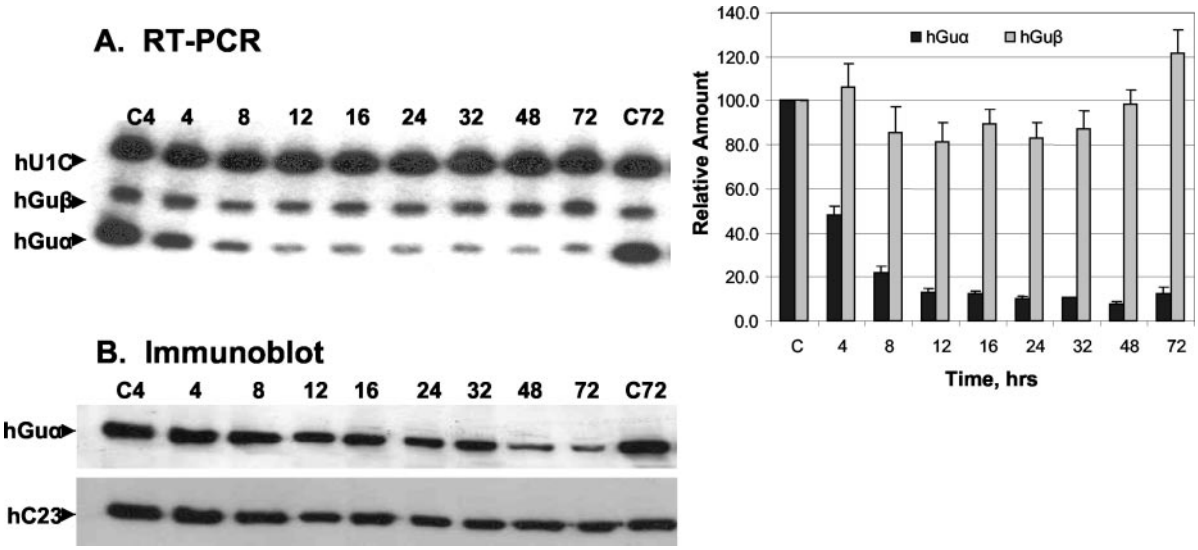

FIG. 2. **Kinetics of changes in the mRNA and protein levels.** HeLa cells were transfected with 10 nM si-934, and total RNA (*A*) and proteins (*B*) were extracted after the indicated time period (4–72 h). *C4* and *C72* refer to mock-transfected samples obtained after 4 and 72 h of transfection. The *graph* shows a quantitative analysis of the RT-PCR products obtained from three independent experiments. Other conditions are similar to those in Fig. 1 except for using nucleolin/C23 as a loading control for immunoblot analysis (*B*).

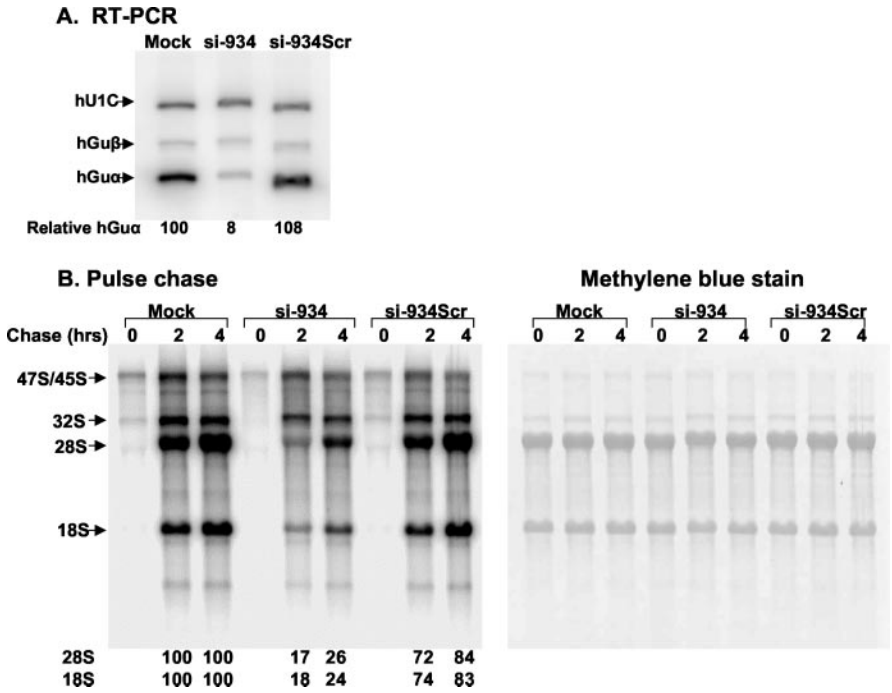

FIG. 3. **Effects of siRNA treatment on rRNA production.** HeLa cells were transfected with 10 nM siRNA for 48 h. Cells were pulse-labeled with [^{32}P]orthophosphate and chased with unlabeled phosphate for 0, 2, or 4 h. *A*, total RNA from the 4-h chase was analyzed by RT-PCR. *B*, ^{32}P-labeled cellular RNAs were resolved on 1% agarose-formaldehyde gel and blotted onto a membrane. After exposure on a phosphorimaging screen, the membrane was stained with 0.2% methylene blue. The levels of 28 and 18 S rRNAs relative to the mock-treated samples are shown at the *bottom*. Results are average of four independent experiments.

18 S rRNA. We sometimes observed an accumulation of the 47 S/45 S rRNA. This inconsistency could be due to inefficient transfer from gel to the membrane. Compared with mock, the use of si-934 resulted in an 83 and 82% decrease in the level of 28 and 18 S rRNA, respectively, after a 2-h chase (Fig. 3*B*). The level of 32 S rRNA decreased to a lesser extent. For example, after a 2-h chase, the level of 32 S decreased by 45% (Fig. 3*B*, *lane 5*). After a 4-h chase, the level decreased by 28%, whereas 28 and 18 S rRNA decreased by 74 and 76%, respectively. The results suggest that the production of 28 and 18 S rRNA was affected by the down-regulation of RH-II/Guα compared with the production of 32 S rRNA. The observed inhibition of rRNA production mediated by down-regulation of RH-II/Guα is similar to the effects caused by inactivation of the *DKC1* gene that encodes dyskerin, a putative pseudouridine synthase that is relevant in rRNA modification (17).

To prove equal loading of RNA, the membrane was stained with methylene blue. Only the final products, 28 and 18 S rRNA, which were present in greater proportion, were stained strongly (Fig. 3*B*, *right panel*).

Wild Type RH-II/Guα but Not Its Helicase Mutant Form Rescues Inhibition of rRNA Production—RH-II/Guα is one of only a few RNA helicases that has RNA unwinding activity *in vitro*. This group includes, but is not limited to, p68 and p72 (28), RNA helicase A/NDH II (29, 30), eIF-4A (31), Prp22 (32), DbpA (33), NPH II (34), NS3 (35), and SV40 large T antigen (36). To determine whether or not the helicase activity of RH-II/Guα was critical for the production of rRNA, we constructed stable LAP3 clones (mouse cells) that expressed wild type and mutant forms of human RH-II/Guα upon IPTG induction. We previously showed that the RH-II/Guα SAT-M1 mutant, wherein the SAT motif was mutated to LET, did not exhibit RNA unwinding activity *in vitro* despite the presence of ATPase activity (21). Cells were first induced with IPTG for

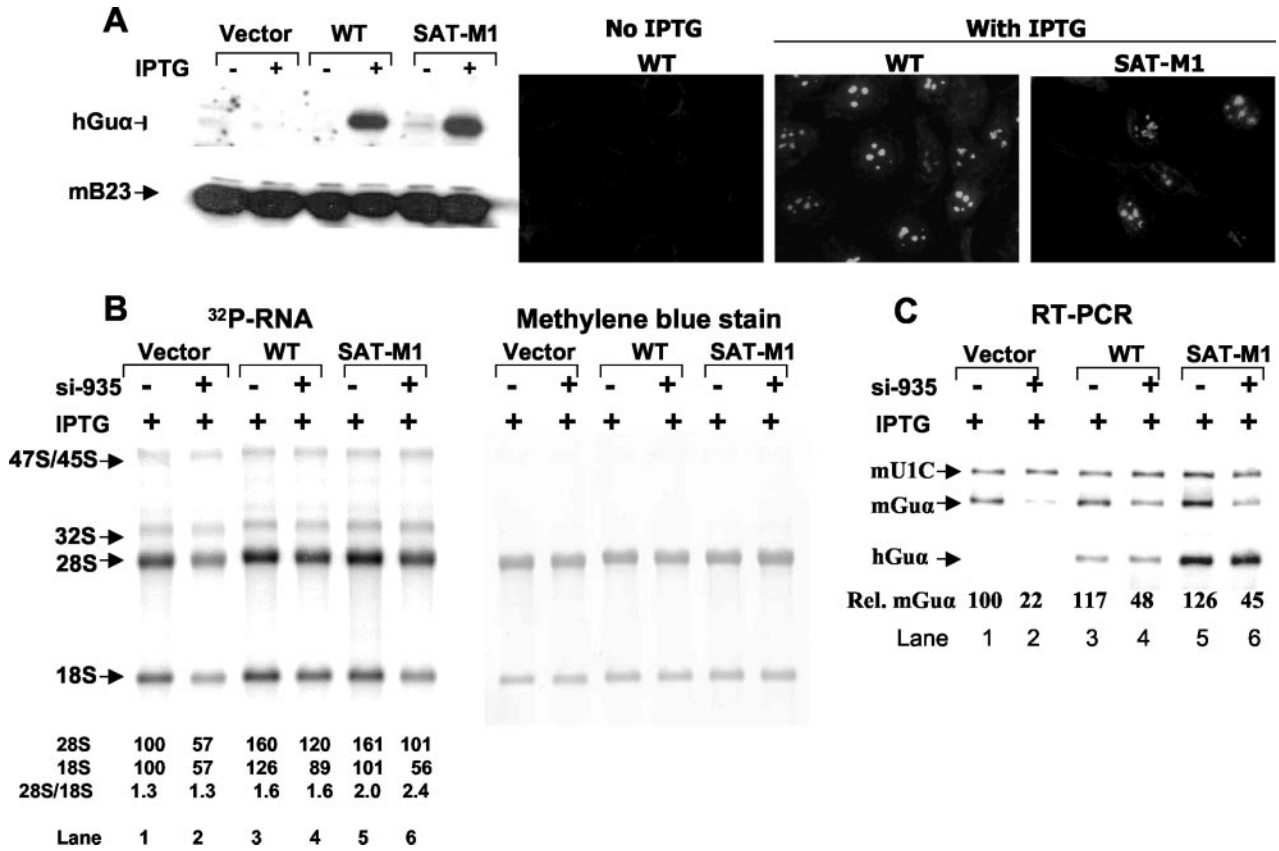

FIG. 4. **Reversal of inhibition of rRNA production.** Stable LAP3 clones were induced with 2 mM IPTG for 48 h to express the exogenous genes. *A*, Western blot analysis using anti-FLAG antibody shows expression of the epitope-tagged human wild type RH-II/Guα (*WT*) and its mutant form (*SAT-M1*) in the presence of IPTG. Anti-B23 antibody was used to compare protein loading. Immunofluorescent staining using anti-FLAG antibody shows nucleolar localization of the exogenously expressed wild type RH-II/Guα. *B*, IPTG-induced cells were treated with 50 nM si-935 for 48 h, and total ^{32}P-labeled RNA was analyzed as described in the legend to Fig. 3. All cells were exposed to 2 mM IPTG throughout the experiment. The *numbers below* show the levels of 28 and 18 S rRNA relative to mock-transfected vector clone (*lane 1*). *C*, the same RNA samples were used for RT-PCR to determine the levels of mouse (*mGuα*) and human (*hGuα*) relative to mouse U1C (*mU1C*) internal control. The levels of mouse Guα relative to mock-transfected vector clone (*first lane*) are shown *below*. Relative levels in *B* and *C* were obtained from three independent experiments.

48 h prior to treatment with si-935, a mouse RH-II/Guα-specific siRNA. Fig. 4*A* shows expression and nucleolar localization of the human RH-II/Guα wild type and SAT mutant upon IPTG induction.

Our purpose was to knock down the endogenous mouse RH-II/Guα while exogenously expressing the human orthologue and look for reversal of inhibition of rRNA production. We already showed in Fig. 1 that si-935 inhibits expression of mouse RH-II/Guα by 75% without a significant effect on human RH-II/Guα. Fig. 4*B* shows that treatment of a vector clone with si-935 resulted in 43% decrease in 28 and 18 S rRNA after a 4-h chase. This effect was reversed by exogenous expression of human wild type RH-II/Guα, suggesting that the human orthologue can functionally replace the mouse enzyme (Fig. 4*B*, *lanes 2* and *4*). To our surprise expression of the human RH-II/Guα SAT-M1 mutant rescued the inhibitory effects on 28 S rRNA but not on 18 S rRNA (compare *lanes 2* and *6* in Fig. 4*B*). This result suggests that the unwinding activity of RH-II/Guα is critical for biosynthesis of 18 S rRNA. Other mechanisms might play a role in the involvement of RH-II/Guα in the production of 28 S rRNA.

One can argue that the differences we observed might be due to unequal RNA loading on the gels, although methylene blue staining shows otherwise (Fig. 4*B*, *right panel*). To circumvent this argument, we compared the ratios of 28 to 18 S rRNA. Whereas these ratios are equal to 1.3 and 1.6 in the control and wild type human RH-II/Guα clones, the SAT-M1 mutant ratios were 2.0 and 2.4 in the absence or presence of siRNA, respectively, suggesting a decrease in 18 S rRNA in the mutant clone relative to 28 S rRNA.

The same RNA samples were analyzed for the levels of mouse and human RH-II/Guα by RT-PCR (Fig. 4*C*). Treatment of the vector clone with si-935 resulted in 78% decrease in mouse RH-II/Guα mRNA (*lane 2*). However, the efficacy of si-935 decreased when human RH-II/Guα (wild type or SAT mutant) was ectopically expressed (compare mouse Guα (*mGuα*) in *lanes 2*, *4*, and *6* in Fig. 4*C*). It is possible that human and mouse RH-II/Guα mRNAs competed for si-935 in a mouse cell line, but Fig. 1*A* shows that si-935 does not have an effect on human RH-II/Guα mRNA in HeLa cells. It is more likely that the ectopically expressed human RH-II/Guα stimulated the expression of the endogenous mouse RH-II/Guα as shown in *lanes 3* and *5* (Fig. 4*C*). Apparently, the helicase activity of human RH-II/Guα is not required for this stimulation, since both wild type (*WT*) and mutant (*SAT-M1*) RH-II/Guα have similar effects.

Down-regulation of RH-II/Guα Blocks Cell Proliferation—After treatment of HeLa cells with si-934, we observed a slowed proliferation rate. We decided to examine the growth rate of the surviving cells. Fig. 5 shows a 55% reduction in relative growth rate of cells treated with si-934, whereas cells treated with si-934Scr showed only 10% reduction relative to the mock-transfected cells. Statistical analyses using Student's t test showed a significant difference ($p < 0.001$) between si-934 treatment and mock or si-934Scr transfection. Examination of the mRNA and protein levels of RH-II/Guα shows almost com-

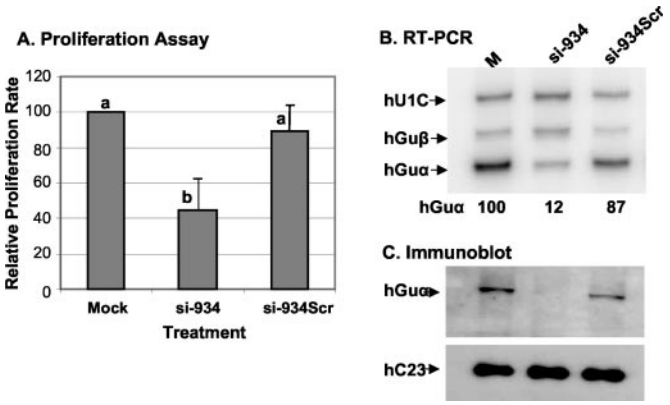

FIG. 5. **Proliferation rate of HeLa cells treated with siRNA.** *A*, cells were treated twice with 10 nM siRNA for 48 h, replated, grown for an additional 72 h, and assayed for cell proliferation as described under "Experimental Procedures." The proliferation rate was measured relative to the mock-treated cells. *Bars* with the *same letter* indicate that the mean values are not significant; *different letters* indicate a highly significant difference ($p < 0.001$). Student's *t* test procedure was used for statistical analysis of results from five independent experiments. *B* and *C*, to determine the mRNA and protein levels of RH-II/Guα in cells used for cell proliferation assay, RT-PCR and Western blot analyses were carried out. The level of nucleolin/C23, another nucleolar protein, was used as a loading control for Western blot analysis.

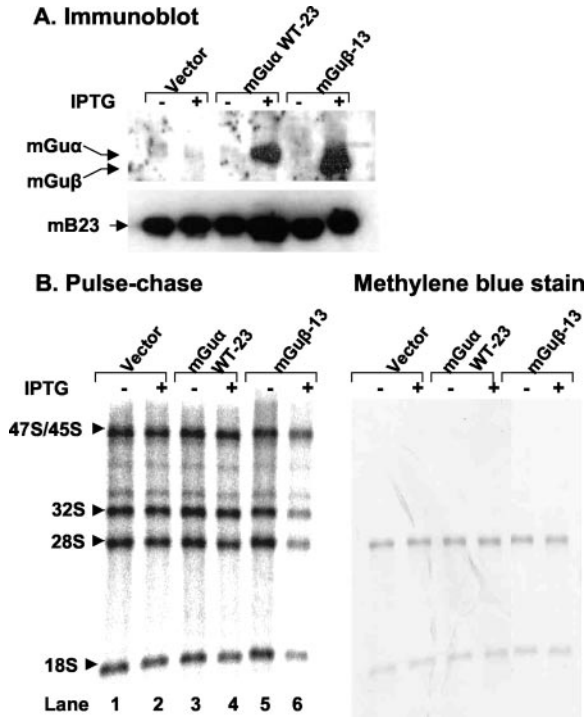

FIG. 6. **Overexpression of RH-II/Guβ blocks rRNA production.** *A*, protein extracts from stable LAP3 clones, either not induced (−) or induced (+) with 2 mM IPTG for 48 h, were analyzed by immunoblotting as described in Fig. 4. The calculated molecular masses of mouse RH-II/Guα and RH-II/Guβ without the FLAG epitope are 94 and 82 kDa, respectively. The level of nucleolar protein nucleophosmin/B23 was used as a loading control. *B*, in a later experiment, the same clones were treated with 2 mM IPTG for 48 h, pulse-labeled with [^{32}P]orthophosphate, and chased for 1 h. Total RNA was extracted and analyzed as described in the legend to Fig. 3. The same results were obtained from three independent experiments.

plete silencing (Fig. 5, *B* and *C*). The difference in magnitude between decreases in growth rate and the level of RH-II/Guα suggests a possible compensatory mechanism.

Analysis by flow cytometry of cells treated with si-934 did not reveal any specific cell cycle arrest (data not shown). The results suggest a slowing down of cellular metabolisms.

Overexpression of RH-II/Guβ Inhibits rRNA Production—It is possible that down-regulation of RH-II/Guα could result in stimulation of expression and/or enhancement of the enzymatic activity of RH-II/Guβ, compensating for the lost activity of RH-II/Guα. After treatment of HeLa cells with si-934, which specifically targets RH-II/Guα, the mRNA level of RH-II/Guβ increased 15% (Fig. 1*A*). We thought that this increase was significant enough to compensate for the decrease in RH-II/Guα, and our plan was to down-regulate expression of endogenous mouse RH-II/Guα with si-935 and look for rescue by expression of exogenous mouse RH-II/Guβ. To our surprise, overexpression of mouse RH-II/Guβ (Fig. 6*A*) inhibited the production of rRNA even without down-regulation of the α form (Fig. 6*B*, *lane 6*). The rRNA production in a clone overexpressing RH-II/Guα is similar to the vector clone treated with IPTG (Fig. 6*B*, *lanes 2* and *4*). This is consistent with the absence of effects on rRNA production when RH-II/Guβ is silenced to 20% with siRNA (data not shown). Another phenotype seen after overexpression of RH-II/Guβ is a decreased cell proliferation (data not shown), which would be expected when rRNA production is inhibited. Overall, up-regulation of the β form, or down-regulation of the α form, inhibits rRNA production and cell proliferation, suggesting an antagonistic relationship between the two paralogues.

DISCUSSION

The general mechanisms of pre-rRNA processing in mammals and frog are similar. Both involve sequential cleavages of the primary and intermediate precursors leading to the production of 28, 18, and 5.8 S rRNA (37, 38). This general mechanism is in fact highly conserved through evolution despite minor differences as far as the functions of the factors involved are concerned.

We recently showed that antisense-mediated down-regulation of RH-II/Guα in *Xenopus* oocyte resulted in inhibition of production of 28 and 18 S rRNAs (18). In that study, accumulation of 20 S rRNA and the appearance of 28 S degradation products were observed, suggesting that RH-II/Guα is involved in the processing of 20 S rRNA to 18 S rRNA and helps stabilize 28 S rRNA. The present study was undertaken to address whether or not mammalian RH-II/Guα has physiological functions similar to the metazoan orthologue. siRNA-mediated down-regulation of the human and mouse RH-II/Guα resulted in a decreased 28 and 18 S rRNA production. However, we did not observe either accumulation of 20 S intermediate product or 28 S degradation in the present study.

It would not surprise us if the mode of actions of human and frog RH-II/Guα are fundamentally different. For example, mammalian nucleolin/C23 is believed to be involved in pre-rRNA processing and assembly of ribosomal proteins and rRNAs (39, 40). Its yeast homologue, Nsr1p, is also required for efficient pre-rRNA processing in yeast (41). Whereas C23 has been shown to be a component of isolated ribonucleoprotein particles involved in pre-rRNA processing (42, 43), Nsr1p has never been identified in yeast preribosomal complexes. Moreover, mammalian nucleolin cannot complement the yeast *nsr1-delta* mutant (44), suggesting differences in their mode of actions.

What, then, could be a possible mechanism for the observed correlation between down-regulation of RH-II/Guα and inhibition of mammalian rRNA production? RH-II/Guα has RNA unwinding activity, and it can also introduce secondary structure to single-stranded RNA, two opposing enzymatic activities exhibited by other RNA helicases including p68 and p72 (28). The RNA unwinding activity of RH-II/Guα, but not its ATPase,

is abolished by mutating its SAT motif to LET, which enhanced its RNA folding activity (21). The same SAT mutant did not rescue the siRNA-mediated inhibition of 18 S rRNA production (Fig. 4*B*), suggesting the relevance of its RNA helicase activity. On the other hand, this mutant did reverse the siRNA-mediated inhibition of 28 S, suggesting that its RNA helicase activity may not be as critical as its ATPase, RNA folding activity, and/or interaction with other nucleolar factors for the production of 28 S rRNA. The ATPase activity of RH-II/Guα might be critical in the formation and reorganization of ribonucleoprotein complexes required for 28 S rRNA production similar to the relevance of ATPase activity in the disruption of U1A-RNA interaction by RNA helicase NPH-II (45). The enhanced RNA folding activity of SAT-M1 mutant might contribute to this reorganization. As we hypothesized in the frog oocyte studies, RH-II/Guα might stabilize 28 S rRNA by maintaining correct secondary structure of 28 S rRNA augmented by its direct interaction with ribosomal protein L4[2] and other unknown factors. This hypothesis is supported by the presence of RH-II/Guα and RPL4 in preribosomal ribonucleoprotein complexes, which were isolated and whose components were identified by mass spectrometry (42, 43).

In addition to directly effecting 28 and 18 S rRNA production, the decreased level of RH-II/Guα might contribute to a decrease in rRNA gene transcription or rRNA turnover. It may be a general phenomenon that when the level of any nucleolar protein related to mammalian rRNA production falls below or raises above a threshold value, the architecture of preribosome processing machineries collapses, sending feedback regulatory signals to RNA polymerase I or rRNA-degrading complexes. It is known that the tightly regulated rRNA production depends on several cellular signals (9, 46). This might explain why down-regulation of RH-II/Guα (this study) or dyskerin (17) or up-regulation of p19^{arf} (15) results in similar inhibitory effects on rRNA production. Although this mechanism would not negate a direct role of RH-II/Guα as suggested by a more significant inhibition of 28 and 18 S rRNA production than the 32 S rRNA biosynthesis (Fig. 3*B*) and the ability of RH-II/Guα SAT-M1 mutant to reverse 28 S but not 18 S rRNA production (Fig. 4*B*), there exists the possibility that both mechanisms co-exist.

Another indirect effect would emanate from the interaction of RH-II/Guα with c-Jun (47). Down-regulation of RH-II/Guα could repress expression of proteins relevant to the processing and stability of rRNAs. Our data, however, support a more direct role of RH-II/Guα in rRNA production as shown by its nucleolar localization, the ability of the SAT mutant to reverse the inhibition of 28 S rRNA production but not 18 S, the RH-II/Guα interaction with RPL4, and its presence in preribosomal RNP particles.

We thought RH-II/Guβ might participate in the processing of rRNA as well. We initially hypothesized a synergistic relationship in the cellular functions of the α and β forms; our data suggest otherwise. Whereas overexpression of RH-II/Guα did not affect rRNA production, overexpression of RH-II/Guβ inhibited this cellular process (Fig. 6), reminiscent of the inhibition caused by RH-II/Guα down-regulation (Fig. 3). This apparently antagonistic relationship is consistent with the marginal *in vitro* RNA unwinding activity of RH-II/Guβ and its lack of RNA folding activity (22). Moreover, the expression level of the β form is lower than the expression level of the α form in monolayer cells (compare RT-PCR signals in Fig. 1) and different human tissues (22). It remains to be shown if the expressions of the two paralogues are cell cycle-regulated or developmentally regulated. We expect to see higher levels of expression of RH-II/Guα in actively growing cells.

A further observation was that overexpression of RH-II/Guβ slowed cell proliferation. This is consistent with our previous report showing higher expression of RH-II/Guβ in normal tissues when compared with matching tumor samples (22). The opposing effects of the two paralogues on rRNA production and cell proliferation suggest that the β form probably evolved as a negative regulator of RH-II/Guα in addition to having other unidentified functions.

The observed repression of cell proliferation when RH-II/Guα was down-regulated or when RH-II/Guβ was up-regulated might be a consequence of reduction in rRNA production. It is known that production of rRNA is tightly coordinated with cell growth and proliferation, and the mechanisms involved are starting to be understood. For example, p19^{arf} was recently shown to suppress tumor formation by inhibiting rRNA production in addition to a p53-dependent mechanism (15). Expression of a dominant-negative mutant of Bop1 inhibited rRNA maturation and induced a reversible p53-dependent cell cycle arrest (48).

On the other hand, the decrease in cell proliferation rate observed during silencing of RH-II/Guα could also be due to inhibition of c-Jun-dependent transcriptions that depend on the interaction between c-Jun and RH-II/Guα (47). The indirect effects of si-934 on c-Jun-mediated transcriptions of genes involved in cell proliferation and death (49) remain to be shown. The effects of RH-II/Guβ on the expression of c-Jun-activated genes are also unknown.

In summary, silencing RH-II/Guα might have double-edged effects, either inclusive or exclusive. A negative effect on rRNA production would result in decreased protein synthesis and, consequently, inhibition of cell growth and proliferation. If the effects of si-934 were through inhibition of the growth-promoting activity of c-Jun, then repression of cell growth and proliferation might send stress signals to down-regulate rRNA biosynthesis and turnover of cytoplasmic ribosomes (50). Both pathways are consistent with our observations. A better understanding of the details of underlying mechanisms should provide us with knowledge of the intricate relationship between ribosome biogenesis and proliferation signals.

Acknowledgment—We thank Dr. Heidi L. Weiss for the statistical analyses.

REFERENCES

1. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* **411,** 494–498
2. McManus, M. T., and Sharp, P. A. (2002) *Nat. Rev. Genet.* **3,** 737–747
3. Paddison, P. J., and Hannon, G. J. (2002) *Cancer Cells* **2,** 17–23
4. Andersen, J. S., Lyon, C. E., Fox, A. H., Leung, A. K. L., Lam, Y. W., Steen, H., Mann, M., and Lamond, A. I. (2002) *Curr. Biol.* **12,** 1–11
5. Scherl, A., Coute, Y., Deon, C., Calle, A., Kindbeiter, K., Sanchez, J-C., Greco, A., Hochstrasser, D., and Diaz, J.-J. (2002) *Mol. Biol. Cell.* **13,** 4100–4109
6. Harnpicharnchai, P., Jakovljevic, J., Horsey, E., Miles, T., Roman, J., Rout, M., Meagher, D., Imai, B., Guo, Y., Brame, C. J., Shabanowitz, J., Hunt, D. F., and Woolford, J. L., Jr. (2001) *Mol. Cell.* **8,** 505–515
7. Venema, J., and Tollervey, D. (1999) *Annu. Rev. Genet.* **33,** 261–311
8. Warner, J. R. (2001) *Cell* **107,** 133–136
9. Leary, D. J., and Huang, S. (2001) *FEBS Lett.* **509,** 145–150
10. Olson, M. O., Hingorani, K., and Szebeni, A. (2002) *Int. Rev. Cytol.* **219,** 199–266
11. Pederson, T. (1998) *Nucleic Acids Res.* **26,** 3871–3876
12. Visintin, R., and Amon, A. (2000) *Curr. Opin. Cell Biol.* **12,** 372–377
13. Wang, C., Query, C. C., and Meier, U. T. (2002) *Mol. Cell. Biol.* **22,** 8457–8466
14. Strezoska, Z., Pestov, D. G., and Lau, L. F. (2002) *J. Biol. Chem.* **277,** 29617–29625
15. Sugimoto, M., Kuo, M-L., Roussel, M. F., and Sherr, C. J. (2003) *Mol. Cell.* **11,** 415–424
16. Meier, U. T. (2003) *Nat. Genet.* **33,** 116–117
17. Ruggero, D., Grisendi, S., Piazza, F., Rego, E., Mari, F., Rao, P. H., Cordon-Cardo, C., and Pandolfi, P. P. (2003) *Science* **299,** 259–262
18. Yang, H., Zhou, J., Ochs, R. L., Henning, D., Jin, R., and Valdez, B. C. (2003) *J. Biol. Chem.* **278,** 38847–38859
19. Flores-Rozas, H., and Hurwitz, J. (1993) *J. Biol. Chem.* **268,** 21372–21383
20. Valdez, B. C., Henning, D., Busch, R. K., Woods, K., Flores-Rozas, H. Hurwitz,

[2] D. Henning and B. C. Valdez, unpublished data.

J., Perlaky, L., and Busch, H. (1996) *Nucleic Acids Res.* **24,** 1220–1224
21. Valdez, B. C., Henning, D., Perumal, K., and Busch, H. (1997) *Eur. J. Biochem.* **250,** 800–807
22. Valdez, B. C., Perlaky, L., and Henning, D. (2002) *Exp. Cell Res.* **276,** 249–263
23. Valdez, B. C., Yang, H., Hong, F., and Sequitin, A. M. (2002) *Gene* (*Amst.*) **284,** 53–61
24. Pestov, D. G., and Lau, L. F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12549–12553
25. Valdez, B. C., Henning, D., Busch, R. K., Srivastava, M., and Busch, H. (1995) *Mol. Immunol.* **32,** 1207–1213
26. Valdez, B. C., and Wang, W. (2000) *Genomics* **66,** 184–194
27. Strezoska, Z., Pestov, D. G., and Lau, L. F. (2000) *Mol. Cell. Biol.* **20,** 5516–5528
28. Rossler, O. G., Straka, A., and Stahl, H. (2001) *Nucleic Acids Res.* **29,** 2088–2096
29. Lee, C-G., and Hurwitz, J. (1993) *J. Biol. Chem.* **268,** 16822–16830
30. Zhang, S., and Grosse, F. (1997) *J. Biol. Chem.* **272,** 11487–11494
31. Pause, A., and Sonenberg, N. (1992) *EMBO J.* **11,** 2643–2654
32. Schwer, B., and Gross, C. H. (1998) *EMBO J.* **17,** 2086–2094
33. Fuller-Pace, F. V., Nicol, S. M., Reid, A. D., and Lane, D. P. (1993) *EMBO J.* **19,** 3619–3626
34. Shuman, S. (1993) *J. Biol. Chem.* **268,** 11798–11802
35. Jin, L., and Peterson, D. L. (1995) *Arch. Biochem. Biophys.* **323,** 47–53
36. Scheffner, M., Knippers, R., and Stahl, H. (1989) *Cell* **57,** 955–963
37. Savino, R., and Gerbi, S. A. (1990) *EMBO J.* **9,** 2299–2308
38. Sollner-Webb, B., Tycowski, K. T., and Steitz, J. A. (1996) in *Ribosomal RNA: Structure, Evolution, Processing, and Function in Protein Synthesis* (Zimmerman, R., and Dahlberg, A., eds) pp. 469–490, CRC Press, Inc., Boca Raton, FL
39. Ginisty, H., Amalric, F., and Bouvet, P. (1998) *EMBO J.* **17,** 1476–1486
40. Shaw, P., and Jordan, E. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 93–121
41. Lee, M-S., Henry, M., and Silver, P. A. (1996) *Genes Dev.* **10,** 1233–1246
42. Hayano, T., Yanagida, M., Yamauchi, Y., Sinkawa, T., Isobe, T., and Takahashi, N. (2003) *J. Biol. Chem.* **278,** 34309–34319
43. Yanagida, M., Shimamoto, A., Nishikawa, K., Furuichi, Y., Isobe, T., and Takahashi, N. (2001) *Proteomics* **1,** 1390–1404
44. Lee, W-C., Zabetakis, D., and Melese, T. (1992) *Mol. Cell. Biol.* **12,** 3865–3871
45. Jankowsky, E., Gross, C. H., Shuman, S., and Pyle, A. M. (2001) *Science* **291,** 121–125
46. Zhao, Y., Sohn, J. H., and Warner, J. R. (2003) *Mol. Cell. Biol.* **23,** 699–707
47. Westermarck, J., Weiss, C., Saffrich, R., Kast, J., Musti, A-M., Wessely, M., Ansorge, W., Seraphin, B., Wilm, M., Valdez, B. C., and Bohmann, D. (2002) *EMBO J.* **21,** 451–460
48. Pestov, D. G., Strezoska, Z., and Lau, L. F. (2001) *Mol. Cell. Biol.* **21,** 4246–4255
49. Shaulian, E., and Karin, M. (2002) *Nat. Cell Biol.* **4,** E131–E136
50. Paule, M. R., and Lofquist, A. K. (1996) in *Ribosomal RNA: Structure, Evolution, Processing, and Function in Protein Synthesis* (Zimmerman, R., and Dahlberg, A., eds) pp. 395–419, CRC Press, Inc., Boca Raton, FL