PROTEOMIC SNAPSHOT ANALYSES OF PRERIBOSOMAL RIBONUCLEOPROTEIN COMPLEXES FORMED AT VARIOUS STAGES OF RIBOSOME BIOGENESIS IN YEAST AND MAMMALIAN CELLS

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Proteomic technologies powered by advancements in mass spectrometry and bioinformatics and coupled with accumulated genome sequence data allow a comprehensive study of cell function through large-scale and systematic protein identifications of protein constituents of the cell and tissues, as well as of multi-protein complexes that carry out many cellular function in a higher-order network in the cell. One of the most extensively analyzed cellular functions by proteomics is the production of ribosome, the protein-synthesis machinery, in the nucle(ol)us—the main site of ribosome biogenesis. The use of tagged proteins as affinity bait, coupled with mass spectrometric identification, enabled us to isolate synthetic intermediates of ribosomes that might represent snapshots of nascent ribosomes at particular stages of ribosome biogenesis and to identify their constituents—some of which showed dynamic changes for association with the intermediates at various stages of ribosome biogenesis. In this review, in conjunction with the results from yeast cells, our proteomic approach to analyze ribosome biogenesis in mammalian cells is described. © 2003 Wiley Periodicals, Inc., Mass Spec Rev 22:287–317, 2003; Published online in Wiley InterScience (www.interscience.wiley.com).
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I. INTRODUCTION

Ribosome biogenesis is one of the major metabolic pathways in all cells. It is necessary for cellular adaptation, growth, and proliferation, and it is a major energetic and biosynthetic demand upon cells. For these reasons, ribosome biogenesis requires precise regulation to balance supply and demand (Leary & Huang, 2001). However, based on the results in reconstituting a functional bacterial small subunit from purified RNA and ribosomal proteins, it had been believed until quite recently that ribosome biogenesis was a simple process *in vivo*, even in mammalian cells; thus, the regulation of ribosome biogenesis was not so intricate and interesting. Currently, several lines of evidences indicate that ribosome biogenesis is closely linked to cell growth and division through the control of cell cycle progression (Thomas, 2000; Volarevic et al., 2000; Jorgensen et al., 2002; Sudbery, 2002). In addition, downstream targets of oncogenes (such as *N*-myc), as well as of growth-factor receptors (such as those for platelet growth factors, fibroblast growth factors, etc.) are shown to be major regulators of the protein synthesis machinery (Nelson et al., 2000; Boon et al., 2001; Kroll, Barth-Baus, & Hensold, 2001). Thus, that focus renewed attention on the regulatory mechanism that underlies ribosome biogenesis. The regulation of ribosome biogenesis has turned out to be far more intricate, and interesting, than originally imagined, and is now becoming one of the major issues in the study of the pathogenesis of various diseases related to disorders in cell growth and cell division, such as premature aging, cancer, etc. (Nadano et al., 2000; David-Pfeuty et al., 2001; Ruggero et al., 2003; Sugimoto et al., 2003).

Ribosome biogenesis is a very complicated process that starts with the transcription of ribosomal DNA (rDNA) repeats by RNA polymerase I, which generates 35S pre-ribosomal RNA (pre-rRNA) in yeast and 47S pre-rRNA in mammalian cells, and by RNA polymerase III, which produces 5S pre-rRNA in yeast and mammalian cells. In addition, approximately 80 ribosomal proteins are translated from mRNAs synthesized by RNA polymerase II. Thus, all types of transcription systems are involved in ribosome biogenesis. Furthermore, the transcribed pre-rRNAs undergo rapid processing to the mature rRNAs (18S, 25S, and 5.8S for yeast 35S pre-rRNA, and 18S, 28S, and 5.8S for mammalian 47S pre-rRNA) by endonucleases and exonucleases, with concomitant modification of the rRNA by pseudouridylation and methylation (Henry et al., 1994; Venema & Tollervey, 1999; Nissan et al., 2002). The order of the cleavage reactions has been well established with the use of genetic mutants of yeast cells coupled with conventional biochemical approaches. The features of the *cis*-acting element within the pre-rRNA, which is critical for correct and efficient removal of the spacer sequences, have been established by the use of systems for mutational analysis of yeast rDNA. The same systems allow a link to be forged between *trans*-acting processing factors and these *cis*-acting elements in the yeast cell (Raue & Planta, 1995). More than 100 *trans*-acting proteins and at least an equal number of small nucleolar RNAs (snoRNAs) involved in ribosome biogenesis are identified by biochemical and genetic analyses of various yeast mutants—mostly those of *Saccharomyces cerevisiae* (*S. cerevisiae*) (Kressler, Linder, & Cruz, 1999; Venema & Tollervey, 1999).

The association of ribosomal proteins with rRNA is believed to begin on the nascent pre-rRNA, and most of the *ca.* 80 known ribosomal proteins are already bound to the rRNA before transport of ribosomal subunits to the cytoplasm. During this assembly process of ribosomal proteins, many *trans*-acting factors are associated with the pre-rRNA and their processing products in the form of a preribosomal ribonucleoprotein (pre-rRNP) complex (Piñol-Roma, 1999; Yanagida et al., 2001; Fujiyama et al., 2002). Each of the *trans*-acting factors is associated with the pre-rRNP complex only transiently, when it is carrying out its given action. Because hundreds of *trans*-acting factors are involved in ribosome biogenesis, the processes must give rise to many steps and opportunities where regulation could take place. Therefore, the actions of *trans*-acting factors must be regulated in a dynamic and precise

coordination, spatially and temporally, that regulates their functions from the transcription of rDNA to the assembly and export of preribosomal particles (Leary & Huang, 2001). However, it is not clear which ribosomal proteins and *trans*-acting factors are assembled—where, how, and at what stages of pre-rRNA processing and assembly.

So far, we are sure that the assembly of the ribosomal subunits at early stages takes place in the nucleolus, one of the most essential and prominent subcellular compartments—the site of rRNA precursor gene transcription, and that in these stages ribosomal and *trans*-acting proteins must be transported from the cytoplasm, and 5S rRNA from the nucleoplasm to the nucleolus. To integrate all of these events (i.e., transcription performed by all types of RNA polymerases, translation of ribosomal proteins and *trans*-acting factors, intracellular transport of protein, etc.), and to ensure the balanced production of individual ribosomal components, the eukaryotic cell, even that of the simplest organism such as yeast, must have developed highly ordered strategies to control ribosome biogenesis in response to varieties of physiological changes. Because higher eukaryotic organisms such as mammals are constructed from a number of differentiated cells, their strategies to regulate ribosome biogenesis must be much more diversified (Larson, Zahradka, & Sells, 1991). However, even in yeast cells, the ordered strategies adopted for ribosome biogenesis are not well understood. Still more, the regulation mechanism in mammalian cells is entirely unknown. Thus, it is evident that some comprehensive experimental methodologies are required to analyze the complexity of ribosome biogenesis, and to determine the initial signals, signal transduction pathways utilized, and the specific targets of these regulatory modifications, and how these modifications are used to control ribosome biogenesis as a whole.

Proteomics is a field of post-genome age science that aims to uncover functional protein networks of biological systems through direct large-scale analysis of proteins expressed in cells. The current field of proteomics comprises two major research areas: ''expression proteomics'' analyzes the dynamics of protein expression that accompanies various biological processes and disorders, and ''functional proteomics'' analyzes the functional aspects of cellular proteins, including localization, modification, and more importantly, interactions among the proteins (Takahashi et al., 2003). A typical proteomic study begins with the ''proteome-wide'' analysis of the protein components in a certain cell or tissue. Differential protein expression analysis results in a protein profile that consists of a cluster of proteins that are characteristic of a certain proteomic phenotype, or of a protein subset that is responsible for the cellular events. Functional proteomics analyzes the functional relationship among proteins in the protein cluster, or directly analyzes protein interactions (Isobe & Takahashi, 2000; Takahashi, 2001). Thus, proteomic studies include the determination of quantitative changes in the expression levels of proteins to assess the effects of a wide variety of perturbations to cells, and the comprehensive analysis of protein interactions and their dynamics during cellular processes.

To analyze the functional relationships among proteins, several strategies are used. One of the strategies is organelle component analysis, which is a basic approach to group functionally related proteins. By this approach, cellular organelles, such as the nucleus, nucleolus, nuclear membrane, Golgi apparatus, mitochondrion, and even large cellular machineries—such as the spliceosome complex, the nuclear pore complex, the anaphase-promoting complex, etc., are prepared as purely as possible by, for instance, ultracentrifugation, and the protein components are identified by proteomic analysis (Peters et al., 1996; Zachariae et al., 1996; Neubauer et al., 1998; Rout et al., 2000; Bell et al., 2001; Dreger et al., 2001; Andersen et al., 2002; Scherl et al., 2002). Another strategy frequently applied to study novel protein partners and interactions is a ''pull-down'' approach (Husi et al., 2000; Takahashi, 2000). In that method, a target gene is attached with a specific ''tag,'' such as a FLAG epitope tag or tandem affinity purification (TAP) tag, to produce a DNA construct designed for a ''bait'' protein (Rigaut et al., 1999; Yanagida & Takahashi, 2001). The gene that encodes the bait protein is transfected into cultured cells to allow the tagged protein to be expressed in the cell and to form physiological complexes with its interacting proteins. The entire multi-protein complex can be isolated by the use of an immobilized antibody against the tag. The protein components in the purified complex are subsequently analyzed by mass spectrometry (MS)-based proteomics technologies. Currently, two groups, the Cellzome Corporation in Germany (Gavin et al., 2002) and MDS Proteomics in Canada (Ho et al., 2002), took this approach for the comprehensive analysis of protein complexes in the budding yeast *S. cerevisiae*. Each group characterized hundreds of distinct multi-protein complexes, and revealed the protein complex network and the groups of connected complexes. They showed that many cellular functions are carried out by proteins bound together in complexes as a functional unit that may be coordinated into a higher-order network of interacting protein complexes, and maybe more interestingly that a certain multi-protein complex is not necessarily of invariable composition nor are all protein components uniquely associated with that specific complex (Kumar & Snyder, 2002). Thus, with several distinct tagged proteins as entry points to purify a complex, it will be possible to identify not only the core components of a functional cellular complex, but also more dynamic, perhaps regulatory, components that may be present differentially in the complex.

Here, we summarize the recent, and often surprising, advances in our understanding of ribosome synthesis in the eukaryotic cell. These advances will underscore the unexpected complexity of eukaryotic ribosome synthesis even in the yeast cell—a single eukaryotic cell. Proteomic technologies are powerful methodologies that provide an extra dimension for analysis of the course of such exceptionally complex process. By use of these proteomic approaches combined with conventional approaches, we are able to determine which proteins and which RNAs are associated at a particular stage of ribosome biogenesis. These analyses are now starting to supply extensive information on components from a series of nascent ribosomes isolated with tagged-proteins as affinity bait, which provide snapshot analyses of the processes of ribosome synthesis in the nucle(ol)us. Indeed, proteomic analysis of ribosome biogenesis in *S. cerevisiae* has succeeded in characterizing a number of synthetic intermediates of ribosomal particles, which are thought to be extremely difficult to isolate because of their expected short lives *in vivo*. We have been trying to apply this proteomic approach to the analysis of mammalian ribosome biogenesis that is expected to be much more diversified process than that of yeast (Yanagida et al., 2001; Fujiyama et al., 2002). Our analysis suggests that ribosome biogenesis in mammalian cells may proceed in a highly integrated way with other events, such as the remodeling of the nucle(ol)ar structure, maturation of mRNA, assembly of translational machinery to mRNA, etc. In this review, a proteomic overview of mammalian ribosome biogenesis is presented in conjunction with that of yeast.

II. TRANSCRIPTION AND PROCESSING OF THE PRE-rRNA IN THE RIBOSOME BIOGENESIS OF YEAST CELLS: A MODEL FOR EUKARYOTIC CELLS

In *S. cerevisiae*, a precursor of rRNA is encoded by a 9.1 kb rDNA unit, which is repeated over 100 times on the long arm of chromosome XII. The principle organization of the rDNA unit is illustrated in Figure 1 (Kempers-Veenstra et al., 1986). The transcription of rDNA is controlled by the rDNA enhancer within the intergenic spacer sequences of *S. cerevisiae* rRNA cistrons (Mestel et al., 1989). In addition, three *cis*-acting sites within the rDNA enhancer, designated the modulator region, mediate the termination

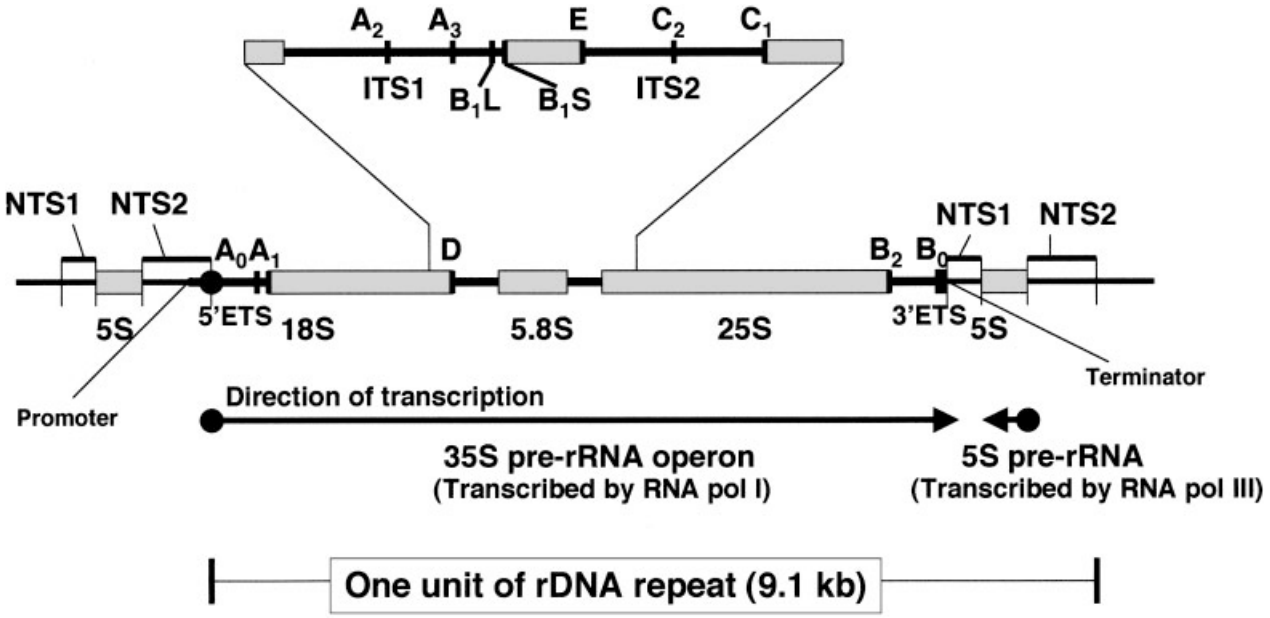


FIGURE 1. Structure of a repeat unit in yeast rDNA. A precursor of rRNA encoded by a 9.1 kb rDNA unit is shown. The one-rDNA unit consists of the 35S pre-rRNA operon and the two non-transcribed spacers (NTS) 1 and 2, interrupted by the 5S rRNA gene. 35S pre-rRNA contains the 18S, 5.8S, and 25S rRNAs sequences that are separated by the two internal transcribed spacer (ITS) 1 and 2 sequences, and are flanked at either end by the two external transcribed spacers (ETS), the 5′-ETS and the 3′-ETS. The processing sites of the 35S pre-rRNA are indicated; A_0, A_1, A_2, A_3, B_1L, B_1S, E, C_2, C_1, and B_2. The entire 35S pre-rRNA operon is transcribed by RNA polymerase I (Pol I) from the 5′-end to 3′-end of rDNA as a large precursor (indicated by a long arrow), whereas the 5S rRNA gene is transcribed in the opposite direction by RNA polymerase III (Pol III) as a precursor of the mature 5S rRNA (indicated by a short arrow).

of transcription by RNA polymerase I, and potentiates the activity of the rDNA enhancer element. Two *trans*-acting factors, Reb1p (rRNA enhancer binding protein 1) and Reb2p (Abf1p), bind independently to sites within the modulator region (Morrow, Johnson, & Warner, 1989), and are required for RNA polymerase I-dependent termination and enhancer function, respectively (Kang, Yokoi, & Holland, 1995). These factors appear to play a crucial role in the efficient transcription of the chromosomal rDNA in yeast cells (Planta, Goncalves, & Mager, 1995).

In mouse cells, it was reported that a homolog of yeast Rrn3p, a polymerase-associated transcription-initiation factor, interacts with the rpa43 subunit of RNA polymerase I and probably regulates rDNA transcription by determining the steady-state concentration of the Rrn3–RNA polymerase I complex within the nucleolus (Cavanaugh et al., 2002). In the case of human rDNA transcription, the rRNA promoter contains two distinct *cis*-control sequences, the core and upstream control elements, that serve as the targets to bind the cellular *trans*-activating proteins that are involved in transcription initiation by RNA polymerase I. The human rRNA promoter appears to be regulated by UBF1 and SL1 (Learned et al., 1986). However, the yeast counterparts (such as Mpl1p, Fun12p, etc.) of human UBF-1 and SL1 as well as Rrn3p are not known to be involved in the transcription of yeast rDNA. In addition, the basic organization of the eukaryotic rRNA operon is similar in all species, including mammals; however, the presence of the 5S rRNA gene within the rDNA repeat is an unusual feature of *S. cerevisiae* among the eukaryotes. Furthermore, a striking difference between the cytoplasmic ribosomes of eukaryotes and prokaryotes is well known to be the presence in eukaryotes of one additional RNA component, the 5.8S rRNA (Nazar, 1984). Thus, some differences in the processes of ribosome synthesis are evident among species, as seen in the rDNA gene configurations and maybe in the *trans*-acting factors involved in rDNA transcription; those differences indicate that those configurations probably cause differences in regulation of the processes at some stages of ribosome biogenesis among eukaryotic cells.

In *S. cerevisiae*, the processing of the pre-rRNA precursor is accompanied by the addition of a large number of covalent modifications, mostly 2′-*O*-ribose methylation and pseudouridylation. Those modifications take place in a coordinated fashion with a series of pre-rRNA processing steps by which external transcribed spacers (ETSs) and internal transcribed spacers (ITSs) are removed by endo- and exo-ribonucleases to produce the three mature rRNAs (18S, 5.8S, and 25S rRNAs) (Fig. 2). Those modifications and processing events proceed in the form of large RNP complexes, and are accompanied by the assembly of ribosomal proteins on the processing intermediates of the mature rRNAs. Hundreds of non-ribosomal proteins and snoRNAs are now identified as *trans*-acting factors that are probably involved in those events in the nucle(ol)us but are not present in the final product of mature ribosome particles after their export to the cytoplasm.

The major intermediates in the pathway of pre-rRNA processing in yeast were originally identified by biochemical analysis; however, the identification of minor processing sites and intermediates that were either too close in size or too short in life to detect during pre-rRNA processing weren't so successful by only biochemical analysis. As a result of the genetic analysis of the mutations in *trans*-acting factors, the yeast pre-rRNA processing pathway is now characterized in far more detail than that of other eukaryotes (Lafontaine & Tollervey, 1995). So far, we know the detail of the processing pathway of the primary 35S pre-rRNA in *S. cerevisiae*, as illustrated in Figure 2 (Kressler, Linder, & Cruz, 1999; Venema & Tollervey, 1999).

III. *TRANS*-ACTING FACTORS INVOLVED IN THE PRE-rRNA PROCESSING AND ASSEMBLY IN YEAST CELLS

A. *Trans*-Acting Proteins That Act with snoRNAs

Coupled with the ongoing transcription of the 35S pre-rRNA, or shortly after the transcription is completed, the processing of the transcript begins with concomitant covalent modification, including the isomerization of uridine to pseudouridine (pseudouridylation) by base rotation, methylation of the 2′-hydroxy group of ribose (2′-*O*-ribosemethylation), and base methylation in the rRNA (Kressler, Linder, & Cruz, 1999; Venema & Tollervey, 1999). A group of nucleolar RNPs that contain small RNAs (small nucleolar RNAs or snoRNAs) are involved in these post-transcriptional modifications. The snoRNAs are present specifically in the nucleolus; thus, the name originates from their localization (Mattaj, Tollervey, & Seraphin, 1993). Many of the identified snoRNAs (∼75 bps) have been proven to be involved in ribosome biogenesis in yeast cells, and are summarized by Samarsky & Fournier; the list of the snoRNAs can be seen through the Internet (http://www.bio.umass.edu/biochem/rna-sequence/Yeast snoRNA Database/mastertable.html). They are classified into three families; most of snoRNAs belong to either the C/D-box, or the H/ACA-box family, and the RNA component of RNase MRP (mitochondrial RNA-processing enzyme), a relative of RNase Ps, constitutes one family by itself. The former two-snoRNA families guide modifications of pre-rRNA by base-pairing near target sites. The latter family is probably involved in cleavage of pre-rRNA by mechanisms that have not yet been identified. Many of the snoRNAs identified have the potential to form a nearly perfect duplex with rRNA. The

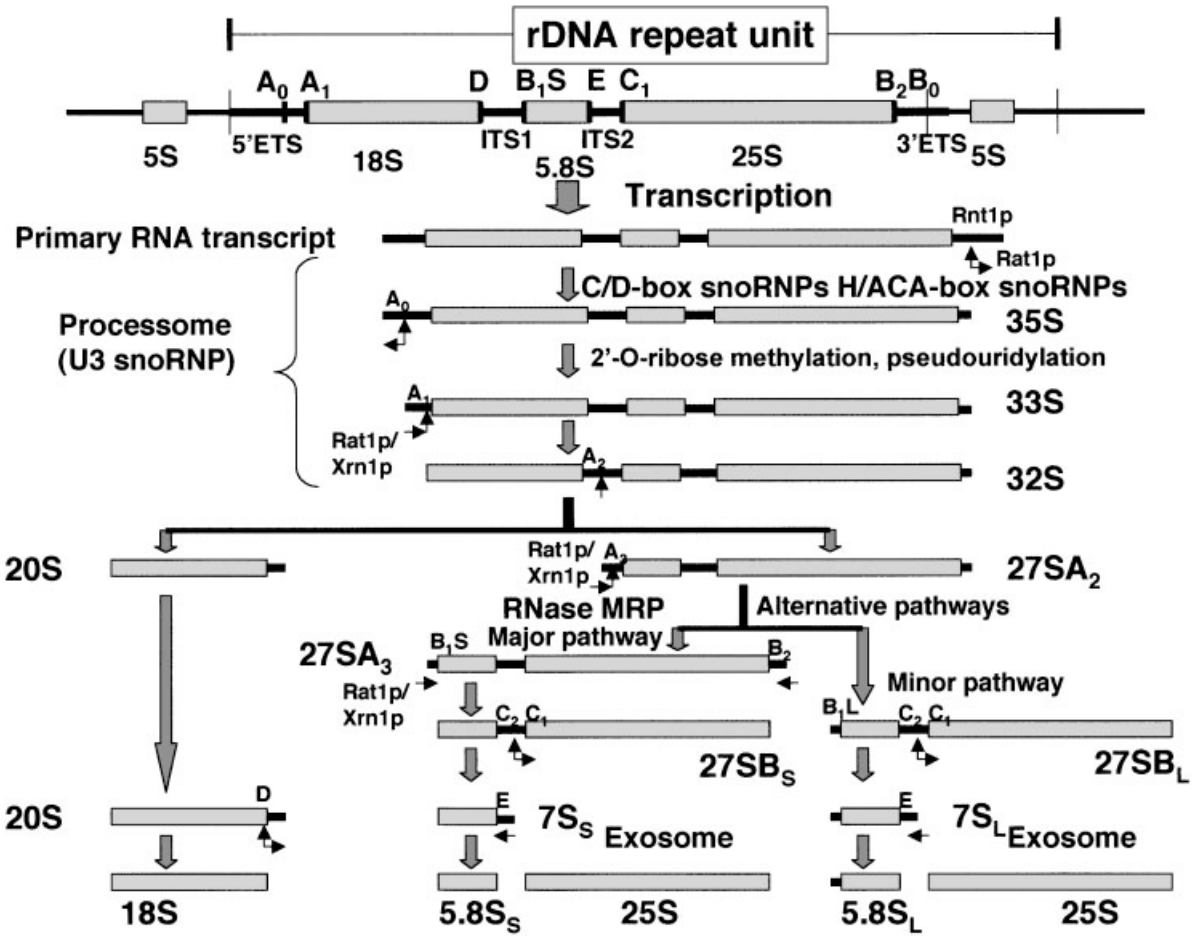


FIGURE 2. The processing pathway of the primary 35S pre-rRNA in yeast. The transcript of the 35S pre-rRNA operon is released from the transcriptional machinery by co-transcriptional cleavage at a site within 3′ETS by endonuclease Rnt1p. The initial processing site of the 35S at A_0, yields 33S, which is further cleaved at site A_1 to produce 32S. By the cleavage at A_2, the 32S is split into 20S and $27SA_2$, which are destined for the 40S and 60S ribosomal subunits, respectively. 20S is processed by cleavage at D to produce the mature small subunit 18S rRNA species. $27SA_2$ is processed by two alternative pathways (major and minor) to result ultimately in mature 5.8S and 25S for the 60S subunit. In the major pathway, cleavage at site A_3 yields $27SA_3$, which is converted to $27SB_S$ by exonucleolytic digestion. In the minor pathway, $27SA_2$ is cleaved at B_1L instead of at A_3 to produce $27SB_L$. Identical steps further process the $27SB_S$ and $27SB_L$ species; those precursors are cleaved at C_2 and are processed to C_1 to release mature 25S and either $7S_S$ or $7S_L$, which is processed from C_2 to the 3′ end of 5.8S by exonuclease digestion to generate $5.8S_S$ or $5.8S_L$. Endonucleolytic cleavage sites are indicated by ↑. Sites and directions of exonucleolytic processing are indicated by → for 5′ to 3′ exonucleolytic processing, and by ← for 3′ to 5′ exonucleolytic processing. Functional complexes (SSU processome, C/D-box snoRNPs, H/ACA-box snoRNPs, exosome, and RNase MRP) are indicated at the steps where they work, or are expected to work.

association of snoRNA disrupts rRNA stems or other important interactions to maintain rRNA structure (Steitz & Tycowski, 1995). Thus, snoRNAs are believed to act mostly as molecular chaperones to assist maturation, modification, and assembly of the rRNA during ribosome biogenesis, even though some snoRNAs may catalyze the cleavage of pre-rRNAs as a ribozyme.

Approximately 50 snoRNAs have been reported to belong to the C/D-box family in yeast. This family of snoRNA is responsible for the methylation of rRNAs. Each C/D-box snoRNA has one or two sets of well-conserved short sequence elements that are called box C (RUGAUGA) and box D (CUGA), and approximately 10–20 nucleotides with sequence complementarity to rRNA.

Forty-one snoRNAs are now identified to correspond to 51 of the 55 sites of 2′-*O*-ribose methylation in the yeast 35S pre-rRNA (Venema & Tollervey, 1999). All members of this family are associated with the proteins Nop56p, Nop5p/Nop58p, and Nop1p (Fig. 3A). Nop1p is probably the methyl-transferase, and is the yeast homolog of the vertebrate fibrillarin. These proteins are thought to form the core of each C/D-box snoRNP (Kressler, Linder, & Cruz, 1999).

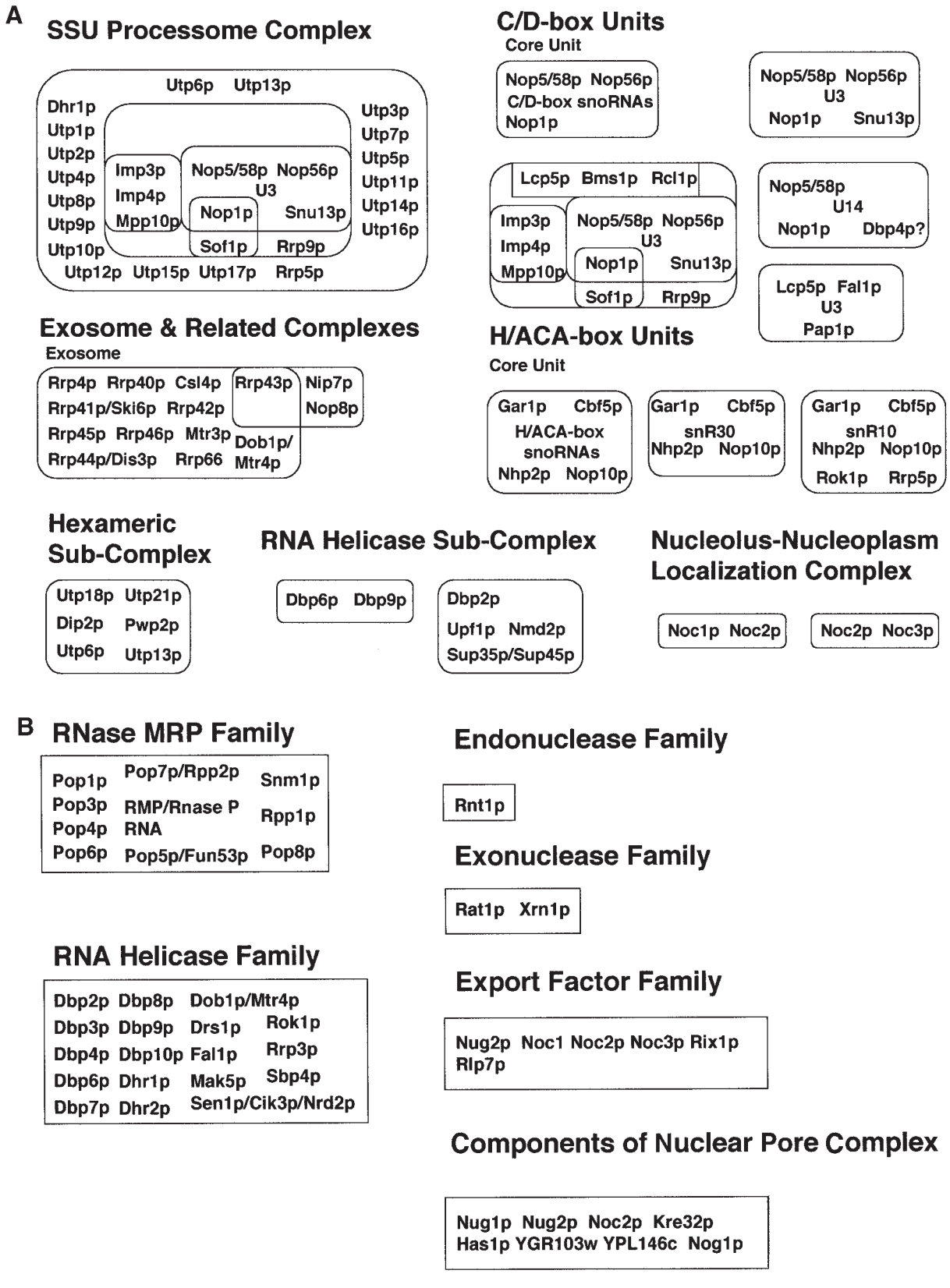
U3 snoRNA, a member of the C/D-box family of snoRNAs, is the most abundant and ubiquitous in eukaryotes, and is required to process pre-18S rRNA. It forms base-pairs with the region in the 5′-ETS of the 35S pre-rRNA that contains the A_0, A_1, and A_2 sites, and also forms base-pairs with the 5′-terminal region of the mature 18S rRNA (Fig. 2). Thus, the U3 snoRNA is though to be required to process at sites A_0, A_1, and A_2 in the 35S pre-rRNA (Venema & Tollervey, 1999). It had been shown by conventional biochemical and genetic analyses that the snoRNP that contains the U3 snoRNA differed in structure from other C/D-box snoRNPs; in addition to the core proteins that are common to C/D-box snoRNPs, U3 snoRNP contained at least eight more proteins-Sof1p, Mpp10p, Rrp9p, Imp3p, Imp4p, Lcp5p, Rcl1p, and Bms1p (Fig. 3A) (Billy et al., 2000; Wegierski et al., 2001). However, the conventional biochemical and genetic analyses have not shown the total picture of this snoRNP until the proteomic approach was applied to the analysis of the U3 snoRNP complexes described in a later section; briefly, the U3 snoRNA was found to form a large 80S RNP complex that contained at least 28 proteins. Of those, 18 proteins were newly identified as the components of the U3 snoRNP by proteomic analysis. This large complex functions in the production of the ribosome small subunit and is named as the ''small subunit (SSU) processome'' (Fig. 3A) (Dragon et al., 2002).

Although most of the C/D-box snoRNAs are in principle responsible for the methylation of rRNAs, U3 snoRNA plays an exceptional role in the rRNA processing as a component of the SSU processome. The only other snoRNA that is required for the cleavage reactions, and that is involved in the processing of the mature 18S rRNA, is U14 snoRNA. This snoRNA is responsible for pre-rRNA processing at A_1 and A_2, and plays a dual role in these processing events; i.e., it has roles in rRNA cleavage, and as a guide for a site-specific 2′-*O*-ribose methylation. However, it is not clear how U3 and U14 snoRNAs are acting coordinately in the processing of the 5′ ETS of the 35S pre-rRNA at sites A_1 and A_2. This U14 snoRNA also forms a snoRNP with Nop1p and Nop5p/Nop58p, and also probably with Dbp4p (Fig. 3A). Dbp4p is a putative ATP-dependent RNA helicase, and genetically suppresses the function of U14 snoRNP (Liang, Clark, & Fournier, 1997). The U14-associating snoRNP has not been characterized yet by a proteomic approach. Still more, the proteomic analysis of RNP complexes for the other snoRNAs is entirely lacking.

Meanwhile, approximately 20 H/ACA-box snoRNAs are known, and they are responsible for the isomerization of uridine to pseudouridine (pseudouridylation) by base rotation at 45 sites in the rRNAs in *S. cerevisiae*. Each H/ACA-box snoRNA pairs to either one or two pseudouridylation sites in the rRNA (Ni, Tien, & Fournier, 1997). The site selection is mediated by short base-pairings between the snoRNA and the pre-rRNA, and pseudouridylation occurs at a fixed distance of approximately 14 nucleotides from the conserved snoRNA sequence elements, called box H and/or ACA triplet, in the pre-rRNA (Kressler, Linder, & Cruz, 1999). One of the well-known H/ACA snoRNAs, snR30, is required for the early pre-rRNA processing reactions at A_1 and A_2. The other only box H/ACA snoRNA that participates in the early pre-rRNA processing, is snR10. However, all of the remaining identified H/ACA-box snoRNAs are not involved in the rRNA processing, but only in pseudouridylation. As is the C/D-box snoRNA, each H/ACA-box snoRNA is associated with the *trans*-acting proteins, Cbf5p, Gar1p, Nhp2p, and Nop10p, and forms a core snoRNP complex (Fig. 3A), which is proposed to contain two copies of each of the four proteins and one H/ACA-box snoRNAs. Of the four core proteins, Cbf5p is probably pseudouridase. At present, however, the detailed architecture of H/ACA-box snoRNPs and the order of their assembly are not established. Thus, again, the proteomic approach is waiting to be applied to the analyses of those snoRNPs.

B. *Trans*-Acting Factors Involved in the rRNA Cleavages

The ETSs and ITSs of 35S pre-rRNA, which is generated by endonucleolytic cleavage of the initial rDNA transcript around nucleotide +30 relative to the 3′-end of the mature 25S rRNA (probably by endo-RNase Rnt1p, a homolog of bacterial RNase III), are removed by subsequent endo- and exonucleases to produce the three mature rRNAs (18S, 5.8S, and 25S rRNAs) during the rRNA processing (Kressler, Linder, & Cruz, 1999). The earliest processing in the 35S pre-rRNA is the endonucleolytic cleavage at site A_0, and is mediated by the SSU processome complex that is characterized by proteomic analysis, and is shown to contain U3 snoRNA and 28 protein components. However, no proteinaceous enzyme (so-called endonuclease) has been found in the SSU processome, and any catalytic ribozyme activity that originates from the *cis*-element of the rRNA or snoRNA has yet been specified. Thus, the mechanism that underlies the cleavage at A_0 is uncharacterized. The only well-characterized endonuclease is RNase MRP that participates in 5.8S ribosomal RNA maturation and is responsible for cleavage of pre-rRNA


A
SSU Processome Complex
Utp6p Utp13p
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Imp3p
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Nop5/58p Nop56p
U3
Nop1p
Snu13p
Sof1p
Rrp9p
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C/D-box Units
Core Unit
Nop5/58p Nop56p
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Nop1p
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U3
Nop1p Snu13p
Lcp5p Bms1p Rcl1p
Imp3p
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Mpp10p
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Nop1p
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Sof1p
Rrp9p
Nop5/58p
U14
Nop1p Dbp4p?
Lcp5p Fal1p
U3
Pap1p
Exosome & Related Complexes
Exosome
Rrp4p Rrp40p Csl4p
Rrp41p/Ski6p Rrp42p
Rrp45p Rrp46p Mtr3p
Rrp44p/Dis3p Rrp66
Rrp43p
Nip7p
Nop8p
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H/ACA-box Units
Core Unit
Gar1p Cbf5p
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Gar1p Cbf5p
snR30
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Gar1p Cbf5p
snR10
Nhp2p Nop10p
Rok1p Rrp5p
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Utp6p Utp13p
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Upf1p Nmd2p
Sup35p/Sup45p
Nucleolus-Nucleoplasm
Localization Complex
Noc1p Noc2p
Noc2p Noc3p
B
RNase MRP Family
Pop1p
Pop3p
Pop4p
Pop6p
Pop7p/Rpp2p
RMP/Rnase P
RNA
Pop5p/Fun53p
Snm1p
Rpp1p
Pop8p
Endonuclease Family
Rnt1p
Exonuclease Family
Rat1p Xrn1p
RNA Helicase Family
Dbp2p Dbp8p Dob1p/Mtr4p
Dbp3p Dbp9p Drs1p Rok1p
Dbp4p Dbp10p Fal1p Rrp3p
Dbp6p Dhr1p Mak5p Sbp4p
Dbp7p Dhr2p Sen1p/Cik3p/Nrd2p
Export Factor Family
Nug2p Noc1 Noc2p Noc3p Rix1p
Rlp7p
Components of Nuclear Pore Complex
Nug1p Nug2p Noc2p Kre32p
Has1p YGR103w YPL146c Nog1p

FIGURE 3.

at site A_3. RNase MRP is a nucleolar ribonucleoprotein particle that contains at least 10 protein components—Pop1p, Pop3p, Pop4p, Pop5p, Pop6p, Pop7p/Rpp2p, Pop8p, Rpp1p, Snm1p, and Xrn1p—all of which (except Snm1p and Xrn1p) share an RNA structural motif with ribonuclease P (RNase P) (Fig. 3A). RNase MRP and RNase P are both located in mitochondria (Lee et al., 1996), and the RNA species present in the RNase MRP is also structurally related to that of RNase P; that relationship suggests that the two RNase complexes are functionally and phylogenetically related to each other. So far, although the involvement of endonuclease in the cleavage at site A_3 of the 35S pre-rRNA is established, we still don't know which endonucleases perform cleavages at the other endonucleolytic cleavage sites, including A_1, A_2, B_1, C_1, and C_2, and even whether a *trans*-acting endonucleotlyic enzyme really participates in the cleavage reaction or the cleavage is catalyzed by *cis*-element in the rRNA or snoRNA as ribozyme.

Contrary to the lack of information about endonucleases in rRNA processing, the enzymes responsible for most of the exonucleolytic processings of pre-rRNAs have been well characterized. For example, the 5′ to 3′ exonucleases, Xrn1p and Rat1p, both have a role in the 5′ to 3′ exonucleolytic digestion of the $27SA_3$ pre-rRNA up to site B_1S, and produce the 5′ end of the major form of 5.8S rRNA (Fig. 2) (Henry et al., 1994; Moy & Silver, 1999). In addition, these enzymes are also known to be required for the degradation of the A_0–A_1, D–A_2, and A_2–A_3 fragments, and are responsible for the formation of the 5′ end of the processing intermediates of the pre-rRNAs that have these spacer fragments. Furthermore, Xrn1p and Rat1p, coupled with Rnt1p, which is involved in the processing of the primary RNA transcript, also participate in the 5′ to 3′ mRNA decay pathway and in the post-transcriptional processing of snoRNAs, such as U14, U18, U24, snR190, etc., from larger precursors. The multifunctional roles of Xrn1p and Rat1p may represent the necessity of the coordinated regulation of those events. The proteomic approach may be useful for the analysis of the coordinated regulation of these proteins.

The well-characterized exonuclease is ''exosome,'' which is a complex of 3′ to 5′ exonucleases involved in RNA processing and degradation. This complex is composed of at least 11 known components: Rrp4p, Rrp6p, Rrp40p, Rrp41p/Ski6p/Ecm20p, Rrp42p, Rrp43p, Rrp44p/Dis3p, Rrp45p, Rrp46p, Cs14p, and Mtr3p (Fig. 3A). The 3′ end of the 5.8S rRNA is generated from site C_2 by this exosome complex (Fig. 2). Each component of the exosome has 3′ to 5′ exoribonuclease activity, and is required for processing. The mode of action of these exonucleases is predicted from their kinetic analyses and/or sequence homology to the other exonucleases, whose modes of action have been characterized (Kressler, Linder, & Cruz, 1999). In addition to these 11 components, Nip7p, which is not an exonuclease and is required for 60S ribosomal subunit synthesis, was shown to interact with one of the exosome components Rrp43 as well as with Nop8—the other *trans*-acting factor involved in the processing of the 35S pre-rRNA and the 25S pre-rRNA (Fig. 3A) (Zanchin & Goldfarb, 1996). This result suggests that the function of the exosome extends beyond its function in the 5.8S rRNA processing as an exonuclease. In fact, the depletion of each individual component inhibits not only the 3′-end processing of the 5.8S rRNA, but also the early pre-rRNA cleavages at sites A_0, A_1, A_2, and A_3, to reduce the levels of the 32S, 20S, $27SA_2$, and $27SA_3$ pre-rRNAs (Allmang et al., 2000). Because none of these processing steps involves 3′ to 5′ exonuclease activities, the requirement for the exosome is probably indirect; that requirement suggests that the action of the exosome affects other *trans*-acting factors. Conversely, the exonucleolytic action itself is affected by other *trans*-acting factors. For example, the putative helicase Dob1p/Mtr4p, which is involved in 60S ribosomal subunit synthesis, functions as a cofactor for the exosome in pre-rRNA processing (Allmang et al., 2000). These results imply that the correct assembly of *trans*-acting factors with the pre-rRNAs may be monitored by a quality control system that inhibits pre-rRNA processing. Since the exosome degrades aberrant pre-rRNAs that arise from such an inhibition, the exosome itself may be involved in a part of the quality control systems. Because the exosome, together with Ski2p that is the cytoplasmic homolog of Dob1p, also functions in the 3′ to 5′ processing of cytoplasmic mRNAs (Anderson & Parker, 1998), the exosome may associate with the other additional components that affect the exonuclease activity of the exosome and/or that work together with the exosome.

FIGURE 3. **A**: Possible assembly units and/or functional complexes formed during yeast ribosome biogenesis. Based on the reported physical association, each assembly unit and functional complex is grouped, and encloses its constituents with a box. Some contain multiple assembly units and functional complexes, each of which is reported to be grouped by physical association in one unit or complex. If the direct interaction between two proteins were known, then it is indicated by a dot between the two proteins' names. **B**: Functionally related protein families involved in yeast ribosome biogenesis. Although a physical association is not known, proteins that are classified as an enzyme family, or that are involved in a related function, are grouped within a square.

However, we still don't know the entire picture of these coordinated actions for exonucleases.

C. *Trans*-Acting Factors Involved in the rRNA Editing and Conformational Rearrangement

Although the precise roles in ribosome biogenesis have not been established, there is another group of *trans*-acting factors, called DEAD-box RNA helicases. They are believed to possess the activity of ATP-dependent RNA helicase-based on their sequence similarity to the known ATP-dependent RNA helicases; therefore, they are collectively referred to as the putative ATP-dependent RNA helicase. So far, their ATP-dependent RNA-helicase activities have not been demonstrated. We now know at least 18 members of the DEAD-box RNA helicase family that are involved in ribosome biogenesis in yeast cells—Dbp2p (Bond et al., 2001), Dbp3p, Dbp4p, Dbp6p, Dbp7p, Dbp8p, Dbp9p, Dbp10p, Dhr1p, Dhr2p, Dob1p/Mtr4p, Drs1p, Fal1p, Mak5p, Rok1p, Rrp3p, Sbp4p, and Sen1p/Cik3p/Nrd2p (Fig. 3B) (Venema & Tollervey, 1999). Of those members, Dbp6p and Dbp9p weakly interact with each other (Daugeron, Kressler, & Linder, 2001), and Dob1p/Mtr4p was found to become a component of the exosome complex in some situations (Fig. 3A) (Allmang et al., 2000). A *trans*-acting factor, Dbp2p, interacts with Upf1p—an essential component of the nonsense-mediated mRNA decay pathway, and also interacts with Nmd2p, and Sup35p/Sup45p that is a ribosome-associating protein (Fig. 3A) (Bond et al., 2001). However, an understanding of the roles of these RNA helicases in ribosome biogenesis is still lacking. Their functions are only speculated to be involved in the association and dissociation of the pre-rRNA with the snoRNAs, in the folding of the rRNAs themselves, as well as in the rearrangement of protein–RNA interactions during the assembly of ribosomal proteins (Warner, 2001). In general, RNA helicases of the DEAD-box proteins form a very large superfamily of proteins that is conserved from viruses to humans. They are associated with all processes that involve RNA molecules, including transcription, editing, splicing, ribosome biogenesis, RNA export, translation, RNA turnover, and organelle gene expression. Thus, those RNA helicases are believed to act as ATP-dependent modulators of RNA structure, whose functions are conserved throughout evolution and in all species (Tanner & Linder, 2001). Functional proteomic approaches may be effective to search the functions of this large family of RNA helicases.

In this section, we have tried to group *trans*-acting factors that are involved in ribosome biogenesis mainly by their physical association to catalog data obtained by proteomic analysis of the intermediates of synthetic ribosome isolated by the use of *trans*-acting factors as affinity bait. There are many other *trans*-acting factors that are not described here, and that involve ribosome biogenesis. For those factors, few data are available in terms of physical association among *trans*-acting factors. Venema and Tollervey (Venema & Tollervey, 1999) and Kressler et al. (Kressler, Linder, & Cruz, 1999) reviewed extensively the detailed involvements of those individual *trans*-acting factors in ribosome biogenesis and their genetic interactions. In addition, the number of the *trans*-acting factors newly found has still been increasing because of the continuing efforts of conventional biochemical and genetic approaches applied to individual proteins or genes. From the results of those analyses, one can speculate endlessly about the role of individual proteins that might be involved in this immensely complicated process. After all of that speculation, it will not be so easy to verify that a specific function can be assigned to any one of them in such complex process, in which hundreds of *trans*-acting factors as well as ribosomal proteins are probably interrelated functionally through a physical association with one another in their complicated interaction web and that function simultaneously in a coordinated fashion. Furthermore, a certain set of the *trans*-acting proteins probably is associated only transiently with the intermediates of the mature ribosome, when it is doing its appointed work. Thus, it is necessary to determine in a systematic order which proteins and which RNAs are associated at a particular stage of the formation of ribosome particles.

IV. ISOLATION AND PROTEOMIC CHARACTERIZATION OF PRE-rRNP COMPLEXES FORMED DURING RIBOSOME BIOGENESIS IN YEAST CELLS

A. Experimental Approaches to Characterize Pre-rRNP Complexes

Ribosome biogenesis encompasses a complicated series of events that involve hundreds of transiently interacting components. Insight into a mechanism for coordinating some of these events is now coming from the characterization of a functional pre-rRNP complex (Peculis, 2002). Early attempts to isolate pre-rRNP complexes by ultracentrifugation analysis with a sucrose density gradient had been made a number of times, and recognized consistently 80S and 55S pre-ribosomes in a number of organisms. However, no pre-rRNP complex had been isolated in a form pure enough to analyze in detail the protein constituents. The main difficulties included the determination of a specific association between the RNA and the co-purifying proteins, and the distinction from other RNP complexes that co-sedimented with pre-rRNP complexes by ultracentrifugation (Yanagida et al., 2001). The use of an

immunopurification approach was also attempted with an antibody specific to a certain *trans*-acting protein involved in ribosome biogenesis to isolate and analyze pre-rRNP complexes. That approach succeeded somewhat in eliminating unwanted contaminations such as heterogeneous nuclear ribonucleoproteins (hnRNPs) that were thought to be major contaminants by ultracentrifugation (Piñol-Roma, 1999). However, on another front, the use of an antibody yielded new contaminations; i.e., other sets of proteins bound nonspecifically to the antibody. That approach allowed the identification of only a few protein components by immunoblot analysis. Because of the limitation in the number of available antibodies for the immunoblot analysis, the characterization of the isolated pre-rRNP complex was still at a primary stage. To isolate the pre-rRNP complexes in a pure form, we have first adopted an epitope affinity tag, an octapeptide FLAG, incorporated into a *trans*-acting factor involved in ribosome biogenesis. The method was applied to a series of mammalian *trans*-acting factors, including nucleolin, to isolate pre-rRNP complexes. The method could have drastically reduced protein contamination in the isolated complexes because the method depended on the highly selective binding between the FLAG-tag and anti-FLAG antibody, and on its specific dissociation with a FLAG peptide (Yanagida et al., 2001). At the time we had applied this approach to isolate pre-rRNPs, the isolation of stable pre-rRNP complexes was thought to be quite difficult because of the speed with which the cell modifies and processes the pre-rRNA. However, we have shown in the first place that, despite the immaturity of the assembly of ribosomal proteins, some pre-ribosomal particles formed in the nucleolus could be isolated as stable intermediates. That approach made possible the large-scale identification of the protein constituents in the isolated pre-ribosomal particles of mammalian origin by mass spectrometry (described below).

Essentially the same approach and the idea as we used were applied to the analysis of a series of pre-ribosomal particles pulled down by the use of various *trans*-acting factors involved in yeast ribosome biogenesis as affinity baits. In those cases, instead of FLAG-tag, a tandem affinity purification tag—known as TAP-tag that is attached to a target protein to create bait protein—was mostly used (Bassler et al., 2001; Harnpicharnchai et al., 2001). In the TAP-method, the complex interacted with a bait protein that was fused with TAP-tag, which has the calmodulin-binding peptide and the IgG-binding unit of protein-A of *Staphylococcus aureus* connected by TEV-protease cleavage sequence, is recovered from cell extract first by affinity selection on an IgG matrix, released by TEV-protease from IgG matrix, and again trapped with calmodulin-coated beads in the presence of calcium (Rigaut et al., 1999). The bait-associating complex was recovered by eluting with EGTA, and purified by ultracentrifugation with a sucrose density gradient. The protein components in the complex (isolated as a single particle in terms of size) were separated by SDS–electrophoresis, each stained protein band was cut, and proteins were digested with either trypsin or lysyl-endopeptidase (Lys-C). The peptides were analyzed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) and/or electrospray ionization (ESI)–tandem mass (MS/MS) spectrometry methods.

So far, there were several reports that dealt with yeast ribosome biogenesis in which ca. two dozen yeast *trans*-acting proteins have been applied as affinity baits fused with the TAP-tag or FLAG-tag to pull down their associated protein complexes (Bassler et al., 2001; Harnpicharnchai et al., 2001; Dragon et al., 2002; Grandi et al., 2002; Nissan et al., 2002). One of the methods that is unique to the proteomic approaches employed in those analyses (we would like to call it a 'double-tagging' approach) relies on the simultaneous epitope tagging of two components (Fig. 4A). The large U3 snoRNP complex was isolated by adopting this approach. The TAP-tagged Mpp10 and the FLAG-tagged Nop5p/Nop58p were co-expressed in the yeast cell to allow the two modified proteins to form physical complexes with other proteins within a cell (Dragon et al., 2002). The first isolation step immobilizes a mixture of U3 snoRNP complexes that contain the TAP-tagged Mpp10 through the protein—A portion of the TAP-tag, which is released by TEV-protease. In the second step, only the U3 snoRNP complex that contains the FLAG-tagged Nop5p/Nop58p is trapped on an anti-FLAG antibody column, and is released with an excess of FLAG peptides. This affinity purification method with two-distinct bait proteins allows the selection of the protein complex that only contains both bait proteins; thus, one can eliminate other protein complexes that are formed by interactions with only one of the bait proteins. With this approach, the U3 snoRNA was found to form a large 80S RNP complex that contained at least 28 proteins, including all of the known proteins except Lcp5p, Rcl1p, and Bms1p. Eighteen of the proteins (Utp1∼17 and Rrp5p) that were present had not been identified in the U3 snoRNP by conventional biochemical and genetic analyses (Fig. 3A). This large complex functions in the production of the mature 18S rRNA, is probably responsible for the cleavages of 35S pre-rRNA at sites A_0, A_1, and A_2, and is proposed to be the SSU processome, which probably corresponds to the terminal knobs present at the 5′-ends of nascent pre-rRNAs that are seen during on-going rDNA transcription (Dragon et al., 2002). Thus, the SSU processome is probably the first machinery to initiate the processing of the nascent 35S rRNA transcript, and thus to trigger the successive pre-rRNA cleavage at sites A_0, A_1, and A_2, and small subunit assembly (Dragon et al., 2002). The finding that Rrp5p is present in the SSU processome is

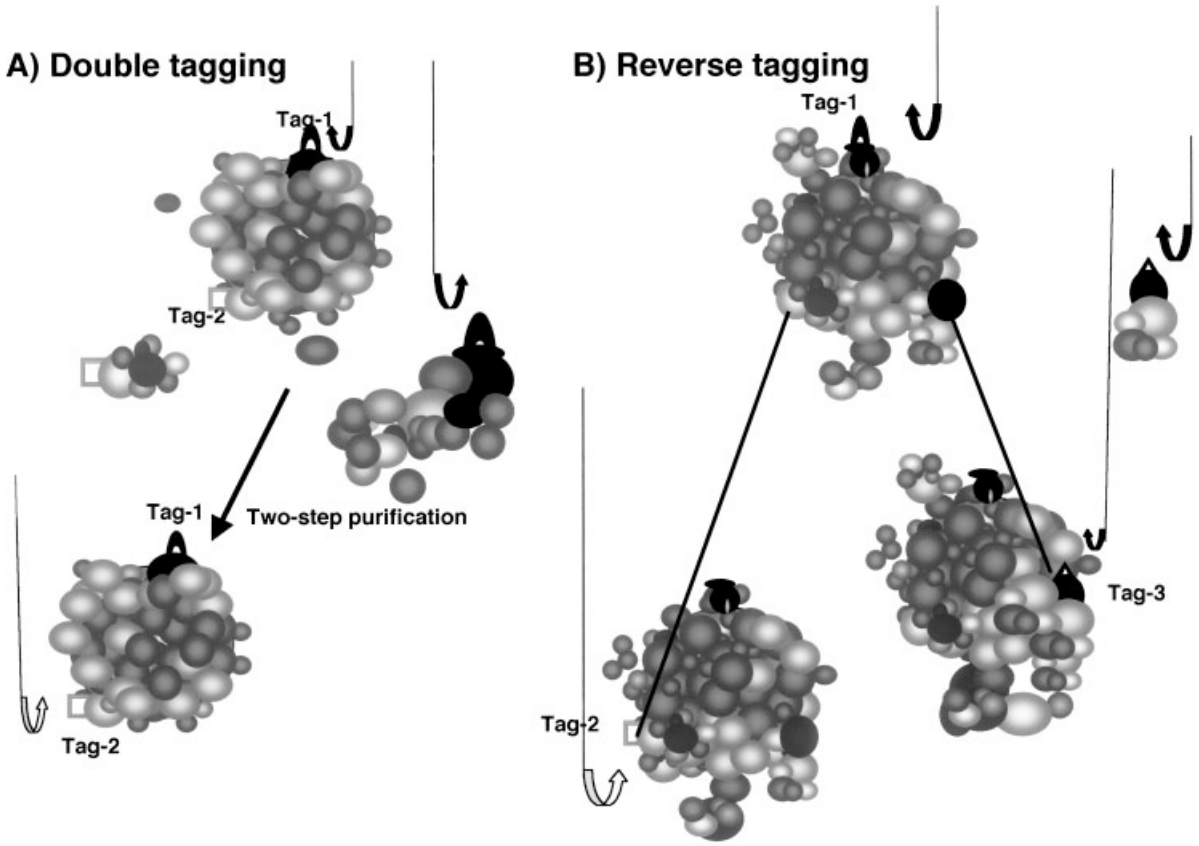

FIGURE 4. Schematic view for isolating protein complexes. **A**: Double-tagging method: Two bait proteins with different tags (tag-1 and tag-2) are expressed in the cell to form physical complexes with other proteins within a cell. The affinity-purification method with two-distinct bait proteins allows selection of the protein complex that only contains both bait proteins; thus, it can eliminate other protein complexes that are formed by interacting with only one of the bait proteins. **B**: Reverse-tagging method: Some of the associated proteins in one complex initially isolated can also be present in other precursor complexes, and thus can allow the purification of pre-ribosomes from different stages of maturation. Thus, protein components present in the first isolated protein complexes are used as an affinity bait to pull down other precursor complexes (tag-1, tag-2, and tag-3).

quite intriguing because Rrp5p is the only known *trans*-acting factor that is essential for the synthesis of 18S rRNA and 5.8S rRNA (Eppens et al., 1999). The presence of Rrp5p suggests that the processing reaction required for 5.8S rRNA is directly linked with that for 18S rRNA carried by the SSU processome. This dual role of the Rrp5p molecule in the synthesis of 18S and 5.8S rRNAs can be attributed separately to its N-terminal and C-terminal domains. Thus, the presence of Rrp5p in the SSU processome explains the coordinate processing reactions that occur at early stages, carried by the U3 snoRNP that is responsible for A_0, A_1, and A_2 cleavages and at a later stage mediated probably by RNase MRP that is responsible for A_3 cleavage of rRNA (Venema & Tollervey, 1999). This example demonstrates the power of the proteomic approach to integrate the independent results obtained by conventional analyses into one plausible explanation.

The other method unique to proteomics is called a 'reverse tagging' methodology (Fig. 4B). The idea behind that methodology is that some of the associated proteins in one initially isolated complex can also be present in other precursor complexes, and thus would allow the purification of pre-ribosomes from different stages of maturation. That method was adapted to the analyses of the Pwp2p-associating pre-rRNP and the Nug1p-associating pre-rRNP complexes, respectively. Eleven proteins were selected as reverse-tagging bait from the components of the former RNP complex (Grandi et al., 2002), and six proteins from the latter pre-rRNP complex (Nissan et al., 2002). By the use of this unique proteomics approach, a number of stable

pre-ribosomal RNP complexes were isolated in series, and were characterized in detail by mass spectrometric analysis coupled with conventional biochemical and genetic analyses. Those analyses showed that each isolated pre-rRNP complex differs in its protein and RNA composition; those differences reflect the presence of a series of the distinct nucleolar and/or nucleoplasmic and/or cytoplasmic pre-ribosomal particles. By those analyses, it also became apparent that a given bait protein can be associated with many different pre-rRNP complexes, yet when purified is preferentially associated with one of the pre-rRNP complexes. That association occurs probably because the concentration of a given bait protein differs in the various pre-rRNP complexes at steady state. Thus, these analyses provided sequential snapshots of the biochemical composition of the pre-ribosomal particles along the various stages of ribosome biogenesis (Grandi et al., 2002; Nissan et al., 2002).

B. 90S Pre-rRNP Complexes Formed at Very Early Stages of Ribosome Biogenesis

The proteomic analyses of the isolated pre-rRNP complexes revealed that pre-ribosomal particles formed at one of the earliest stages of ribosome biogenesis are, so far, the ones isolated by the use of Pwp2p as the affinity bait as well as by the use of each of the 12 *trans*-acting proteins (Rrp9p, YDR449cp, Krr1p, Noc4p, Kre31p, Bud21p, Nop58p, YHR196wp, YGR090wp, Enp1p, YJL109cp, and Nop14p) selected from the components of the Pwp2p-associating pre-rRNP complex as the reverse tagging baits (Grandi et al., 2002). Each isolated pre-ribosomal particle is located in the nucleolus, is approximately 90S in size, and contains the 35S pre-rRNA and U3 snoRNA. A notable feature of the isolated 90S pre-rRNP complexes, in terms of the protein composition, is the predominant presence of the *trans*-acting factors involved in the 40S subunit formation and the components of the SSU processome complex.

Out of the 40 non-ribosomal proteins identified in the 90S pre-rRNP complexes, fourteen proteins (Gar1p, Rrp12p, Krr1p, Kre31p, Kre33p, Utp20p, Utp22p, Noc4p, Nop14p, Emg1p/Nep1p, Enp1p, Enp2p, Bfr2p, and YKR060wp) are the *trans*-acting factors involved in 18S rRNA processing and assembly (Fig. 5A) and 22 proteins (Bms1p, Nop1p, Nop5/58p, Nop56p/Sik1p, Sof1p, Rrp5p, Rrp9p, Dhr1p, Imp3p, Imp4p, Mpp10p, Utp1p, Utp2p, Utp4p, Utp6p, Utp8p, Utp9p, Utp10p, Utp12p, Utp13p, Utp15p, and Utp17p) are the proteins reported to be associated with the U3 snoRNA. Twenty-one of those proteins are components of the SSU processome complex (Dragon et al., 2002; Grandi et al., 2002); the presence of those components indicates that the 90S pre-rRNP complexes are principally the SSU processome-associating pre-ribosome particles. However, the seven components (Snu13p, Utp3p, Utp5p, Utp7p, Utp11p, Utp14p, and Utp16p) identified in the SSU processome have not been found in any of the isolated 90S pre-rRNP complexes (Figs. 3 and 5). Instead, Bms1p (a GTP-binding protein, which is known to associate with U3 snoRNA and to be involved in 40S subunit formation but is not a component of the SSU processome) is present in the 90S pre-rRNP complexes. Because, upon depletion of Snu13p, C/D-box snoRNAs failed to accumulate in the nuclear body and delocalized to the nucleoplasm (Verheggen et al., 2001), the replacement of Snu13p by Bms1p in the 90S pre-rRNP complexes may trigger the successive disassembly of the SSU processome components from the 90S pre-rRNP complexes. It would be very interesting to know whether the replacement of Snu13p by Bms1p causes the dissociation of the six SSU processome components from the 90S pre-rRNP complexes.

The other feature of the 90S pre-rRNP complexes is the presence of the hexameric sub-complex, which is present in a stable 600 kDa complex, composed of six proteins: Pwp2p, Dip2p, Utp6p, Utp13p, Utp18p, and Utp21p (Fig. 3A). Four of those proteins (Pwp2p, Dip2p, Utp13p, and Utp21p) have WD-repeats, which are known to be involved in protein–protein interactions in general. Although it must be determined whether this complex is a part of the 90S pre-rRNP complexes, or not, it is possible that this sub-complex helps to nucleate the assembly of the pre-ribosomal particle (Grandi et al., 2002) because two proteins (Utp6p and Utp13p) are the components of the SSU processome. The hexameric sub-complex may bridge between the SSU processome complex and 40S subunit-processing factors during the assembly of the 90S pre-rRNP complexes (Fig. 5A).

Above all else, the most striking feature of the isolated 90S pre-rRNP complexes is the lack of the *trans*-acting factors known to be involved in the 60S subunit synthesis (Fig. 5A). Because most of the protein factors associated with the 90S pre-rRNP complexes are those required for 40S synthesis and the initial processing of 35S pre-rRNA at A_0, A_1, and A_2, the ribosome biogenesis is probably started from the formation of 40S small subunit in yeast cells. Thus, we named the 90S pre-rRNP complex as the very early nucleolar pre-90S (VEN pre-90S) particle (Fig. 5A). The presence of that particle is somewhat contradictory to the earlier ''processome model,'' in which it was envisaged that the 40S and 60S synthesis machineries bound simultaneously to the 35S pre-rRNA to ensure that all components were present to carry out rapid and efficient processing (Grandi et al., 2002). However, proteomic analysis of the pre-rRNP complexes indicates that the 40S synthesis machinery predominately associates with the 35S pre-rRNA factors at an initial step, whereas those factors required for 60S subunit synthesis largely bind

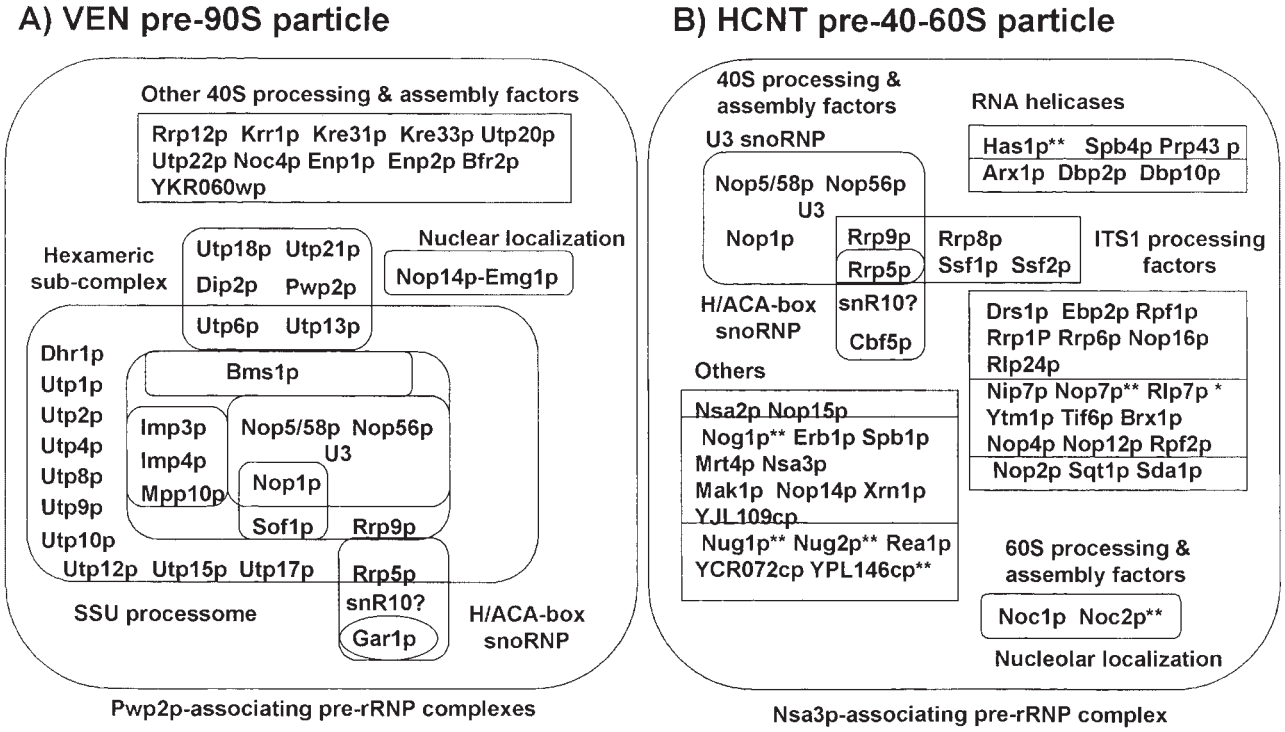

FIGURE 5. Proteomic classification of the pre-rRNP complexes isolated from yeast cells. Boxes and squares are used, as indicated in Figure 3. Factors involved in the export of the pre-rRNP complex are indicated by *, and components of the nuclear pore complex by **. **A**: VEN (very early nucleolar) pre-90S particle. The 90S particle/is formed at the initial stages of ribosome biogenesis in the nucleolus, and is characterized by the lack of the *trans*-acting factors involved in the 60S subunit synthesis but contains a number of 40S-processing and -assembly factors, and many of the 28 SSU processome components. The Pwp2p-associating and its reverse-tagging bait-associating pre-rRNP complexes exemplify VEN pre-90S particles. Some of the SSU processome components are not present in any of the isolated complexes classified into VEN pre-90S particle. **B**: HCNT (highly complex nucleolar transitional) pre-40–60S particle; The particle, which contains a number of 60S-processing and -assembly factors, functions probably in the production of 60S ribosomal subunit in the nucleolus; however, it also contains a high proportion of *trans*-acting factors involved in 40S subunit formation, including the core components of U3 snoRNP and several ITS1-processing factors. This particle contains the nucleolar-localization Noc1p–Noc2p hetero-dimer. The Nsa3p-associating pre-rRNP complex exemplifies an HCNT pre40–60S particle. The HCNT pre40–60S particle corresponds to very early pre-60S particles and/or possibly to 90S particle that is formed at late stages of 40S production in the nucleolus. HCNT pre-40–60S is probably formed as a transitional particle from a VEN pre40–60S particle to a HCN pre-60S particle during ribosome synthesis. **C**: HCN (highly complex nucleolar) pre-60S particle; the particle functions in the production of a 60S ribosomal subunit in the nucleolus, and contains the *trans*-acting proteins involved only in 60S subunit formation but not in 40S subunit formation. This particle also contains the nucleolar-localization Noc1p–Noc2p hetero-dimer. **D**: CNN (complex nucleolar/nuclear) pre-60S particle; the particle has the nucleoplasm localization Noc2p–Noc3p hetero-dimer, and localizes within the nucleoplasm as well as in the nucleolus. It functions in the production of the 60S ribosomal subunit, and represents the transport intermediate for 60S subunit export from the nucleolus to the nucleoplasm. The particle has Rea1p, a putative AAA-type ATPase, which probably functions in the late nucleoplasmic 60S pre-ribosome to dissociate non-ribosomal proteins at, or prior to, export. The Nug1p-associating pre-rRNP complex exemplifies a CNN pre-60S particle. **E**: ICN (intermediate complex nucleoplasmic) pre-60S particle; the particle functions in the maturation of 60S subunit in the nucleoplasm. It also has Rea1p as the CNN pre-60S particle. The Rix1p- and Sda1p-associating pre-rRNP complexes exemplify an ICN pre-60S particle. **F**: ICNC (intermediate complex nuclear-cytoplasmic) pre-60S particle; the particle has a dual localization in the nucleoplasm and cytoplasm, and has relatively few 60S-processing and -assembly factors. It no longer has any Noc1p∼Nop3 factors, but begins to associate with new export factors (Nmd3p and Mtr2p) and a number of proteins that have unknown function in ribosome biogenesis. The Arx1p-associating pre-rRNP exemplifies an ICNC pre60S particle.

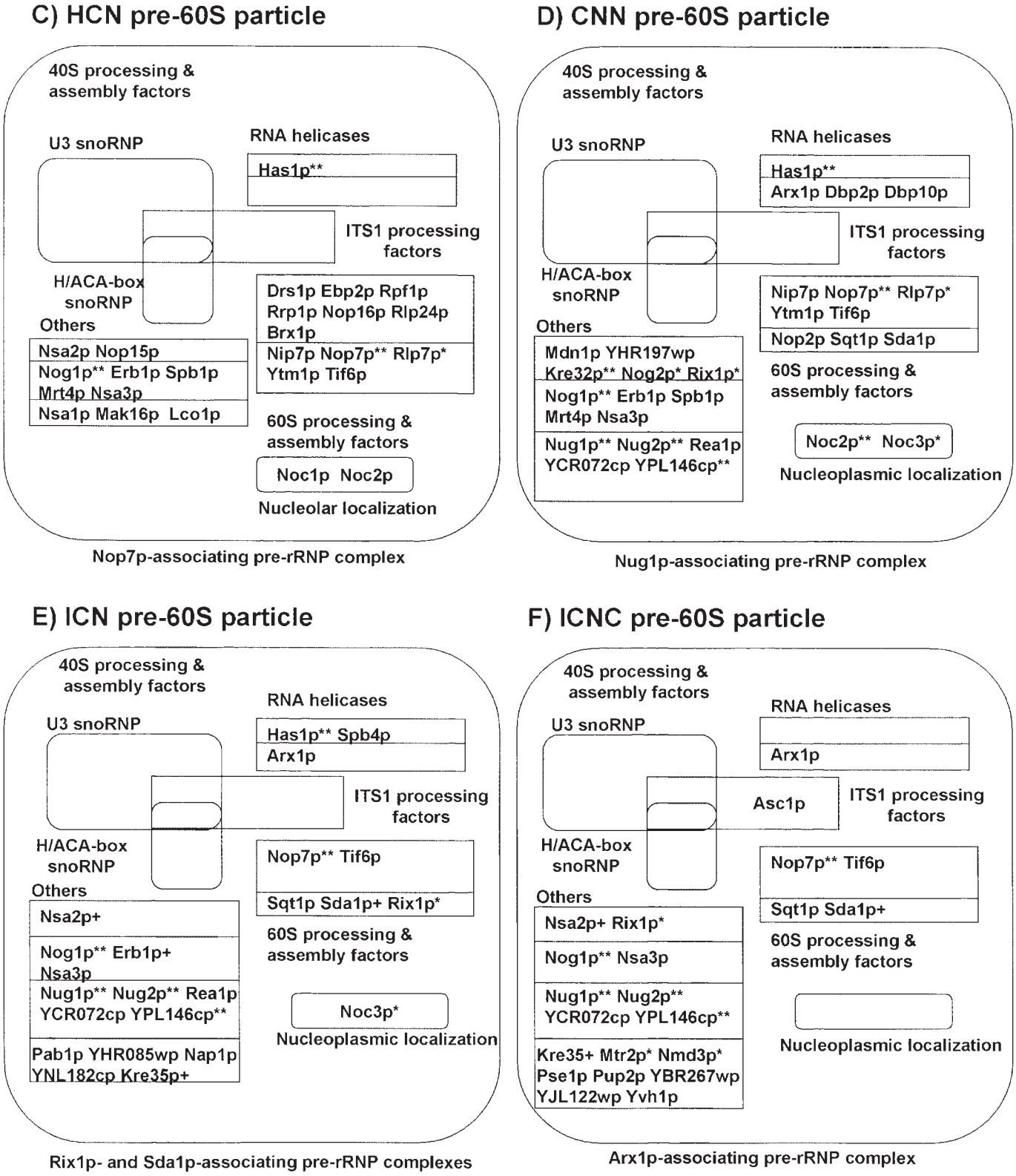

FIGURE 5. (*Continued*)

later, and show an unexpected dichotomy in binding. In the isolated 90S pre-ribosome particles, the fact that many ribosomal proteins of the 40S subunit are already assembled, whereas only few ribosomal proteins are found in the 60S subunit, supports the dichotomy in binding (Grandi et al., 2002).

The idea of the dichotomy in binding of the *trans*-acting factors involved in the synthesis of the small and the large subunits is further supported in the first place by the isolation of the 60S pre-ribosomal particle associated with TAP-tagged Nug1p that contained only the *trans*-acting factors that are (or are predicted to be) involved in the formation of the 60S subunit of ribosome particle but not those of 40S formation. That Nug1p-associating 60S pre-ribosomal particle also contained late precursors to the 25S and 5.8 rRNAs as well as non-ribosomal proteins that are implicated in 60S subunit export and/or that interacted with nuclear-pore complexes (described below). The presence of a Noc2p–Noc3p hetero-dimer also suggests that the Nug1p-associating 60S pre-ribosomal particle represents that the particle formed at late stages of maturation. Because Nug1p is located in the nucleolus and the nucleoplasm, the Nug1p-associating pre-rRNP complex probably represents the transport intermediate for 60S subunit export (Bassler et al., 2001).

C. Pre-rRNP Complexes Formed at Early/Middle Stages of Ribosome Biogenesis

Reverse-tagging methodology was also applied to the isolation of a series of pre-rRNP complexes by the use of the six protein components of the Nug1p-associating pre-rRNP complex (Nsa3p, Nop7p, Sda1p, Rix1p, Arx1p, and Kre35p) (Nissan et al., 2002). The pre-rRNP complexes that were pulled-down were characterized by their particle size, cellular localization, composition of pre-rRNAs, and protein components. Those characterizations revealed that the given bait protein could bind to distinct pre-ribosomal particles, and that the isolated pre-rRNP complexes were involved mostly in the formation of 60S pre-ribosome from assembly in the nucleolus until export to the cytoplasm (Nissan et al., 2002). Based on the compositional analyses of the isolated pre-rRNP complexes, Nissan et al. (2002) named those complexes as 60S preribosomal particles, and proposed that there are five different classes of the isolated 60S pre-ribosome particles that represent five distinct maturation stages of 60S pre-ribosomes during ribosome biogenesis (Fig. 5): i.e., (1) highly complex nucleolar pre-60S particles (HCN pre-60S); (2) complex nucleolar/nuclear pre-60S particles (CNN pre-60S); (3) intermediate complex nucleoplasmic pre-60S particles (ICN pre-60S); (4) intermediate complex nuclear-cytoplasmic 60S pre-ribosome (ICNC pre-60S); and (5) simple cytoplasmic 60S pre-ribosome (SC 60S).

Nissan et al. (2002) exemplified the Nsa3p-associating pre-rRNP complex as the HCN 60S pre-ribosome particle, which is the earliest pre-60S particle among the pre-60S ribosomal particles that have been isolated by the reverse-tagged methodology. It contained mostly $27SA_2$, 27SB, and 7S pre-rRNAs, and contained the *trans*-acting factors involved in 60S and 40S subunit formation. At least 21 known 60S biogenesis factors (Brx1p, Drs1p, Ebp2p, Nip7p, Noc1p, Noc2p, Nop2p, Nop4p, Nop7p, Nop12p, Nop16p, Rlp7p, Rlp24p, Rpf1p, Rpf2p, Rrp1p, Rrp6p, Sda1p, Sqt1p, Tif6p, and Ytm1p) were identified. Of ten identified 40S biogenesis factors, six proteins participate in rRNA methylation and pseudouridylation; five core factors of C/D-box snoRNP (Nop56p/Sik1p, Rrp5p, Rrp9p, Nop5p/Nop58p, and Nop1p) and one core factor (Cbf5p) of H/ACA-box snoRNP. Most of the proteins involved in ITS1 processing (Rrp5p, Rrp8p, Rrp9p, Ssf1p, and Ssf2p) are also found in the Nsa3p-associating pre-rRNP complex. Thus, Nsa3p binds to very early pre-60S particles and possibly to a 90S particle that is formed for the formation of pre-40S particle at the beginning stages of ribosome biogenesis (Fig. 5B; also, see later discussion). Despite the prematurity of the particle, most of the ribosomal proteins of large subunit are already assembled onto the Nsa3p-associating pre-rRNP complex. Judging from those results, the Nsa3p-associating pre-rRNP complex is formed probably at a stage later than that for the formation of the Pwp2p-associating 90S pre-rRNP complex that contains some of the SSU processome components and the *trans*-acting factors involved in 40S pre-ribosome formation.

Nissan et al. (2002) also denoted the Nop7p-associating pre-rRNP complex as the HCN 60S pre-ribosome particle. It represents the one formed probably at a stage next to the formation of the Nsa3p-associating pre-rRNP complex. Nop7p is associated primarily with the 66S pre-ribosome that contains either 27SB or 25.5S plus 7S pre-rRNAs (Harnpicharnchai et al., 2001). It has the size of 66S, and functions in the production of the 60S ribosomal subunit in the nucleolus. This 66S pre-ribosome contains the *trans*-acting proteins involved only in 60S subunit formation but not in 40S subunit formation. Twenty-three *trans*-acting proteins (Nop7p, Tif6p, Nip7p, Brix1p, Nog1p, Mak16p, Has1p, Drs1p, Erb1p, Rpf1p, Ebp2p, Rlp7p, Rrp1p, Spb1p, Ytm1p, Nsa3p, Nsa1p, Nsa2p, Rlp24p, Nop15p, Lcp1p, Mrt4p, and Nop16p) were identified as the components of the Nop7p-associating pre-rRNP complex by Harnpicharnchai et al., 2001 (Fig. 5C). Of those proteins, eight proteins (Nsa3p, Nsa1p, Nsa2p, Rlp24p, Nop15p, Lcp1p, Mrt4p, and Nop16p) were newly shown to be involved in the formation of the 60S subunit by gene-disruption analyses (Harnpicharnchai et al., 2001). Two additional proteins, Noc1p and Noc2p, which are together present in early nucleolar pre-ribosomes and are the markers for the nucleolar precursor

of ribosome particles, were also present in the Nop7p-associating pre-rRNP complex—as revealed by the analysis by Nissan et al. (2002) even though they had not been identified in the complex analyzed by Harnpicharnchai et al. (2001). Both of the factors were also present in the Nsa3p-associating pre-rRNP complex. The presence of Noc1p and Noc2p, and the absence of Noc3p in the Nsa3p- and Nop7p-associating pre-rRNP complexes, indicate that those complexes represent pre-ribosome particles formed at early stages of ribosome biogenesis in the nucleolus; thus, these two complexes are classified as an HCN pre-60S particle by Nissan et al. (2002). In that connection, Noc3p replaces Noc2p upon release of the pre-ribosome particles to the nucleoplasm (Milkereit et al., 2001). However, despite the presence of Noc1p and Noc2p factors, more than half of the *trans*-acting factors found in the Nsa3p-associating pre-rRNP complex are missing in the Nop7p-associating pre-rRNP complex. Only three proteins with unknown function (Mak16p, Nsa1p, and Lco1p) are uniquely present in the Nop7p-associating pre-rRNP complex. In addition, the Nsa3p-associating pre-rRNP complex combines the features of pre-40S and pre-60S particles; however, the Nop7p-associating pre-rRNP complex does not have those features of pre-40S particle (Fig. 5B,C). Although Nissan et al. (2002) categorize the Nsa3p- and Nop7p-associating pre-rRNP complexes into the same HCN pre-60S class, the former complex is distinctly different from the latter complex, and thus they should not be classified into the same HCN pre-60S. We propose to allocate the Nsa3p-associating pre-rRNP complex to a new class of pre-ribosomal particle; i.e., highly complex nucleolar transitional (HCNT) pre-40–60S particles, which are characterized by the presence of a high proportion of 40S processing and assembly factors despite its probable major role in the production of the 60S subunit (Fig. 5B). The HCNT pre-40–60S particle seems to be a transitional complex formed between the formation of the VEN pre-40S and HCN pre-60S particles during the progression of ribosome synthesis in the nucleolus of yeast cells.

Meanwhile, because of the dual localization in the nucleolus and the nucleoplasm, and of the presence of Noc1p and Noc3p but not Noc2p, the stage of the formation of the Nug1p-associating pre-rRNP complex is probably ordered after the stages of the formation of the Nop7p-associating pre-ribosome particle in ribosome biogenesis, and is classified into the complex nucleolar/nuclear pre-60S particles (CNN pre-60S) (Fig. 5D). A large compositional change is observed between the pre-rRNP complexes associated with Nop7p and Nug1p-possibly as a consequence of the replacement of Nop2p by Nop3p. One set of the *trans*-acting factors (Rpl24p, Nsa2p, Nop16p, Brx1p, Rrp1p, Ebp2p, Rpf1p, Nop15p, and Drs1p) present in the Nop7p-associating pre-rRNP complex seems to be replaced by two other sets of 60S processing and assembly factors present in the Nug1p-associating pre-rRNP complex; one set contains the *trans*-acting proteins once presented in the Nsa3p-associating pre-rRNP complex (Arx1p, Dbp2p, Dbp10p, Nop2p, Sqt1p, Sda1p, Nug1p, Nug2p, Rea1p, YCR072cp, and YPL146cp) and the other set contains the *trans*-acting proteins that are not components of the Nsa3p-associating pre-rRNP complex (Mdn1p, YHR197wp, Kre32p, Nog2p, Rix1p, and Noc3p). The notable feature of the Nug1p-associating pre-rRNP complex is that it is proposed to represent the transport intermediates for 60S subunit export based on its protein constituents. The nucleopore complex-associating proteins (Nug1p, Nug2p, Noc2p, Kre32p, Has1p, Nop7p, YPL146cp, and Nog1p) as well as of proteins required for 60S ribosome transport (Rix1p, Noc2p, Noc3p, and Rix9p) are present in the Nug1p-associating pre-rRNP complex (Rout et al., 2000; Milkereit et al., 2001). To be fair, some of those factors are also present in the Nop7p- and Nsa3p-associating pre-rRNP complexes that are formed at earlier stages of ribosome maturation in the nucleolus; therefore, they may have roles not only in the export of the pre-ribosome particles but also in the processing and assembly of the pre-rRNA and ribosomal proteins (Fig. 5).

D. Pre-rRNP Complexes Formed at Later Stages of Ribosome Biogenesis

In later stages of ribosome biogenesis, it becomes clearer that the more the pre-ribosome particles matured, the simpler the pre-ribosome particles seem to become in their *trans*-acting protein composition. This tendency becomes increasingly prominent in the Rix1p-, Sda1p-, Arx1p-, and Kre35p-associating pre-rRNP complexes, approximately in this order (Nissan et al., 2002). All of these complexes still contain 60S processing and assembly factors, but the number of their factors decreases from 19 to 7. Accordingly, their predominant cellular localizations change from the nucleoplasm (pre-rRNP complexes associated with Rix1p and Sda1p) through the nuclear pore (Arx1p-associating pre-rRNP complex) to the cytoplasm (Kre35p-associating pre-rRNP complex). The preribosomal particles associated with Rix1p and Sda1p are very similar in their protein contents; they still have at least 19 and 17 of 60S *trans*-acting factors, respectively. Of those factors, both pre-ribosomal particles share 13 of the *trans*-acting factors with each other; they include Arx1p, Nap1p, Nog1p, Nop7p, Nug1p, Nug2p, Rea1p, Rix1p, Sqt1p, Tif6p, YCR072cp, YNL182cp, and YPL146cp (Fig. 5E), and no longer possess Noc1p, Noc2p, or Noc3p. In addition, the *trans*-acting factors, including Dbp2p, Dbp10p, Nop2p, Spb1p, Ytm1p, and many other proteins that are present in the Nug1p-associating pre-rRNP complex, are removed upon the translocation from the nucleolus to the nucleo-

plasm. Concomitant with the removal of those protein factors, other components are recruited onto the Rix1p- and Sda1p-associating pre-rRNP complexes. Among those components, Rea1p (a putative AAA-type ATPase that is speculated to function in the late nucleoplasmic 60S pre-ribosome to dissociate non-ribosomal proteins at, or prior to, export), Pab1p [poly(A)-binding protein], Nap1p (nucleosome assembly protein), and YHR085wp and YNL182p (both unknown proteins) are recruited to the Rix1p-associating pre-rRNP complex, and Kre35p (putative GTPase) is recruited to the Sda1p-associating pre-rRNP complex (Fig. 5E). The Rix1p- and Sda1p-associating pre-rRNP complexes are similar in their protein compositions, and both are predominantly localized in the nucleoplasm; thus, they are classified as ICN pre-60S particles (Fig. 5E).

The Arx1p- and Kre35p-associating pre-rRNP complexes can be placed at the later stages just prior and after export into the cytoplasm, respectively. Because the former complex has dual location in the nucleoplasm and cytoplasm, and still contains 22 non-ribosomal proteins, it is classified as ICNC pre-60S particle (Fig. 5F). However, although there are relatively few *trans*-acting factors known to be involved in 60S subunit formation (Nop7p, Sda1p, Sqt1p, and Tif6p), the Arx1p-associating pre-rRNP complex associates with at least two export factors (Nmd3p and Mtr2p) and with a number of proteins that have a known function other than ribosome biogenesis [a nuclear transport factor (Pse1p), four putative GTPases (Kre35p, Nog1p, Nug1p, and Nug2p), a DEAD-box RNA helicase (Arx1p), three proteasome assembly factors (Nsa2p, Nsa3p, and Pup2p)], and that have an unknown function (YPL146cp, YJL122wp, YCR072cp, YBR267wp, Asc1p, and YVH1p). When the Arx1p-associating pre-rRNP complex reaches the cytoplasm, most of the factors (except Arx1p, Kre35p, Nmd3p, Sqt1p, YBR267wp, YCR072cp, and Yvh1p) are dissociated, and become a much simpler 60S pre-ribosome particle, such as that represented by the Kre35p-associating pre-rRNP complex (Nissan et al., 2002). Thus, the pre-rRNP complex localized in the cytoplasm is classified as an SC pre-60S particle.

V. ISOLATION AND PROTEOMIC CHARACTERIZATION OF PRE-rRNP COMPLEXES INVOLVED IN MAMMALIAN RIBOSOME BIOGENESIS

A. Ribosome Biogenesis in Mammalian Cells

Basic features of the pathway of the rRNA processing and assembly are believed to be conserved evolutionarily within eukaryotes. The genetic and proteomic approaches available in yeast research should provide considerable information that will be useful to understand higher eukaryotic systems, such as those of mammals. However, despite general features of ribosome structure and function that are highly conserved between bacterial and eukaryotic systems, the basic strategy in ribosome biogenesis is distinctly different (Eichler & Craig, 1994). Thus, we must independently decipher the detailed molecular mechanisms that underlie rRNA processing and assembly in ribosome biogenesis to elucidate its unique features and regulation in mammalian cells.

In mammalian cells, rRNA is transcribed as a large precursor of 47S, which undergoes multiple post-transcriptional nucleotide modifications and nucleotic-processing steps to yield the mature 18S, 5.8S, and 28S rRNA species (Fig. 6) (Ginisty, Amalric, & Bouvet, 1998). The 47S precursor is rapidly cleaved at the 5′ETS and at the 3′ETS to produce the 45S pre-rRNA. Further processing at the 5′ETS takes place and produces a 41S pre-rRNA, which is rapidly processed to the 18S rRNA by eliminating ITS1 and a 36S pre-rRNA that contains 5.8S and 28S rRNAs with an ITS2. The 36S pre-rRNA undergoes cleavage at the 5′ end to give rise to a 32S pre-rRNA, which is processed to the 28S rRNA and a 12S pre-rRNA, which is further processed to form the 5.8S rRNA. The processing events occur, in general, from the 5′ to the 3′ end of the nascent transcript (Strezoska, Pestov, & Lau, 2000). However, differences in the order of processing events and intermediates of the rRNA have been reported for different mammalian cell types (Bowman, Rabin, & Friesen, 1981; Hadjiolova et al., 1993). Although, as in the case of yeast cells, the association of pre-rRNA with non-ribosomal proteins as RNP complexes persists during the maturation of 18S, 5.8S, and 28S rRNAs, and through assembly of ribosomal subunits in the nucleus (Piñol-Roma, 1999), only a few of the *trans*-acting proteins that are involved in ribosome biogenesis have been characterized in mammalian systems. The well-characterized *trans*-acting proteins are nucleolin (NCL), B23, fibrillarin (FIB), Mpp10, Bop1, and dyskerin (Ochs et al., 1985; Ginisty, Amalric, & Bouvet, 1998; Westendorf et al., 1998; Strezoska, Pestov, & Lau, 2000; Ruggero et al., 2003). Among those proteins, NCL, B23, and FIB have been actually found in the pre-rRNP complexes that were isolated from human HeLa cells by pull-down analysis with an anti-NCL antibody. B23 has no yeast counterpart; thus, it should have roles in ribosome biogenesis unique to mammalian cells. There are known yeast counterparts for the rest of the proteins; yeast counterparts of mammalian NCL, FIB, Mpp10, Bop1, and dyskerin are Nsr1p (also known as She5p/Pab1p), Nop1p, Mpp10p, Erb1p, and Cbf5p, respectively. Although their basic functions in ribosome biogenesis are probably conserved between yeast and mammals, they seem to have more diversified functions than yeast counterparts (Srivastava & Pollard, 1999). For example, NCL and FIB seem to have some roles in the virus

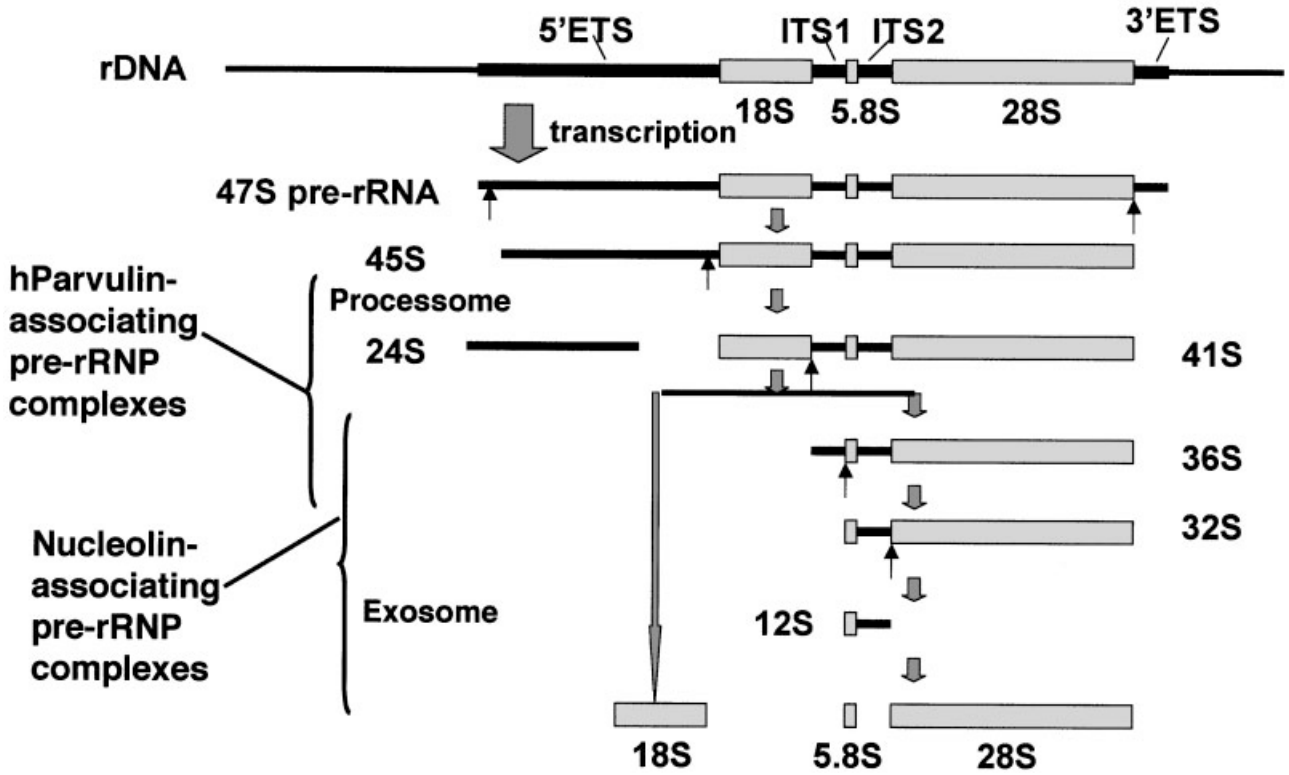

FIGURE 6. A known rRNA processing pathway in mammalian cells. rRNA is transcribed as a large precursor of 47S, which is rapidly cleaved at the 5′ETS and at the 3′ETS to produce the 45S pre-rRNA. By processing at the 5′ETS of the 45S, 41S is produced, and is rapidly processed to the 18S by eliminating ITS1 and a 36S that contains 5.8S and 28S rRNAs with an ITS2. The 36S undergoes cleavage at the 5′ end to give rise to a 32S, which is processed to the 28S and a 12S that is further processed to form the 5.8S rRNA. The processing sites are indicated by ↑. A possible step that is performed by a processome or exosome is indicated. The processes in that hParvulin- and nucleolin-associating pre-rRNP complexes are involved are indicated.

infection and pathogenesis of some infectious diseases of mammals, and mutations in dyskerin (that results in an impairment in ribosomal RNA pseudouridylation in hypomorphic dyskerin gene mutant cells) cause dyskeratosis congenita, a disease characterized by premature aging and an increased tumor susceptibility (Ruggero et al., 2003). Therefore, we have been trying to analyze pre-rRNP complexes associated with those mammalian *trans*-acting proteins by the proteomic approach with a hope of finding their new roles in ribosome biogenesis and/or in its related cellular function in mammalian cells.

B. Proteomic Analysis of the Pre-rRNP Complexes Associated With Nucleolin: A Major Nucleolar Protein

We first selected human NCL as affinity bait to pull down pre-rRNP complex because it is the best-characterized protein among the known *trans*-acting proteins involved in mammalian ribosome biogenesis, and it is believed to play an essential role in regulating multi-processes of ribosome biogenesis, such as rDNA transcription, pre-rRNA processing, and assembly of ribosomal proteins and rRNAs (Yang et al., 1994). NCL is also well-known as an abundant nuclear phosphoprotein and a principal protein constituent of the nucleolar-organizing region. This protein has a molecular weight of 110 kDa, and consists of three functional domains; i.e., an amino (N)-terminal domain, an RNA-binding (RBD) domain, and a carboxyl-terminal arginine–glycine-rich domain (RGG domain). The N-terminal domain contains multiple phosphorylation sites (Belenguer et al., 1990) and a nuclear-localization signal. The specific interaction of the RBD domain with 5′ETS sequences is required for the first step of pre-rRNA processing, and is consistent with a result in that the yeast counterpart Nsr1p is required for efficient pre-rRNA processing at sites A_0 to A_2 (Ginisty, Amalric, & Bouvet, 1998). In addition to the binding to the 5′ETS sequence, the RBD domain is also able to interact specifically with the rRNA at a number of the nucleolin-recognition element

(NRE) sequences present throughout the pre-rRNA molecule. Because the RBD and RGG domains of NCL bind directly to a dozen ribosomal proteins (Bouvet et al., 1998), NCL possibly acts as a carrier for ribosomal proteins from the cytoplasm to the nucleolus, and as an adapter for the specific binding of ribosomal proteins to rRNA (Borer et al., 1989). Thus, the NCL molecule is believed to set up the scaffolding of many proteins that participate in the rRNA processing and assembly of ribosomal subunits at different stages of ribosome biosynthesis. Because of the expected multifunctions of NCL in mammalian ribosome biogenesis, an attempt to isolate pre-rRNP complexes by immunoprecipitation with anti-NCL antibody was made. The data showed that the NCL-binding complexes contained B23, fibrillarin, ribosomal protein S6, many unknown proteins, as well as 18S and 28S rRNAs (Piñol-Roma, 1999). However, the purification was still at a primary stage because numerous proteins interacted non-specifically with the antibody and the immobilized-beads used for affinity purification of pre-rRNP complexes. In addition, identification of the constituents of pre-rRNP complexes was difficult in mammals because the genetic analysis cannot be routinely carried out, and only a limited amount of the sample was available, so that the number, identity, and function of the proteins present in the pre-rRNP complexes in the nucleolus remained largely unknown.

We used an affinity tag, an octa-peptide FLAG, fused to NCL to pull down the pre-rRNP complexes from human cells (kidney 293EBNA cells) (Yanagida et al., 2001). The method drastically reduced the protein contamination in the NCL-binding complex, because the method depends on the highly selective binding between the FLAG-tag and the anti-FLAG antibody, and on its specific dissociation with a FLAG peptide. We also contrived a way to obtain the NCL-associating pre-rRNP complexes in pure form by using an RNA oligonucleotide that corresponds to the NRE sequence, and that is able to dissociate specifically the pre-rRNP complexes from NCL. Because the verification of the specificity of the binding between bait protein and its binding complexes, and the purity of the isolated complexes were key to success in its proteomic characterization, we further confirmed their specificity and purity by isolating the NCL-associating pre-rRNP complexes with the domain mutants of NCL, and showed the RNA integrity for retaining its protein constituents. Because those approaches ensure the specificity and purity of the isolated NCL-associating pre-rRNP complexes, the large-scale identification of the protein constituents in the NCL-binding complexes by mass spectrometry analysis was enabled (Yanagida et al., 2001).

Initially, we applied the peptide mass fingerprinting (PMF) method after protein separation by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of the NCL-associating complex (Fig. 7) (Yanagida et al., 2001). Each

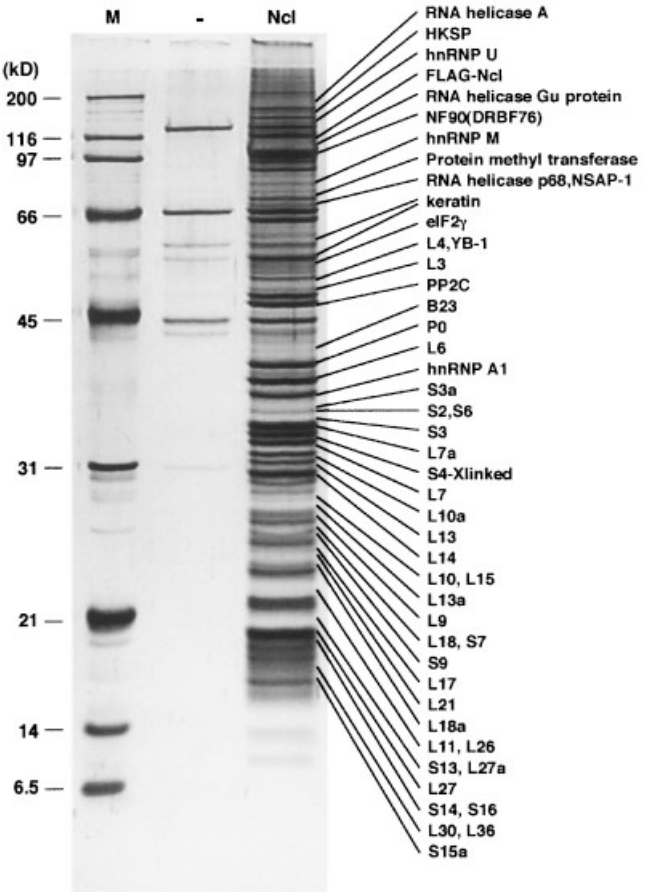

FIGURE 7. Identification of protein components of the nucleolin (NCL)-associating pre-rRNP complex by the gel electrophoresis-based method. The isolated NCL-associating pre-rRNP complex was subjected to 11% SDS–PAGE gel. The gel was silver-stained. The proteins identified by MALDI-TOF are partly indicated at the right side of the gel. Names with a gray tone indicate the proteins identified in the control. Lane 1; molecular weight markers, -; mock, NCL; NCL-binding complexes. (Reprinted from Yanagida et al., 2001, with permission from Wiley-VCH, copyright © 2001.)

stained protein spot was excised from a SDS–PAGE gel, and the proteins were in-gel-digested with a protease, such as trypsin. The peptides were extracted, and were analyzed with a MALDI/TOF mass spectrometer to obtain the mass values of the peptides. A set of the peptide mass values was searched against a protein sequence database by using the search program, MS-Fit, which was developed by Dr. Baker and Dr. Clauser and is available through the Internet (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). With this approach, we showed that the NCL-associating pre-rRNP complexes contained at least 60 proteins, including forty ribosomal proteins from the large and small subunits, and twenty non-ribosomal proteins. However, only several known *trans*-acting proteins involved, or expected to be

involved, in ribosome biogenesis were identified in the isolated pre-rRNP complexes with this gel electrophoresis-based analysis (Yanagida et al., 2001).

Protein identification by the PMF method depends on the information capacity of the protein sequence database because only proteins with entire sequences are archived. In addition, protein separation by SDS–PAGE largely affects protein identification by the PMF method; i.e., an incomplete protein separation on a SDS–PAGE gel causes ambiguous protein identification by the PMF method, reduces the number of proteins identified, and erroneously identifies proteins. Furthermore, some proteins may be lost during the multi-step operation of SDS–PAGE. To overcome some of those problems, we adopted a liquid chromatography (LC)-based technology to serve as an alternative proteomics technology that, in combination with the ESI–MS/MS, allows an on-line automation of the protein analysis. The identification is based on the ''peptide sequence tag'' method, in which an internal amino acid sequence is considered for a more reliable peptide assignment and protein identification.

Instead of separating by SDS–PAGE, the protein components of the isolated NCL-associating pre-rRNP complex were directly digested with trypsin or Lys-C, and the peptide mixture was separated with a direct nano-flow-LC-system (Natsume et al., 2002). The nano-flow LC-system is combined with data-dependent collision-induced dissociation MS/MS (Q-Tof2, Micromass, Manchester, UK), and the computer-assisted retrieval of spectra to identify proteins from which the retrieved peptides had originated. The LC–MS system has a unique flow system equipped with a novel split-less nano-flow gradient elution device, which produces almost linear profiles of a solvent gradient at extremely low flow rates (less than 100 nL/min) (Fig. 8). A highly efficient analysis can be achieved with LC on a miniaturized electrospray interface (ESI) column without any frit for reversed phase (RP) LC and our gradient device (Natsume et al., 2002). The ESI column we used was a tiny needle column (150 μm i.d. × ∼30 mm length) with a tip size of 0.3–0.5 μm. That column design had the advantages that one could use high-resolution RP separation media of very small particle size (1 μm diameter) without frit, and could form small droplets during the spray ionization to increase the sensitivity of MS analysis by accelerating the ionization of peptides (Link et al., 1999). However, the conventional ''split-flow'' LC system became unsuitable to operate this spray-tip column in terms of pressure-resistance and flow-rate capability; namely, the flow rate at micro- to several hundreds-nL/min level, produced by the ''split-flow'' LC system, was too fast to operate the column and reduced the chance of the probability-based, data-dependent MS/MS analysis of eluted peptides. We expected that the slower the flow-rate became, many more eluted peptides had a chance to be analyzed by MS/MS; thereby the number and the coverage of the identified proteins in a sample protein mixture were increased. Thus, we designed a novel, direct, nano-flow solvent-delivery system, equipped with a high-pressure nano-flow pump and a unique gradient device, to operate the spray-tip column at a flow rate less than 50 nL/min without a splitter (Fig. 8). In that gradient device, different solvents were filled into separate reservoirs and, by sequentially rotating the electric valves for programmed time duration, each solvent was transferred step-by-step to the fused silica capillary, where a linear gradient was generated by the diffusion of solvent boundaries during the transfer to the column. By controlling the speed of the valve rotation and/or the flow rate of the solvent delivery, any type of solvent gradient could easily be formed to maximize the peptide separation. In fact, the number of identifications increased dramatically by decreasing the flow rate. For example, when an *E. coli* protein extract was analyzed, the analysis identified 48 proteins at 100 nL/min, whereas 136 proteins were identified at 50 nL/min (Natsume et al., 2002). We consider that the reason for this improvement would be that (1) the peptide samples are introduced into MS at a higher concentration, and (2) the number of peptides selected for the data-dependent MS/MS analysis increases under the reduced flow rate. Thus, without the use of multi-dimensional LC system, in a typical single run of this direct nano-flow LC–MS analysis with a single spray-tip column, ∼2,000 MS/MS spectral data were acquired in a data-dependent mode that, in general, resulted in assignments of ∼400 peptides and the identification of 100∼200 proteins by searching a genome sequence database with the MASCOT algorithm (Matrix Science Ltd., London, UK).

One of the difficulties that was encountered with the LC-based large-scale protein identification was that the evaluation of final data set was time-consuming and labor-intensive, mainly because of the lack of unifying specification of protein/gene entries in the current genome/gene/protein database. In many cases, there were more than 10 entries for one single protein, and each one had different accession numbers in the database. Far more confusing was that one could not distinguish whether a slight difference in amino acid sequence between two protein entries might indicate different proteins or artifacts because of experimental error, etc. Clearly, the informatics technology and the refinement of a background database will be necessary for future advances in genome science, including proteomics (Isobe, Yamauchi, & Takahashi, 2002; Mawuenyega et al., 2002).

Although 60 proteins of the nucleolin-associating pre-rRNP complexes were identified by the gel electrophoresis-based analysis, we could have identified 141 proteins in the same protein complexes with this direct-nano-LC–MS/MS system; they included 75 non-ribosomal proteins

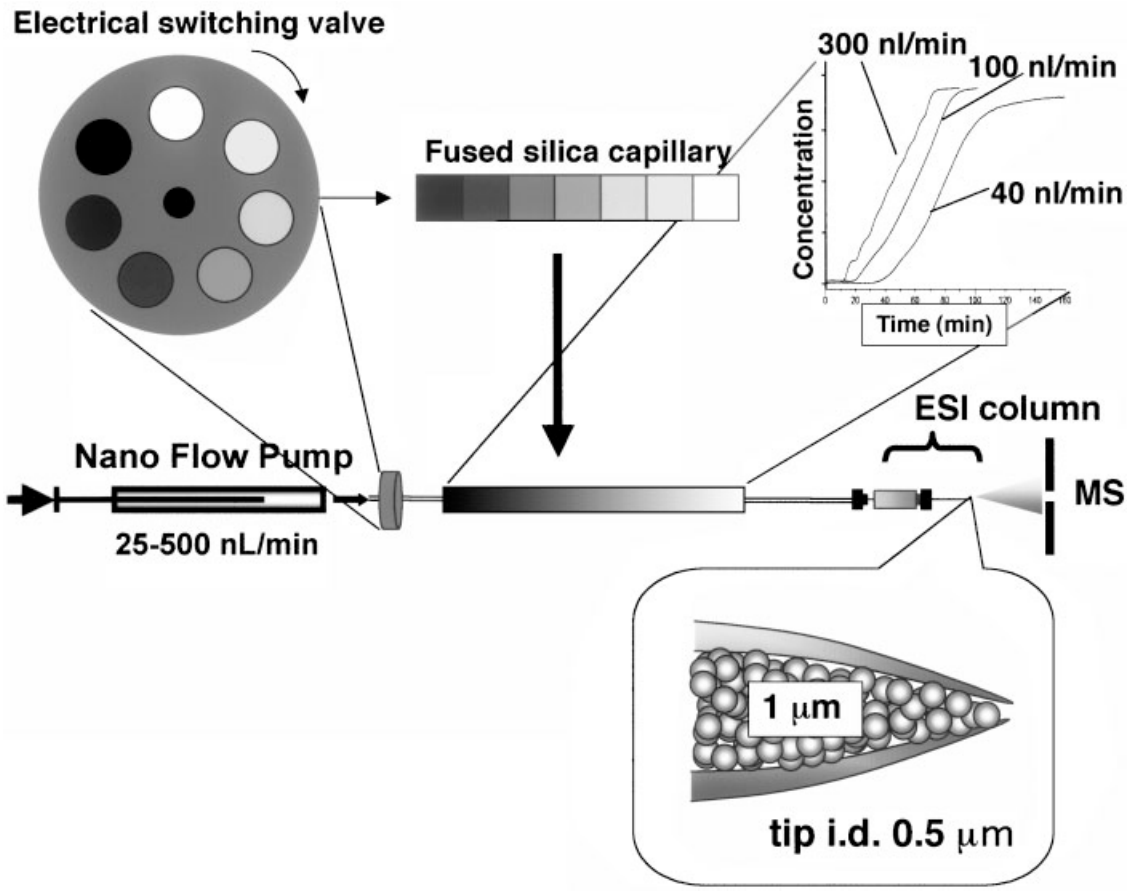

FIGURE 8. Direct nano-LC System (Natsume et al., 2002). The design of a gradient device, the revolving nano connection (ReNCon) system for direct nano-LC (DNLC), is illustrated. The solvent reservoirs are connected between an electrical-switching valve and a manifold. By switching the electrical valves sequentially for a programmed-time duration, each solvent was transferred step-by-step to the fused silica capillary, where a linear gradient was generated by the diffusion of solvent boundaries during the transfer to the column. Three gradient curves generated by the DNLC system at flow-rates of 40, 100, and 300 nL/min are shown at the upper right. A miniaturized electrospray interface (ESI) column without frit is shown at the lower right. Peptides separated by the ESI column are introduced directly into mass spectrometry (MS). (Modified from Natsume et al., 2002, reproduced with permission from American Chemical Society, copyright © 2002.)

and 66 ribosomal proteins (Natsume et al., 2002; Yanagida, 2003). All proteins identified by the gel electrophoresis-based analysis were included in the proteins identified by LC–MS/MS analysis. So far, 27 possible *trans*-acting proteins were assigned to the NCL-associating pre-rRNP complexes, which have sequence homology to the yeast *trans*-acting proteins involved in ribosome biogenesis (Yanagida, 2003; Yanagida et al., in review). Of those proteins, 10 yeast counterparts assigned as 60S processing and assembly factors (Brx1p, two homologs of Ebp2p, Nip7p, Nop2p, Nop4p, Nop7p/Yph1p, Rrp1p, Rrp6p, and Spb1p) and other 7 factors (Nsa3p/Cic1p, Dbp2p, Nug1p, Nug2p, and 3 homologs of Pab1p) were found mostly in the HCN pre-60S and/or partly in HCNT pre-40–60S particles (Figs. 5 and 9A). Only four yeast counterparts, Nop1p (component of C/D-box snoRNP), Ssf1p (ITS1-processing factor), Kre33p, and Srp40p (participated in C/D-box snoRNA production) are 40S-processing factors (Fig. 9A). The remaining seven (Dbp3p, Csl4p, two homologs of Rrp4p, and three homologs of Rrp45p) yeast counterparts have not been found in any pre-rRNP complex isolated from yeast cells. However, of those protein, six proteins (Csl4p, two homologs of Rrp4p, and three homologs of Rrp45p) belong to the components of the exosome complex (Fig. 3A). Combined with one other possible component of the ''exosome'' (Rrp6p homolog identified as a

60S-processing and -assembly factor), the NCL-associating pre-rRNP complexes contain seven counterparts of the 11 yeast exosome components (Yanagida, 2003; Yanagida et al., in press), which participate mostly in the production of 5.8S and 25S rRNAs in yeast ribosome biogenesis (Figs. 2, 6, and 9A). Because the yeast HCNT pre-40–60S and HCN pre-60S particles contain few exosome components, the presence of the seven of the 11-exosome components in the NCL-associating pre-rRNP complexes was quite striking. Based on those results, and coupled with its predominant localization in the nucleolus, we propose that the NCL-associating pre-rRNP complex might be classified as a mammalian HCN pre-60S particle (Yanagida et al., in press) (Fig. 9A). However, the NCL-associating pre-rRNP complexes still have at least four 40S processing factors; thus, it may be ordered between the HCNT pre-40–60S and HCN pre-60S particles in the maturation stages of pre-ribosome particles (Fig. 5). Despite those results, the counterparts of Noc1p and Noc2p, which determine the nucleolar localization of the preribosomal particle, were not found in the NCL-associating pre-rRNP complex, unlike the yeast HCNT pre-40–60S and HCN pre-60 particles. We must clarify why they were not found in the NCL-associating pre-rRNP complexes.

In addition to the possible assigned *trans*-acting proteins, the isolated NCL-associating prerRNP complexes contained 46 more non-ribosomal proteins, including B23, DNA–topoisomerase, Myb-binding protein, poly-ADP–

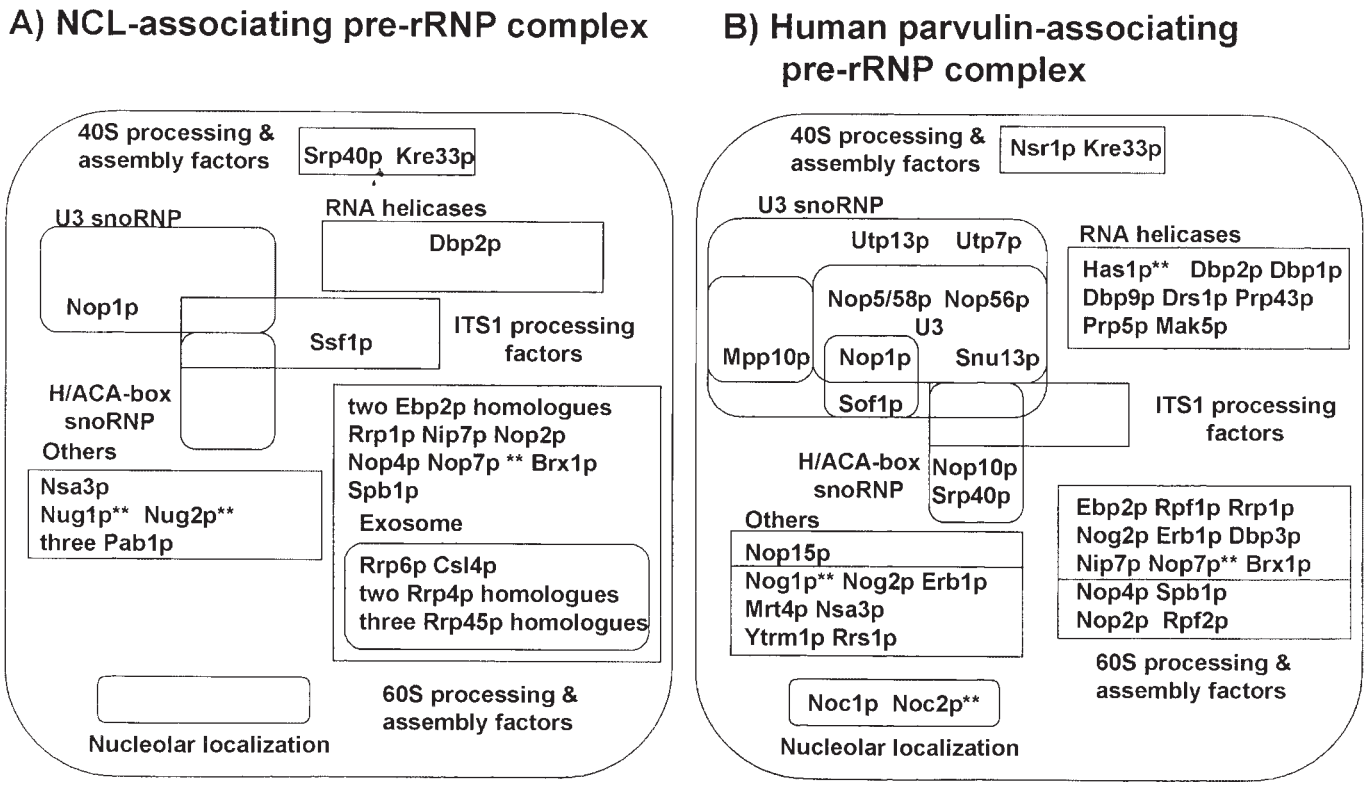

FIGURE 9. Proteomic classification of the pre-rRNP complexes isolated from mammalian cells. Possible *trans*-acting factors assigned in the mammalian pre-rRNP complexes isolated are shown as the protein names of their yeast counterparts in this figure. Boxes and squares are used, as indicated in Figs. 3 and 5. Factors involved in the export of the pre-rRNP complex are indicated by *, and components of the nuclear pore complex by **. **A**: NCL-associating pre-rRNP complex; Because the NCL-associating pre-rRNP complex is mainly composed of 60S-processing and -assembly factors, it is probably involved in the production of the 60S subunit; thus, it may be classified as the mammalian counterpart of the yeast HCN pre-60S particle (Yanagida, 2003). However, the NCL-associating pre-rRNP complex uniquely contains seven out of the eleven known components of exosome; therefore, that complex may be called the exosome-recruiting HCN pre-60S particle. **B**: Human parvulin-associating pre-rRNP complex; because the hParvulin-associating pre-rRNP complex has more 40S subunit-processing factors than the HCNT pre-40–60S particle, it probably belongs to the pre-HCNT pre-40–60S particle, which may be formed at a stage between the formation of the VEN pre-90S and HCNT pre-40–60S particles. The particle has a complete set of the core proteins of the U3 snoRNP and the Noc1p–Noc2p heterodimer complex.

ribose polymerase, Y-box binding protein, putative RNA helicases, components of DNA PK, arginine–serine-rich splicing factors, components of a signal–recognition particle, heterogeneous nuclear ribonucleoproteins (hnRNPs), nuclear matrix proteins, many hypothetical/putative/unknown proteins, etc. (Yanagida, 2003; Yanagida et al., submitted in review). It is not clear yet how those non-ribosomal proteins are involved in the process of ribosome biogenesis. However, many of those proteins are localized in the nucleolus and/or the nucleus, and are also found in other pre-rRNP complexes that were isolated with other reverse-tagging bait proteins.

C. Reverse-Tagging Methodology Applied to Human *Trans*-Acting Proteins Involved in Ribosome Biogenesis

As discussed above, a particular complex is not necessarily of an invariable composition, nor are all of its protein components uniquely associated with that specific complex (Kumar & Snyder, 2002). With several distinct tagged proteins as entry points to purify a complex, core components can be identified and validated, whereas more dynamic, perhaps regulatory, components may be present differentially. We applied the direct-nano-LC–MS/MS technology to the analysis of the dynamics of complex composition of pre-rRNP complexes that were isolated with reverse-tagging baits. In addition to nucleolin, we selected B23, fibrillalin, and many other proteins that are found in the NCL-associating pre-rRNP complexes as affinity bait to adopt the reverse-tagging methodology. We first compared the protein components present in the pre-rRNP complexes isolated by the reverse-tagging approach by SDS–PAGE analyses, and confirmed that many of the protein bands were present commonly in the isolated protein-complexes, and that some were uniquely present in each protein complex (Yanagida, 2003; Yanagida et al., in press). The protein components of each isolated complex were analyzed by the direct nano-LC–MS/MS system after Lys-C protease digestion. We identified approximately 110∼170 proteins in each protein complex. The advantage of the use of the LC-based approach over the gel electrophoresis-based one is the ease to repeat many times the analysis for protein identification, and thus to rapidly confirm the results of protein identification. In addition, the LC-based technology was a highly sensitive method; thus, it has been successful to identify a large number of protein components of the RNP complexes, for which only limited amounts were available in mammalian cells. With the gel electrophoresis-based technology, we identified 60 protein components in the NCL-associating pre-rRNP complexes obtained from human cells, which is very powerful if compared with the conventional methods of classical protein chemistry. However, the gel electrophoresis-based MS analysis is still tedious and labor-intensive, and takes many weeks and sometimes several months even for the analysis of a single protein-complex. On the contrary, with our LC-based approach, we could identify *ca.* 90 core components of the protein complexes and 20∼80 proteins uniquely present in each protein complex with less amount of the sample than that used for the gel electrophoresis-based analysis, and within only a few days (Yanagida, 2003). Thus, with several distinct tagged proteins as entry points to purify complexes, core components can be identified and validated rapidly. In addition, more dynamic, perhaps regulatory, components that are present differentially can also be identified easily by our LC-based approach.

By comparing the results with those obtained from the analyses of yeast pre-rRNP complexes, we tried to correlate the isolated mammalian pre-rRNP complexes to the yeast pre-ribosomal particles. We found that most of the mammalian pre-rRNP complexes contained mainly *trans*-acting proteins for 60S processing and assembly, and thus, might be classified principally as pre-60S particles. The detailed results will be published elsewhere (Yanagida M. et al.; Hayano T. et al., J Biol Chem, in press). Essentially, the same approaches as those adopted for yeast ribosome biogenesis were equally effective for the analyses of the mammalian ribosome biogenesis process. However, we encountered some difficulties in expressing a bait protein in mammalian cells; i.e., some bait proteins were expressed too little to recover its associating complex because of unknown reasons, to an expression of some bait proteins that showed toxicity to the cell, etc. Those difficulties tend to be encountered often when probable 40S-processing and -assembly factors are expressed in the cell. In addition, there are always some concerns that address some disadvantages of the tag-expression method, such that protein overexpression may not be possible for heteromeric complexes of unknown composition or may lead to the assembly of overexpressed protein in non-physiological complexes; thus, those concerns must be considered in each individual case (Rigaut et al., 1999). In some cases, it was necessary to use approaches other than the tag expression method for the successful isolation of pre-rRNP complexes.

D. Isolation and Proteomic Analysis of Human Parvulin-Associating Pre-rRNP Complexes

To overcome some of those difficulties, we used an affinity tag, glutathione-*S*-transferase (GST)-tag with a thrombin cleavage site fused to a bait protein at the N-terminus, to precipitate its associating proteins from cell extracts of mammalian cells. In that approach, the pre-purified GST-fused bait protein was pulled down with glutathione-beads after mixing with cell extracts to form a protein complex with other proteins, so that it can be applied to isolating its

protein complex regardless of its low expression level or toxicity when it is expressed in the cell. The thrombin cleavage allowed us to elute gently the protein complex from the glutathione-beads without any unwanted contaminations of GST-associating and/or glutathione-beads-binding proteins. That approach was applied to human parvulin (hParvulin), which belongs to the third family of peptidyl prolyl *cis–trans* isomerases that exhibit an enzymatic activity of inter-converting the *cis–trans* conformation of the prolyl peptide bond, but that has no known function. The hParvulin-associating protein complexes were pulled down mostly from the nuclear extract of all of the mammalian cells that were used. Sixty-two proteins had been identified by a gel electrophoresis-based PMF analysis; the 26 proteins were counted as ribosomal proteins, another 26 proteins were considered as *trans*-acting factors involved in ribosome biogenesis, and the remaining 10 proteins were those whose involvements in ribosome biogenesis were unknown. Based on the protein composition, together with the presence of pre-rRNAs such as 45S, 34S, 32S, and 12S, the isolated complex was concluded to be an intermediate of ribosomal particles (Fujiyama et al., 2002). This hParvulin-associating pre-rRNP complex was also analyzed by direct nano-LC–MS/MS analysis after its Lys-C protease digestion. After making allowance for proteins duplicated in the gel electrophoresis-based and LC-based analyses, 102 proteins were identified in the hParvulin-associating pre-rRNP complex; of those proteins, 45 were ribosomal proteins and 57 were non-ribosomal proteins (Fujiyama et al., 2002; Fujiyama et al., manuscript in preparation). Of 57 non-ribosomal proteins, we assigned 35 proteins as *trans*-acting proteins thought to be involved in ribosome biogenesis of mammalian cells based on their sequence homologies to the yeast *trans*-acting proteins involved in yeast ribosome biogenesis.

The association of hParvulin with the pre-rRNP complex is quite striking in terms of its protein constituents. That complex contained not only a number of 60S-subunit-processing and -assembly factors, but also a high proportion of 40S-subunit-processing and -assembly factors. The former includes at least 13 counterparts of yeast *trans*-acting proteins, such as Ebp2p, Rpf1p, Rix1p, Nog2p, etc. (Fig. 9B). The latter includes yeast counterparts of eight possible components of the ''SSU processome'', two components of H/ACA-box snoRNP, Nsr1p, Kre33p, etc. (Fujiyama et al., 2002; Fujiyama et al., in press). In addition, the hParvulin-associating pre-rRNP complex contained homologs of Noc1p and Noc2p, but not of Noc3p (Fig. 9B). Because Noc1p–Noc2p is present in early nucleolar pre-ribosomes, but Noc2p is replaced by Noc3p upon the transport to the nucleoplasm (Milkereit et al., 2001; Nissan et al., 2002), the presence of Noc1p and Noc2p counterparts suggests that the hParvulin-associating pre-rRNP complex represents the ones formed at an early stage of ribosome biogenesis in the nucleolus of mammalian cells. The Noc1p–Noc2p containing pre-rRNP complex that has been isolated from yeast cells is the one associated with Nsa3p and Nop7p, which were classified as HCNT pre-40–60S and HCN pre-60S particles, respectively. Although the HCNT pre-40–60S particle contained not only 60S-processing and -assembly factors, but also a high proportion of 40S-processing and -assembly factors, the HCN pre-60S particle contains principally only 60S-processing and -assembly factors (Fig. 5). Because the hParvulin-associating pre-rRNP complex has more 40S subunit-processing factors that the HCNT pre-40–60S particle represented by the Nsa3p-associating pre-rRNP complex (Figs. 5B and 9B), it seems to represent one formed at an earlier stage than one formed on the HCNT pre-40–60S particle in the nucleolus. Therefore, the hParvulin-associating pre-rRNP complex may be classified as a mammalian pre-HCNT pre-40–60S particle, which may be formed at a stage between that for the formations of VEN pre-90S and HCNT pre-40–60S particles (Fig. 5A,B).

In addition to the *trans*-acting factors, we have co-purified approximately 20 non-ribosomal proteins of previously unknown function in ribosome biogenesis along with the rRNP complex by the use of hParvulin as affinity bait. Of those factors, at least half of the proteins were reported or expected to be present in the nucleolus. Based on the protein constituents, we have proposed that the isolated hParvulin-associating rRNP complexes represent those formed during post-mitotic nucleolar reformation before rDNA transcription, or premitotic nucleolar disassembly, and that accordingly, hParvulin may play a role in those processes (Fujiyama et al., 2002; Fujiyama et al., manuscript in preparation). In addition, hParvulin can interact with DNA and/or RNA at the amino-terminal domain, and act as a peptidyl-prolyl *cis–trans* isomerase at the carboxyl-terminal domain with preferential substrate specificity for positively charged residues that precede proline. Thus, it is very intriguing to speculate that hParvulin may have roles in recruiting and/or selecting proteins that must be assembled on the pre-rRNP complex at just the right timing during the early stages of ribosome biogenesis. Because there is no yeast counterpart of hParvulin, its role in ribosome biogenesis must be unique to higher eukaryotes.

The use of the pre-purified tagged protein as affinity bait leads to the successful isolation of the hParvulin-associating pre-rRNP complex that is possibly the earliest pre-40–60S particle in the pre-rRNP complexes that have previously been isolated from yeast and mammalian cells. That approach may be used as an alternative approach for the isolation of a protein complex if the expression of a tagged protein by transfecting its expression vector into mammalian cells is not successful. In addition, the pre-

purified tagged protein can be used as the second bait protein for the ''double-tagging methodology.'' In another independent experiment, a new large human co-activator complex necessary for the estrogen receptor alpha (ERα) transactivation, which contains GCN5, HAT, c-Myc-interacting protein TRRAP/PAF400, TAF (II) 30, and other subunits, was purified by the use of the double-tagging methodology. The co-activator complex was first pulled down with pre-purified GST–ERα from the extract of HeLa cells that were transfected with the FLAG-tagged *GCN5* gene; it was further pulled down by FLAG-antibody-beads, and was eluted with FLAG peptides (Yanagisawa et al., 2002). Thus, the full use of the methodologies currently available in proteomics, combined with well-established biochemical approaches, made it possible to isolate and to characterize almost any protein complex formed in the cell, so that the analysis of cellular process seems to be no longer limited by the inability to isolate the synthetic intermediates of cellular machinery or functional protein complexes.

VI. PROTEOMIC ANALYSIS OF THE NUCLEOLUS OF MAMMALIAN CELLS

Recently, the proteomic analyses of the nucleolus, the main site of ribosome biogenesis, of the human HeLa cell have been performed on the largest scale in the sub-cellular compartments that have ever been analyzed by the proteomics approach (Andersen et al., 2002; Dundr & Misteli, 2002; Scherl et al., 2002). A total of *ca.* 350 different proteins has been listed as the catalog complements of the nucleolar proteins; those proteins include the well-known nucleolar proteins, such as NCL, B23, FIB, nucleic acid/nucleotide-binding proteins, RNA-dependent helicases, and proteins involved in mRNA metabolism, as well as proteins involved in ribosome biogenesis. In addition, many novel proteins that are annotated as hypothetical/putative/unknown proteins were listed in the catalog. Among the proteins unexpected to be present in the nucleolus, a set of translational factors, signal-recognition particles, chaperones, and endoplasmic reticulum-localized proteins that are involved in the folding of newly synthesized proteins were found in the nucleolus. In our analyses, we also found many translation factors, including translation-initiation factors, and signal-recognition particles in the NCL-, B23-, FIB-associating pre-rRNP complexes, etc. (Yanagida et al.; Hayano et al., J Biol Chem, in press). Although we can't exclude the possibility that those proteins might have been contaminants during the isolation processes of the complexes, those results, in turn, support a possibility that protein synthesis takes place in the nucle(ol)us. Because the isolated pre-rRNP complexes contained almost all sets of ribosomal proteins, but still had some *trans*-acting proteins involved in intermediate stages of ribosome biogenesis as well as almost all sets of translation machineries, that result raised another intriguing possibility that intermediates of ribosome particles synthesized in the nucle(ol)us may be checked for that translational performance before transporting to the cytoplasm and/or to the nucleoplasm. Furthermore, we found transcriptional regulator proteins, splicing factors, hnRNPs, etc. in the isolated pre-rRNP complexes (Yanagida et al., 2001; Hayano et al., J Biol Chem, in press; Fujiyama et al., manuscript in preparation). Because almost all of those proteins were also present in the protein catalog of the mammalian nucleolus, an additional possibility is implied that the processes of ribosome biogenesis may also progress coordinately with events such as those that occur during mRNA metabolism, including transcription, splicing, and transport. The detailed proteomic analysis of each highly organized nuclear structure may clarify how DNA replication, transcription, pre-mRNA processing, ribosome biogenesis, and RNA transport are coordinated in the nucle(ol)us.

VII. CONCLUSIONS

Based on the vast amount of information for the processes of ribosome biogenesis that have been obtained from biochemical and genetic analyses as well as proteomic analyses of yeast cells, a new model has been proposed from the proteomics point of view for the pathway of pre-ribosome maturation and export in yeast cells (Nissan et al., 2002; Milkereit et al., 2003). We have been trying to characterize pre-rRNP complexes in comparison with those complexes described in yeast with a hope of finding new aspects of the regulation mechanisms that underlie ribosome synthesis in mammalian cells. By applying the proteomic analyses to the characterization of the pre-rRNP complexes isolated from mammalian cells, we found that a large proportion of the *trans*-acting factors described in yeast have counterparts in mammalian organisms; thus the fundamental mechanisms that underlie ribosome biogenesis have been conserved throughout evolution. In fact, we have demonstrated the presence of a series of distinctly different intermediates of ribosome particles in the nucle(ol)us of mammalian cells, and could correlate those intermediates with the corresponding yeast pre-ribosome particles. However, in the isolated mammalian pre-rRNP complexes, we have also identified a number of non-ribosomal proteins, which do not have a counterpart in the yeast pre-rRNP complexes. Those proteins could function uniquely in the process of mammalian ribosome biogenesis and/or its related processes of mammalian cells, and might be downstream targets of some of the oncogenes and growth-factor receptors, which are shown to be major

regulators of the protein-synthesis machinery (Nelson et al., 2000; Boon et al., 2001; Kroll, Barth-Baus, & Hensold, 2001). We suspect that regulatory mechanisms that underlie ribosome biogenesis of mammalian cells are much more diversified than those of yeast cells because adaptation, growth, and proliferation of distinctly different cell types, each of which is dependent on different growth factors and other stimuli, are directly coupled with the regulatory mechanisms of ribosome biogenesis in mammalian cells. Failure of some of those regulatory mechanisms may result in some specific disorders that are only observed in mammals. In fact, during the course of our analyses, we have found that the function of a protein that is responsible for the cause of a genetic craniofacial disorder could have been connected to ribosome biogenesis (Hayano et al., J Biol Chem, in press). In addition, human disease caused by abnormal ribosome biogenesis has also been reported; i.e., an impairment in ribosomal RNA

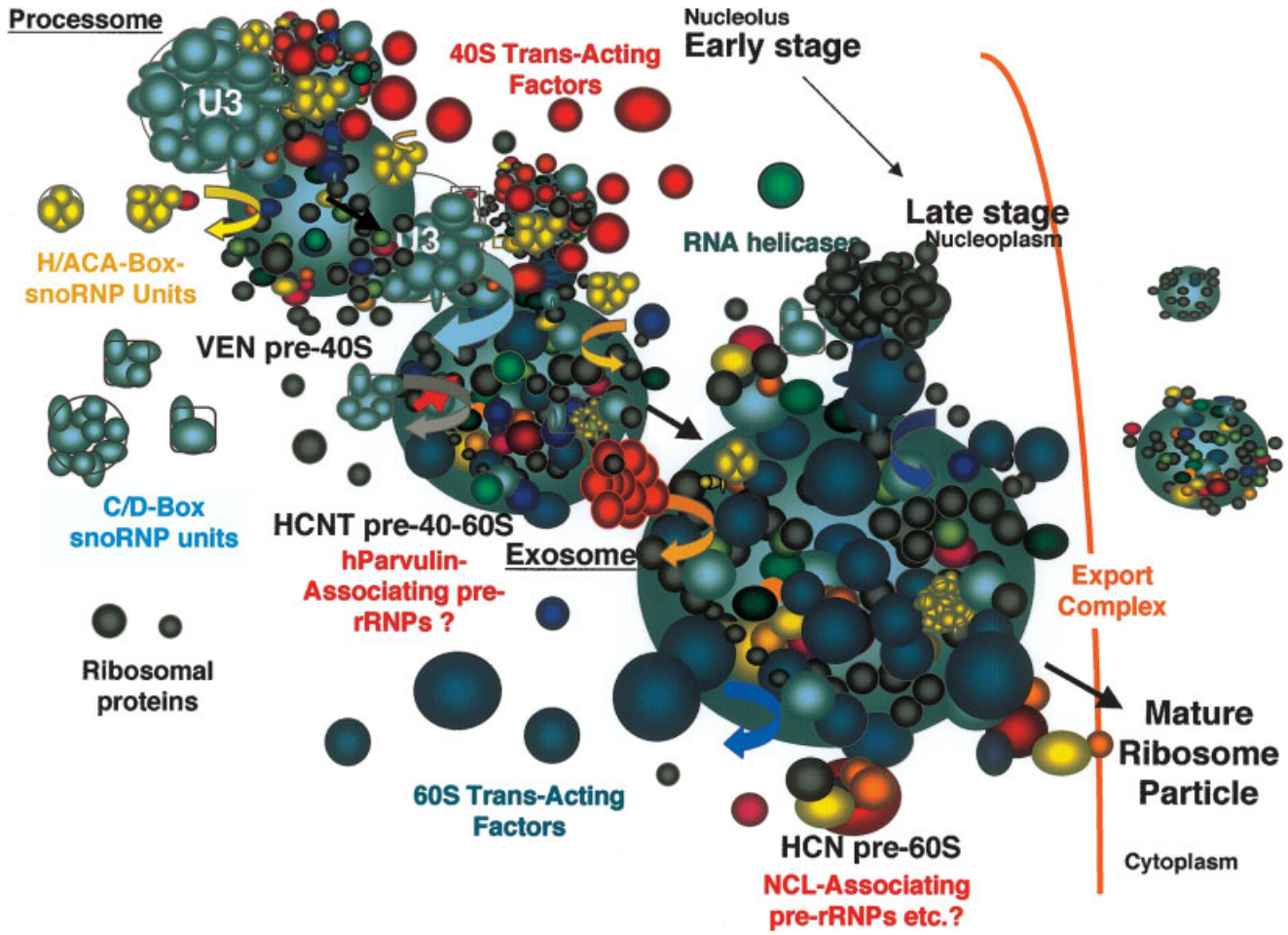

FIGURE 10. An image of the ''Dynamome'' of ribosome biogenesis. Ribosome biogenesis is a series of dynamic processes in that many *trans*-acting factors (colored circles) and ribosomal proteins (dark gray circles) are associated with pre-rRNAs (large gray circles) and their processing products in the form of pre-rRNP complexes (VEN pre-40S, HCNT pre-40–60S, HCN pre-60S, etc.), and each *trans*-acting factor is associated only transiently with the pre-rRNP complex when it is carrying out its given action. Hundreds of *trans*-acting factors (40S *trans*-acting factors, red circles; 60S *trans*-acting factors, blue circles; RNA helicases, green circles; snoRNAs, U3, etc.) are involved in the process, and some carry out their work as functional protein complexes (processome, gray complex; exosome, orange complex, etc.) and some are assembled as assembly units on the pre-rRNP complex (C/D-box snoRNPs, gray complex; H/ACA-box snoRNPs, yellow complex, etc.) or may leave as disassembly units from the pre-rRNP complex. The Dynamome of ribosome biogenesis is shown as an image to express a comprehensive molecular set, including *trans*-acting proteins and RNAs (snoRNAs, RNA component of RNase MRP, etc.), ribosomal proteins, pre-rRNAs, rRNAs, and others, all of which are involved in a series of dynamic processes of ribosome biogenesis in the cell.

pseudouridylation causes dyskeratosis congenita, a disease characterized by premature aging, nail dystrophy, mucosal leukoplakia, interstitial fibrosis of the lung, and an increased susceptibility to cancer (Ruggero et al., 2003). Through our continuing proteomic analysis of mammalian ribosome biogenesis, we are hoping to find out new connections between ribosome biogenesis and some other diseases whose pathogenesis has not been understood.

Recently, several lines of evidence suggest that all events that are necessary for protein synthesis, including not only transcription and mRNA editing but also translation, can take place in the nucle(ol)us. We also identified a number of protein factors that participated in almost all of those events, along with the possible *trans*-acting factors in the pre-rRNP complexes isolated by the use of human *trans*-acting factors that were probably involved in ribosome biogenesis as affinity baits; those experiments support the concept that protein synthesis takes place in the nucle(ol)us. Those results, in turn, imply that we might have succeeded in isolating the functional machinery that is involved in such highly coordinated events in the nucle(ol)us of mammalian cells. Thus, the methodology of the use of tagged proteins as affinity baits, coupled with mass spectrometric identification, is very powerful in terms of the characterization of pre-rRNP complexes that may represent snapshots of nascent ribosomes at particular stages of ribosome biogenesis; thus, those experiments are applicable to the analysis of the dynamics of ribosome biogenesis. Finally, we propose a word ''Dynamome'' to express a comprehensive molecular set that participates in the whole dynamic process of a series of cellular events, such as ribosome biogenesis (Fig. 10).

VIII. ABBREVIATIONS

CNN pre-60S	complex nucleolar/nuclear pre-60S particle
ER	estrogen receptor
ESI	electrospray ionization
ETS	external transcribed spacer
FIB	fibrillarin
GST	glutathione-*S*-transferase
HCN pre-60S	highly complex nucleolar pre-60S particle
HCNT pre-40–60S	highly complex nucleolar transitional pre-40–60S particle
hnRNP	heterogeneous nuclear ribonucleoprotein
hParvulin	human parvulin
ICN pre-60S	intermediate complex nucleoplasmic pre-60S particle
ICNC pre-60S	intermediate complex nuclear-cytoplasmic 60S pre-ribosome
ITS	internal transcribed spacer
LC	liquid chromatography
Lys-C	lysyl-endopeptidase
MALDI-TOF	matrix-assisted laser desorption ionization-time-of-flight
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NCL	nucleolin
NRE	nucleolin recognition element
PMF	peptide mass fingerprinting
pre-rRNA	preribosomal RNA
RBD	RNA-binding
rDNA	ribosomal DNA
Reb1p rRNA	enhancer binding protein 1
RGR	arginine-glycine rich
RNP	ribonucleoprotein
RP	reversed phase
SC 60S	simple cytoplasmic 60S pre-ribosome
SDS–PAGEsodium	dodecyl sulfate–polyacrylamide gel electrophoresis
snoRNA	small nucleolar RNA
SSU	small subunit
TAP	tandem affinity purification
VEN	very early nucleolar pre 90S

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