Critical Role of Nucleostemin in Pre-rRNA Processing*

Luidmila Romanova, Anthony Grand, Liying Zhang, Samuel Rayner, Nobuko Katoku-Kikyo, Steven Kellner, and Nobuaki Kikyo

From the Stem Cell Institute, Division of Hematology, Oncology and Transplantation, Department of Medicine, University of Minnesota, Minneapolis, Minnesota 55455

Nucleostemin is a nucleolar protein widely expressed in proliferating cells. Nucleostemin is involved in the regulation of cell proliferation, and both depletion and overexpression of nucleostemin induce cell cycle arrest through the p53 signaling pathway. Although the presence of p53-independent functions of nucleostemin has been previously suggested, the identities of these additional functions remained to be investigated. Here, we show that nucleostemin has a novel role as an integrated component of ribosome biogenesis, particularly pre-rRNA processing. Nucleostemin forms a large protein complex (>700 kDa) that co-fractionates with the pre-60 S ribosomal subunit in a sucrose gradient. This complex contains proteins related to pre-rRNA processing, such as Pes1, DDX21, and EB2, in addition to several ribosomal proteins. We show that the nucleolar retention of DDX21 and EB2 is dependent on the presence of nucleostemin in the nucleolus. Furthermore, the knockdown of nucleostemin delays the processing of 32 S pre-rRNA into 28 S rRNA. This is accompanied by a substantial decrease of protein synthesis as well as the levels of rRNAs and some mRNAs. In addition, overexpressed nucleostemin significantly promotes the processing of 32 S pre-rRNA. Collectively, these biochemical and functional studies demonstrate a novel role of nucleostemin in ribosome biogenesis. This is a key aspect of the role of nucleostemin in regulating cell proliferation.

Nucleostemin (NS)* is a nucleolar protein preferentially expressed in actively proliferating cells. The structure of NS is characterized by two GTP-binding domains, which are involved in the regulation of its dynamic shuttling between the nucleolus and nucleoplasm (1). NS was originally identified as a nucleolar protein prominently expressed in rat neural stem cells and down-regulated during differentiation of these cells in vitro (2). The same authors also found that NS is widely expressed in neural precursor cells in early mouse embryos as well as in a variety of cancer cells and stem cells, including embryonic stem cells and a hematopoietic stem cell-enriched fraction. NS is generally down-regulated in the early stage of differentiation before exit from the cell cycle. In addition, knockdown of NS significantly inhibits proliferation of cortical stem cells and cancer cells. These initial observations led to suggestions that NS is involved in multipotency in stem cells as well as in the regulation of cancer and stem cell proliferation (2).

Recent work, however, has demonstrated that NS is in fact widely expressed in many types of normal proliferating cells at levels similar to those in malignant cells. For instance, NS is expressed in normal kidney cells and renal carcinoma cells at comparable levels as detected in histological sections (3). The expression of NS is significantly up-regulated when normal T lymphocytes are activated by concanavalin A (3) and when bone marrow stem cells are stimulated by fibroblast growth factor 2 (4). Cells in NS-null mouse embryos fail to enter the S phase, resulting in embryonic death at the blastocyst stage (5, 6). In early Xenopus embryos NS is also expressed in the sites of active cell proliferation and local depletion of NS results in a decrease in proliferating neural progenitor cells (6). Based on these observations, it was proposed that expression of NS is more closely linked with cell proliferation than with the malignant state or differentiation status of a cell.

Several studies have provided evidence that the p53 signaling pathway is involved in the G1 arrest of the cell cycle induced by the down-regulation of NS. Physical interaction between NS and p53 was initially reported by Tsai and McKay (2). Later, it was shown that the G1 arrest requires the presence of p53 (7). In the most recent study Dai et al. (8) showed that knockdown of NS enhances the interaction between the p53-binding protein MDM2 and the ribosomal protein L5 or L11, preventing MDM2 from inducing ubiquitylation-based p53 degradation. However, other studies have also suggested that NS may have a p53-independent role in the regulation of cell proliferation. For instance, the depletion of p53 from NS-null blastocysts did not rescue them from the embryonic lethality (6). In addition, NS partial loss-of-function in mouse fibroblasts did not result in any change in the p53 level (5). Furthermore, knockdown of L5 and L11 only partially rescued the G1 arrest in NS knockdown cells (8). Finally, the fact that NS is primarily localized in the nucleolus, whereas the p53-mediated mechanism occurs in the nucleoplasm, suggests that NS might have an additional role more directly relevant to nucleolar functions.

To identify novel functions of NS, we purified an endogenous NS complex from HeLa cell extract and investigated whether NS interacts with other proteins not described previously. Identification of the components of this complex and the alter-
ations of the expression level of NS in HeLa cells led us to uncover a novel role of NS in the processing of rRNA. Our findings not only provide supporting evidence for the hypothesis that NS has a p53-independent function but also demonstrate that NS is critical for ribosome biogenesis, one of the most fundamental processes common for all cell types.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells were cultured in minimum essential medium containing 10% fetal bovine serum (FBS), nonessential amino acids, and 1 mM sodium pyruvate.

**Isolation of the NS Complex from HeLa Cell Extract**—Cytoplasmic extract and nuclear extract were sequentially prepared from a 10-liter suspension culture of HeLa S3 cells (National Cell Culture Center) and mixed to prepare whole cell extract as described previously (9). All of the chromatographic columns were purchased from GE Healthcare. The extract was applied to an SP Sepharose cation exchange column, followed by elution of bound proteins with a linear gradient of sodium chloride to 1M in Buffer A (10 mM HEPES, pH 7.8, 4 mM MgCl2, 1 mM dithiothreitol, 10% glycerol, 0.003% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1.5 mM peptatin A). Western blotting showed that NS was eluted in the fractions containing 570–870 mM NaCl. These fractions were pooled, and ammonium sulfate was added to a final concentration of 1M before application to a phenyl-Sepharose hydrophobic column. Bound proteins were eluted from the phenyl-Sepharose column with a linear gradient of ammonium sulfate from 1 to 0 M in Buffer A. The NS-positive fractions, eluted by 600–300 mM ammonium sulfate, were pooled and dialyzed against Buffer A with 75 mM NaCl as preparation for application to a HiTrap Heparin column. Protein elution from the Heparin column was carried out using a linear gradient of NaCl from 75 mM to 1 M in Buffer A. NS was eluted in fractions containing 680–850 mM NaCl. These fractions were dialyzed against Buffer A with 75 mM NaCl and applied to a Mono Q 5/5 anion exchange column. Bound proteins were eluted with a gradient from 75 mM to 1 M NaCl in Buffer A. NS was eluted from this column by 450–550 mM NaCl. The NS-positive fractions were applied to a Superdex 200 gel filtration column for size estimation of the NS complex. NS-positive Mono Q fractions were also analyzed by mass spectrometry. For this purpose, the proteins were resolved on a 12% SDS-PAGE, and the gel was stained with Deep Purple (GE Healthcare). Prominent protein bands were excised from the gel and applied to a ThermoFinnigan LTQ mass spectrometer (Thermo Fisher Scientific). The obtained data were searched using the software Sequest (ThermoFinnigan) and X! Tandem for protein identification. Scaffold (Proteome Software Inc.) was used to validate tandem mass spectrometry-based peptide and protein identification.

**Fractionation of HeLa Cell Nuclear Extract by a Sucrose Gradient**—Two hundred μl of HeLa S3 cell nuclear extract (equivalent to 1 × 10^7 cells) were layered on top of a sucrose gradient solution (10–40% sucrose in 50 mM HEPES, pH 7.8, 100 mM NaCl, 10 mM magnesium acetate, 0.2 mM phenylmethylsulfonfonyl fluoride, 2 mM leupeptin, 1.5 mM peptatin A, and 10 units/ml RNasin purchased from Promega) and centrifuged at 210,000 × g for 12 h at 4 °C using an SW41 rotor (Beckman Coulter). The resulting gradient solution was fractionated, and each fraction was divided into half for separate isolation of proteins and RNA. Total protein was precipitated in each fraction by adding 3 volumes of ethanol, 20 μg of glycogen, and sodium acetate (pH 6.0) to a final concentration of 0.1 M and analyzed by Western blotting. Total RNA was isolated from each fraction with TRIzol (Invitrogen), and rRNA was detected with a SuperScript III One-Step RT-PCR system (Invitrogen). The following PCR primers were used: 28 S rRNA, 5′-GTTCACCCCACTAATAGGGAACG-3′ and 5′-GGATTCTGACTTAAAGGGGT-3′ (10); and 18 S rRNA, 5′-TTGGTGGTTTCGGAGACTGAG-3′ and 5′-ATTGCTTCAATCTCCGGTGCTG-3′ (11).

**Antibodies**—The sources of the antibodies and the dilutions used in this study are as follows (W indicates Western blotting, and I indicates immunofluorescence staining): NS (Millipore; 1:2000 for W and 1:1200 for I), NS (Santa Cruz Biotechnology; 1:200 for W and 1:100 for I), DDX21 (Aviva Systems Biology; 1:1000 for W and 1:400 for I), Pes1 (provided by Dr. Elisabeth Kremmer; 1:1000 for W and 1:400 for I) (12), EBP2 (gift from Dr. Lori Frappier; 1:1000 for W and 1:500 for I) (13), nucleasein (Santa Cruz Biotechnology), 1:50 for I), β-tubulin (Sigma, 1:1000 for W), TATA-binding protein (Santa Cruz Biotechnology, 1:1000 for W), B23 (Santa Cruz Biotechnology, 1:500 for W), Hisp (Santa Cruz Biotechnology, 1:300 for I), and rabbit normal IgG for immunoprecipitation (Santa Cruz Biotechnology).

For immunofluorescence staining, the following secondary antibodies were used: Cy3-conjugated anti-mouse IgG, anti-rabbit IgG and anti-goat IgG (Jackson ImmunoResearch Laboratories; 1:1000), Alexa Fluor 488-conjugated anti-rabbit IgG and anti-mouse IgG (Invitrogen; 1:400), and rhodamine-conjugated anti-rat IgG (Santa Cruz Biotechnology; 1:300). For Western blotting, horseradish peroxidase-conjugated anti-IgG of appropriate species (Jackson ImmunoResearch Laboratories, 1:1000) were used.

**Immunofluorescence Staining**—HeLa cells were grown on cover glasses and fixed with 4% formaldehyde in phosphate-buffered saline (PBS). The cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min and washed three times with a washing solution containing 10% PBS and 0.2% Tween 20 in PBS before application of primary antibodies. Double immunostaining was carried out sequentially. The cells were incubated with primary antibodies for 1 h at 25 °C, washed with the washing solution, and subsequently incubated with secondary antibodies for 1 h at 25 °C. Finally, the cells were washed with the washing solution, and the nuclei were stained with Topro 3 (Invitrogen) for 15 min. The mounting solution for the cover glasses contained 50 mg/ml 1,4-diazabicyclo[2.2.2]octane (Sigma) and 50% glycerol in PBS. The fluorescence images were captured with a Radiance 2000 confocal system attached to an Axioskop 2 Plus microscope (both Carl Zeiss). A 488-nm argon laser and a 543-nm green helium-neon laser were used with a 63 × oil Plan Apochromat objective (numerical aperture, 1.4). All of the images were processed with Photoshop 7.0 (Adobe Systems).

**Western Blotting**—Whole cell extract was prepared from 2 × 10^5 cells unless stated otherwise. Extracted proteins were
resolved in a 12% SDS-PAGE gel and transferred onto an Immobilon P membrane (Millipore) for immunodetection. The membrane was blocked in a blocking solution containing 5% skim milk and 0.2% Tween 20 in PBS for 1 h. Incubation time with primary and secondary antibodies was 1 h each. Signal was detected by using SuperSignal West Dura (Pierce) as a substrate for peroxidase.

**Immunoprecipitation**—HeLa cell nuclear extract prepared from 1 × 10⁷ cells as described previously (9) was used for each immunoprecipitation. The extract was precleared by incubation with 2.5 μg of normal rabbit IgG and 60 μl of the GammaBind G Sepharose beads (GE Healthcare) for 2 h at 4 °C with rotation. The precleared extract was incubated with 5 μg of rabbit anti-NS antibody or rabbit normal IgG for 1 h at 4 °C and subsequently with 20 μl of the GammaBind G Sepharose for additional 1 h at 4 °C. Following extensive washing, immunoprecipitated proteins were eluted with 2× SDS-PAGE sample buffer (14) by incubation for 5 min at 98 °C and analyzed by Western blotting.

The antibodies against DDX21, EB2P, and Pes1 did not precipitate the target proteins under the abovementioned conditions, potentially because of epitope masking. To overcome this problem, a combination of a denaturing buffer and the protein cross-linker dithiobis[succinimidylpropionate] (Pierce) was used in (15). HeLa cells were treated with 2 mM dithiobis[succinimidylpropionate] (Pierce) in PBS for 2 h at 4 °C, and cross-linking was terminated with 20 mM Tris-HCl, pH 8.0, at 25 °C. The cells were resuspended in 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1.5 mM pepstatin A) and overlaid onto Buffer 3 (10 mM Tris-HCl, pH 8.0, 0.5 mM MgCl₂, 0.5 mM dithiothreitol, 2 mM leupeptin, and 1.5 mM pepstatin A). The plasma membrane was then disrupted with a tissue grinder before centrifugation at 230,000 × g for 5 min at 4 °C. The supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in Buffer 2 (10 mM HEPES, pH 7.8, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1.5 mM pepstatin A). The nuclear fraction was further disrupted with a needle and collected as the nucleoplasmic fraction. The pellet was washed and cultured in Met-free Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed FBS, and the cells were cultured for 1 h. 

HeLa cells were transfected with siRNA or plasmid as described above. The culture medium was replaced with phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed FBS, and the cells were cultured for an additional 1.5 h. The culture medium was then added to a final concentration of 20 μCi/ml, and the cells were incubated for an additional 3–6 h before harvest. Total RNA was isolated from these cells using an RNasea Mini kit (Qiagen). Two μg of total RNA from each sample were resolved in a 1% agarose formaldehyde gel. The gel was dried, and radioactive bands were detected by autoradiography. The developed films were scanned with an image scanner, and the intensity of each band was monitored using the software ImageJ. The ratios of the band intensities were calculated with the software Excel 2002 (Microsoft).

**Metabolic Labeling of Protein with [³⁵S]Methionine**—HeLa cells were metabolically labeled with [³⁵S]Met after transfection with siRNA or plasmid as described above. The cells were first washed and cultured in Met-free Dulbecco’s modified Eagle’s medium containing 5% dialyzed FBS for 1 h. After the addition of [³⁵S]Met to a final concentration of 15 μCi/ml, the cells were incubated for 3–6 h. Whole cell extracts prepared from these cells were loaded onto a 12% SDS-polyacrylamide gel at 2 × 10⁵ cells equivalent/well. The gel was dried and analyzed by autoradiography. Total radioactivity in each lane was measured with a scintillation counter LS 6500 (Beckman Coulter).
Flow Cytometry—After transfection with siRNA HeLa cells were harvested, washed in PBS, resuspended in ice-cold 70% ethanol, and fixed for at least 30 min. The cells were collected by centrifugation and stained in a solution containing 1% FBS, 50 μg/ml propidium iodide, and 100 μg/ml RNase A in PBS for 30 min. Flow cytometry was performed on a FACSCalibur cytometer, and the data were analyzed using the CellQuest Pro software (both BD Biosciences).

Purification of Polysomes—Cytoplasmic extract was prepared as described previously (9) from 1 × 10^7 HeLa cells transfected with control or NS siRNA. Polysomes were purified by sedimentation at 260,000 × g for 2.5 h through a sucrose cushion (1 mM sucrose in 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, and 10 units/ml RNasin) essentially as described previously (16). The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 1.5 mM MgCl₂, and polysome concentration was evaluated by UV absorbance at 260 nm, which is a commonly used method to quantify ribosome concentration (16). In addition, the amounts of 18 and 28S rRNA were measured by quantitative RT-PCR using a HotStart-IT SYBR Green One-Step quantitative RT-PCR kit (USB) and the primer pairs described above.

Nuclear Run-on Assay for rRNA Synthesis—HeLa cells transfected with control and NS siRNA were harvested and washed twice with ice-cold PBS. 5 × 10^6 cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40) and incubated on ice for 5 min. The lysed cells, called nuclei hereafter, were precipitated by centrifugation at 500 × g for 5 min and resuspended in 200 μl of cold storage buffer (20 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA, and 40% glycerol). The nuclei were then mixed with 200 μl of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, 10 μl of [α-32P]UTP, and 0.1 μl each of ATP, CTP, and GTP) and incubated for 30 min at 37°C. This step was followed by addition of 200 μl of proteinase K solution (500 mM Tris-HCl, pH 7.4, 5% SDS, 0.125 mM EDTA, and 0.1 μg/μl proteinase K) and incubation at 42°C for 30 min. RNA was isolated from the reaction mixture using TRI reagent LS (Molecular Research Center) and hybridized to the 5’ half of the rDNA sequence provided by Dr. J. E. Sylvester (17) and the control plasmid pcDNA3.1 with ExpressHyb hybridization solution (Clontech). Hybridization signal was detected by autoradiography, and the radioactivity of each band was quantified by Cher- enkov counting.

RESULTS

Identification of NS-binding Proteins—To begin our investigation into the function of NS, we examined whether or not NS existed as a protein complex within cells by estimating the molecular mass of the possible NS-containing entity. For this purpose we applied whole cell extract prepared from HeLa S3 cells to a Superdex 200 gel filtration column and analyzed the elution pattern of NS by Western blotting. NS (62 kDa) was eluted in the fractions typical for proteins with molecular masses ranging from 200 kDa to over 2 MDa, with a major peak appearing between 700 kDa and 2 MDa (Fig. 1A, upper panel).
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This result suggested that NS potentially forms heterogeneous multi-protein complexes. To identify the binding partners of NS, we purified the NS complexes using the four steps of column chromatography outlined in Fig. 1B. Western blotting was performed after each step to detect fractions containing NS. When the NS-positive fractions obtained from the last column, Mono Q, were analyzed by gel filtration, the elution pattern of NS was similar to that observed for HeLa cell extract described above (Fig. 1A, lower panel). This observation indicated that our purification of NS had not resulted in significant disassembly of the NS complexes. SDS-PAGE and silver staining of the Mono Q fractions revealed more than 20 protein bands (Fig. 1C), and this number did not substantially decrease after additional steps of column chromatography (data not shown). Therefore, further purification of the NS complexes was not pursued, and prominent protein bands observed with the Mono Q fractions were analyzed by mass spectrometry. Identified proteins included five ribosomal subunits (RPS6, RPS8, RPS24, Q fractions were analyzed by mass spectrometry. Identified

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DDX21, Pes1, and EBP2 are involved in the late steps of pre-rRNA processing (see Fig. 4D). The RNA helicase DDX21 (also called RHI1/Guo) is necessary for the processing of 20 S pre-rRNA into 18 S rRNA as well as for the stability of 28 S rRNA in Xenopus (18). Human DDX21 has also been shown to be critical for the production of 28 and 18 S rRNA (19). Mammalian Pes1 is important for multiple steps of processing, including processing of 36, 32, and 12 S pre-rRNA (20). A yeast orthologue of human EBP2 is involved in the processing of 27SA3 into 27SB pre-rRNA (corresponding to human 36 S into 32 S pre-rRNA) (21). Therefore, the co-fractionation of DDX21, Pes1, and EBP2 with NS suggested a potential role for NS in pre-rRNA processing.

We examined physical interactions between NS and the three proteins mentioned above by immunoprecipitation with antibody against NS. As shown by Western blotting, Pes1, DDX21, and EBP2 were all co-precipitated with anti-NS antibody from HeLa cell extract but not with control rabbit IgG, verifying their molecular interactions (Fig. 1D). In addition, reciprocal immunoprecipitation using antibodies against DDX21, Pes1, and EBP2 could all co-precipitate NS (Fig. 1, E–G). Consistent with the co-immunoprecipitation of these proteins, immunofluorescence staining demonstrated that NS, DDX21, Pes1, and EBP2 were all co-localized within the nucleoli (Fig. 2, A–C, yellow signals in the merged images).

Pes1 is known to be associated with the pre-60 S ribosomal subunit as shown by its sucrose gradient co-fractionation with 28 S rRNA, a core component of the pre-60 S subunit (20, 22). To test whether NS was also associated with the pre-60 S subunit, we prepared extract from HeLa cell nuclei to enrich nucleolar components and resolved the extract in a sucrose gradient. Western blotting and RT-PCR with rRNA-specific primers showed that all the four proteins were co-fractionated with 28 S rRNA but only partially co-fractionated with 18 S rRNA, a core component of the pre-40 S subunit (Fig. 2D). To test whether 28 S rRNA is an integrated component of the NS complex, we immunoprecipitated NS and examined co-precipitation of 28 S rRNA by RT-PCR. However, it was not detectable above the background level (not shown). This result could be due to a weak interaction between 28 S rRNA and the components in the NS complex. We also attempted to immunoprecipitate NS after formaldehyde cross-linking to preserve RNA-protein interactions; however, we could not precipitate NS under this condition. To further investigate a potential interaction between NS and ribosomes, we prepared a cytoplasmic polysome fraction with a sucrose cushion, but NS was not detectable in the fraction (not shown).

Nucleolar Localization of DDX21 and EBP2 Is Dependent on NS—To study the functional significance of the interaction of NS with DDX21, Pes1, and EBP2, we tested how the nucleolar localization of these proteins was affected when NS was knocked down in HeLa cells using siRNA. Western blotting demonstrated highly efficient depletion of NS in HeLa cells by NS siRNA (Fig. 3A, top panel). Immunofluorescence staining confirmed the disappearance of NS from the nucleoli in these cells (Fig. 3, B–E). Double immunostaining also revealed that DDX21 and EBP2 became almost undetectable in the nucleoli following transfection with NS siRNA (Fig. 3, B and C), although Pes1 remained in the nucleoli without a significant
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The effects of NS knockdown on the amount and nucleolar localization of NS-interacting proteins and the cell cycle. A, Western blotting of the extract prepared from HeLa cells (2 × 10⁶ cells equivalent) treated with NS siRNA and control siRNA. Antibodies against NS and DDX21 (B), NS and EBP2 (C), NS and Pes1 (D), and NS and nucleolin (E) were used. DNA was counterstained with Topro 3. Bars, 15 μm in all panels in this figure. F, Western blotting demonstrating subcellular distribution of DDX21, EBP2, and Pes1 after NS knockdown. β-tubulin, TATA-binding protein (TBP), and B23 are markers for cytoplasmic (Cy), nucleoplasmic (Np), and nucleolar (No) fractions, respectively. G, Western blotting of the extract prepared from HeLa cells treated with EBP2 siRNA and control siRNA. H, double immunofluorescence staining of the HeLa cells transfected with EBP2 siRNA and control siRNA. The cells were stained with antibodies against EBP2 and Pes1. I, Western blotting demonstrating subcellular redistribution of Pes1 in EBP2 knockdown cells. J, cell cycle analysis of the HeLa cells transfected with NS siRNA and control siRNA. The histograms show DNA contents of the cells stained with propidium iodide. The percentage of the cells in each cell cycle phase is indicated under each histogram. The sub-G₁ population represents fragmented nuclei.

FIGURE 3. The effects of NS knockdown on the amount and nucleolar localization of NS-interacting proteins and the cell cycle. A, Western blotting of the extract prepared from HeLa cells (2 × 10⁶ cells equivalent) treated with NS siRNA and control siRNA. Used antibodies are listed on the left. Histone H2B was used as a loading control. B–E, double immunofluorescence staining of the HeLa cells transfected with NS siRNA and control siRNA. Antibodies against NS and DDX21 (B), NS and EBP2 (C), NS and Pes1 (D), and NS and nucleolin (E) were used. DNA was counterstained with Topro 3. Bars, 15 μm in all panels in this figure. F, Western blotting demonstrating subcellular distribution of DDX21, EBP2, and Pes1 after NS knockdown. β-tubulin, TATA-binding protein (TBP), and B23 are markers for cytoplasmic (Cy), nucleoplasmic (Np), and nucleolar (No) fractions, respectively. G, Western blotting of the extract prepared from HeLa cells treated with EBP2 siRNA and control siRNA. H, double immunofluorescence staining of the HeLa cells transfected with EBP2 siRNA and control siRNA. The cells were stained with antibodies against EBP2 and Pes1. I, Western blotting demonstrating subcellular redistribution of Pes1 in EBP2 knockdown cells. J, cell cycle analysis of the HeLa cells transfected with NS siRNA and control siRNA. The histograms show DNA contents of the cells stained with propidium iodide. The percentage of the cells in each cell cycle phase is indicated under each histogram. The sub-G₁ population represents fragmented nuclei.

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**FIGURE 4. Inhibition of pre-rRNA processing and protein synthesis by knockdown of NS.** A, Western blotting of the extract prepared from HeLa cells treated with NS siRNA and control siRNA. B, autoradiogram of rRNA pulse-labeled with [32P]orthophosphate and chased for the indicated time. HeLa cells transfected with NS siRNA and control siRNA were compared. The arrowhead and the arrow indicate the positions of 32 S pre-rRNA and 28 S rRNA bands, respectively. Ethidium bromide staining of 28 S rRNA is shown in the bottom panel as a loading control. C, graph of the band intensity ratios between 28 S rRNA and 32 S pre-rRNA during the pulse-chase shown in B. Means ± S.D. obtained from three independent experiments are shown. The difference of the mean values at 6 h is statistically significant (Student’s t test, *p* < 0.05, asterisk). D, schematic summary of pre-rRNA processing steps in mammalian cells. Steps that are disrupted by the loss of NS, Pes1, DDX21, and EBP2 are indicated. Data obtained with human NS and Pes1, Xenopus DDX21, and Saccharomyces cerevisiae EBP2 are compiled. Although the processing steps in *S. cerevisiae* and human differ in the details, the yeast data with EBP2 was extrapolated to the human system. E, total signal intensity of each lane shown in B. The difference of the mean values at 6 h is statistically significant (*p* < 0.05, asterisk). F, autoradiogram of nuclear run-on assay. RNA prepared from the same number of nuclei from NS knockdown and control cells was used for hybridization with a rDNA fragment and the pcDNA3.1 plasmid. G, autoradiogram of total protein that incorporated [35S]Met, comparing HeLa cells transfected with NS siRNA and control siRNA. Total radioactivity in each lane was measured by a scintillation counter and presented as the means ± S.D. from three independent experiments. The difference in values was statistically significant (*p* < 0.05, asterisk).

siRNA (Fig. 3G), nucleolar Pes1 became undetectable (Fig. 3H), unlike with the knockdown of NS. It is possible that our NS knockdown did not completely eliminate nucleolar EBP2 and thus the residual EBP2 retained Pes1 within the nucleoli. The nucleolar loss of Pes1 upon depletion of EBP2 was confirmed by the subcellular fractionation of EBP2 knockdown cells (Fig. 3F). The loss of EBP2 did not affect nucleolar localization of NS or DDX21 (data not shown). We also attempted to knockdown DDX21 and Pes1 using three siRNA sequences for each but could not substantially deplete these proteins.

**Effect of NS Depletion on the Cell Cycle**—Depletion of NS is known to cause the G1 arrest of the cell cycle (6, 7), but a delay in cell proliferation was not obvious in our microscopic observation of the NS-depleted HeLa cells. This is likely to be due to the lack of p53 in HeLa cells (see “Discussion”) (23, 24). Similar to this observation, no decrease in proliferating cells was detected after knockdown of NS in Saos-2 cells (7), which do not express p53. To confirm that the loss of NS did not affect cell cycle progression, we analyzed the control and the knockdown cells by flow cytometry. The results in Fig. 3F show no increase of the cells in the G0/G1 phase after knockdown of NS. Instead, we observed a decrease of the G2/G0 phase cells and an increase of the sub-G1 phase cells, which indicates higher number of apoptotic cells in the NS-depleted cells. This result demonstrates that depletion of NS in HeLa cells does not cause an immediate cell cycle arrest. We repeated siRNA transfection two additional times to observe a longer term effect of NS knockdown. Proliferation was gradually slowed down for both control and NS knockdown cells, and eventually more than 50% of both cells died because of the toxicity of LipoLfectamine 2000 and/or siRNA. Therefore we could not observe a specific effect of NS knockdown on the cell cycle or induction of apoptosis in long term culture.

**NS Is Essential for the Processing of 32 S Pre-rRNA and Protein Synthesis**—To further understand the functional implications of the interactions between NS and the above three proteins, we investigated whether processing of pre-rRNA was disrupted by the loss of NS. First, NS was knocked down in HeLa cells by siRNA (Fig. 4A), and the cells were incubated with [32P]orthophosphate for 1.5 h to pulse label newly synthesized pre-rRNA. After the original culture medium was replaced with nonradioactive medium, the progress of pre-rRNA processing was chased for up to 6 h. Autoradiography of RNA purified from these cells demonstrated that the processing of 32 S pre-rRNA into 28 S rRNA was clearly delayed in the cells transfected with NS siRNA. This was shown by lower ratios of the band intensity between 28 S rRNA (Fig. 4B, arrow) and 32 S pre-rRNA (Fig. 4B, arrowhead) in the HeLa cells transfected with NS siRNA than that in the cells transfected with control siRNA throughout the chase for up to 6 h (Fig. 4C). This is the
first evidence that NS is functionally involved in ribosome biogenesis.

Fig. 4D represents a simplified scheme of mammalian pre-rRNA processing marked with the steps that can be disrupted by depletion or mutations of human NS and Pes1, Xenopus DDX21, and yeast EBP2 (18, 20, 21, 25, 26). Unlike after depletion of DDX21 in Xenopus, relocalization of DDX21 by NS depletion did not cause an accumulation of 20 S pre-rRNA in our experiments with HeLa cells. Another group also did not observe an accumulation of 20 S pre-rRNA when DDX21 was knocked down in HeLa cells (19). Although the lack of EBP2 in yeast results in an accumulation of 36 S-equivalent pre-rRNA, this was not apparent in our case despite substantial release of EBP2 from the nucleoli. This discrepancy may be explained by the species difference or by the incomplete loss of EBP2 from the nucleoli by NS siRNA.

We also found that the total signal intensity of each lane in Fig. 4B was lower in the cells transfected with NS siRNA than that in the control cells (Fig. 4E). This observation prompted us to compare the level of newly transcribed rRNA between NS knockdown cells and control cells by a nuclear run-on assay. When radiolabeled RNA prepared from an equal number of nuclei was hybridized to a DNA fragment on a membrane, the intensity of the hybridization signal with the RNA prepared from NS knockdown cells was 77 ± 13% of the control RNA (Fig. 4F). Quantitative PCR demonstrated that the levels of not only rRNAs but also mRNAs encoding two housekeeping genes were decreased by NS knockdown as follows: total RNA (78 ± 6%), 28 S rRNA (73 ± 5%), 18 S rRNA (78 ± 7%), glyceraldehyde-3-phosphate dehydrogenase (71 ± 3%), and β-actin (82 ± 6%). Although the mechanism for the overall drop of RNA levels remains unclear, this could be relevant to the impaired protein synthesis described below.

To understand how the disruption of rRNA transcription and processing affect the level of translationally competent ribosomes, we isolated cytoplasmic polysomes with ultracentrifugation through a sucrose cushion. The polysome contents, measured by UV absorption at 260 nm, became 57 ± 13% in NS knockdown cells and 77 ± 13% in the control cells as compared to 100% in control HeLa cells. These data indicate that rRNA synthesis is indeed impaired in NS knockdown cells, in agreement with the run-on assay described above. This is also consistent with previously reported results in Xenopus and yeast (18, 20, 21, 25, 26).

The decreased polysome level by NS knockdown was expected to impair protein synthesis. This possibility was evaluated by metabolic labeling of newly synthesized proteins with [35S]Met in HeLa cells in which NS had been depleted. As expected, treatment with NS siRNA significantly decreased the uptake of [35S]Met compared with the control siRNA (Fig. 4G). Quantification of the total radioactivity in each lane in Fig. 4G indicated that the amount of [35S]Met uptake in the NS knockdown cells was reduced to 75 ± 6% in contrast to control cells, although the qualitative pattern remained unchanged. This finding is consistent with the decreased polysome level in the NS knockdown cells described above.

Overexpression of NS Facilitates the Processing of 32 S Pre-rRNA—As a complementary approach to the depletion of NS, we studied whether overexpression of NS could facilitate pre-rRNA processing and protein synthesis. First, plasmids encoding NS tagged with His6 and EGFP were separately transfected into HeLa cells. Western blotting confirmed a significantly higher expression level of NS in the cells transfected with the NS gene than in the cells transfected with the EGFP gene (Fig. 5A). Using immunofluorescence staining with anti-His6 antibody, we estimated that ∼90% of the cells transfected with the NS gene expressed the His tag (Fig. 5B). As we confirmed the overexpression of NS, we proceeded to the two types of metabolic labeling experiments described earlier. For pulse labeling of newly synthesized RNA, the cells were incubated with [32P]orthophosphate for 1.5 h and then chased in nonradioactive medium for 6 h. The autoradiograph indicated that the ratio of the band intensity ratios between 28 S rRNA and 32 S pre-rRNA observed during the pulse-chase shown in C. The values are ± S.D. obtained from three independent experiments. The difference of the mean values at 6 h is statistically significant (p < 0.05, asterisk). E, autoradiogram of total protein that incorporated [35S]Met, comparing HeLa cells transfected with pcDNA3.1-NS-His6 and EGFP-C1. Total radioactivity in each lane was measured by a scintillation counter and presented as the means ± S.D. from three independent experiments. The arrowhead indicates the band corresponding to EGFP.
expression level of NS is critical for the processing of 32 S pre-rRNA to 28 S rRNA.

**DISCUSSION**

In this work, purification of the NS complex led us to discover a critical role of NS in ribosome biogenesis. We showed that NS interacts with three nucleolar proteins involved in pre-rRNA processing and that NS is essential for the nucleolar localization of some of these proteins. Functionally, both knockdown and overexpression of NS altered the rate of the processing of 32 S pre-rRNA into 28 S rRNA. In the case of the knockdown, total RNA level and protein synthesis were reduced as a consequence. Co-fractionation of NS and its protein partners with 28 S rRNA in a sucrose gradient provided additional supporting evidence that NS is involved in the biogenesis of the 60 S preribosomal subunit. Collectively, our work demonstrated that NS is involved in the canonical function of the nucleolus, ribosome biosynthesis.

The potential role of NS in ribosome biogenesis has been previously suggested based on the structural similarity of NS with members of a nucleolar GTPase family that are involved in different stages of ribosome biogenesis (6, 27). Additionally, a recent work demonstrated that a deletion of NS substantially decreases the amounts of 18 and 26 S rRNAs in *Caenorhabditis elegans* (28). However, impairment of pre-rRNA processing by NS depletion has not been previously reported, and our work represents the first study to directly link NS with ribosome biogenesis. A previous microscopic analysis suggested that NS may not be involved in ribosome biogenesis because it appeared to be concentrated in rRNA-deficient areas within the nucleolus (29). Yeast studies also have not substantially contributed to our understanding of the functions of NS. NS and another human nucleolar protein GNL3L share the same orthologue Grn1p in yeast (30) but only GNL3L can rescue a deletion mutant of Grn1p (27, 30). This result emphasizes the necessity of using mammalian species as a model to understand the process of human ribosome biogenesis.

DDX21, Pes1, and EBP2 are all involved in the late processing steps of pre-rRNAs that are eventually incorporated into the pre-60 S ribosomal subunit. DDX21 is a member of the DEx(D/H) box RNA helicases that unwind RNA structure or disrupt RNA-protein interactions by using ATP as an energy source (31). Although precise structural alterations in rRNA brought about by DDX21 have not been characterized, this helicase is likely to create a specific conformation of the rRNA-protein complex favorable for proper processing. Unlike DDX21, the protein structures of NS, Pes1, and EBP2 do not suggest specific enzymatic activities; perhaps they function as adaptors or regulators of pre-rRNA processing.

Our current work focused on the function of NS in ribosome biogenesis; however, it is also possible that NS plays another uncharacterized role that is completely independent of ribosome biogenesis as shown to exist for other processing proteins. For example, the yeast orthologue of Pes1 interacts with the origin recognition complex of DNA replication and is required for normal S phase progression (32). EBP2 is involved in mitotic segregation of Epstein-Barr virus in mammalian cells (33). The possibility of additional functions for NS is also supported by our gel filtration results, which indicated a potential heterogeneity of NS complexes.

When the current study was initiated, it was not established that p53 is necessary for the cell cycle arrest induced by the depletion of NS. HeLa cells, which we chose for our study, express p53 at a very low level because of the degradation by the oncoprotein E6 (23, 24). Although not intended, this choice of HeLa cells allowed us to demonstrate that the observed disruption of ribosome biogenesis and the resulting protein synthesis were not secondary effects of the cell cycle arrest. It is known that various perturbations in nucleolar structure or ribosomal biogenesis can cause nucleolar stress that leads to p53 activation (34). However, it was not clear until now which type of stress NS depletion induces to the nucleoli. A previous morphological (phase contrast images) and immunofluorescence study could not detect a clear sign of structural disruption of the nucleoli after depletion of NS (7). Our biochemical work linked NS and ribosome biogenesis, which indicates that the NS-p53 connection is not an isolated event but is a part of a larger context of the well-established nucleolar stress signaling pathway.

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