

## COMMENTARY

# Structure and functions of nucleolin

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## SUMMARY

**Nucleolin is an abundant protein of the nucleolus. Nucleolar proteins structurally related to nucleolin are found in organisms ranging from yeast to plants and mammals. The association of several structural domains in nucleolin allows the interaction of nucleolin with different proteins and RNA sequences. Nucleolin has been implicated in chromatin structure, rDNA transcription, rRNA maturation, ribosome assembly and nucleo-**

**cytoplasmic transport. Studies of nucleolin over the last 25 years have revealed a fascinating role for nucleolin in ribosome biogenesis. The involvement of nucleolin at multiple steps of this biosynthetic pathway suggests that it could play a key role in this highly integrated process.**

Key words: Nucleolin, Ribosome biogenesis, Nucleolus, Ribosomal RNA, RNA-binding protein

## INTRODUCTION

Since the discovery of the nucleolus, many works have focused on understanding the function of this dynamic nuclear structure. Ribosome biogenesis is the major function known to be associated with the nucleolus (Hadjiolov, 1985), although recent work suggest that this nuclear compartment might be involved in other cellular processes (Pederson, 1998).

The nucleolus is the site of ribosomal RNA (rRNA) transcription, rRNA modification, maturation and assembly with ribosomal proteins to form the pre-ribosomal particles that are then exported to the cytoplasm to form the mature ribosomes. Ribosome biogenesis is probably one of the most complex pathways of ribonucleoparticle synthesis, involving a complex rRNA maturation and an ordered assembly of about 80 ribosomal proteins in eukaryotes. In addition to the component found in the mature ribosomes, the nucleolus contains many other RNAs and proteins, most of which transiently associate with pre-ribosomal particles and appear to be involved in various aspects of ribosome biogenesis (transcription, maturation, modification and ribosome assembly). In this review we focus on one of the major and most studied nucleolar proteins, nucleolin, whose multiple functions in ribosome biogenesis are beginning to be characterized.

### Properties of the different domains of nucleolin and 'nucleolin-like proteins'

Nucleolin was first described by Orrick et al. (1973) and was initially called C23 because of its mobility on a two-

dimensional gel (Prestayko et al., 1974). The same protein was then described and purified from Chinese Hamster Ovary cells (Bugler et al., 1982) and several other eukaryotic cells. The name nucleolin is now widely used for this nucleolar protein, which can represent as much as 10% of total nucleolar protein in CHO (Bugler et al., 1982). This protein is also often described as a 100-110 kDa protein; however, cloning of the cDNA of hamster nucleolin revealed that it contained 713 amino acids, giving rise to a predicted molecular mass of 77 kDa (Lapeyre et al., 1987, 1985). This discrepancy was latter attributed to the amino acid composition of the N-terminal domain of nucleolin. Homologous proteins were then identified in man (Srivastava et al., 1989), rat (Bourbon et al., 1988), mouse (Bourbon et al., 1988), chicken (Maridor and Nigg, 1990) and *Xenopus laevis* (Caizergues-Ferrer et al., 1989; Rankin et al., 1993). Nucleolin is highly phosphorylated (Olson et al., 1975; Rao et al., 1982) and methylated (Lischwe et al., 1982), and could also be ADP-ribosylated (Leitinger and Wesierska-Gadek, 1993).

The human nucleolin gene is present at one copy per haploid genome. The human gene consists of 14 exons and 13 introns on chromosome 2q12-qter (Srivastava et al., 1990). The four acidic stretches of the N-terminal domain lie within exons 2 to 4 and the nuclear localization signal (NLS) is encoded in exon 5. Interestingly, each of the four RNA-binding domains is encoded by two independent and consecutive exons. For the four RNA-binding domains, the intron position is similar and interrupts the conserved RNP1 sequence of each RNA-binding domain, suggesting that these RNA-binding domains are duplications of the same ancestor RBD domain. This genomic

organization is extremely well conserved in hamster, mouse and rat (Bourbon and Amalric, 1990; Bourbon et al., 1988). Another intriguing feature of nucleolin gene organization (in tetrapod and vertebrates) is that two snoRNAs (U20 and U23 snoRNA) are encoded in introns 11 and 12, respectively (Nicoloso et al., 1994; L. H. Qu and J. P. Bachellerie, personal communication). U20 and U23 belong to the two large families of snoRNAs, the box C/D antisense snoRNAs and the H/ACA snoRNAs, respectively, which guide the two major types of rRNA nucleotide modifications, namely ribose methylation and pseudouridine formation (Bachellerie and Cavaillé, 1998; Smith and Steitz, 1997). In vertebrates, most modification-guiding snoRNAs are intron-encoded and processed from pre-mRNA introns, and their host genes are usually directly involved in ribosome biogenesis (Bachellerie and Cavaillé, 1998; Bachellerie et al., 1995).

Analysis of the amino acid sequence of nucleolin reveals the presence of three different structural domains (Fig. 1). The N-terminal domain is made up of highly acidic regions interspersed with basic sequences and contains multiple phosphorylation sites. The central domain contains four RNA-binding domains called RBD or RRM. The C-terminal domain called GAR or RGG domain is rich in glycine, arginine and phenylalanine residues. This domain contains high levels of  $N^G,N^G$ -dimethylarginines (Lischwe et al., 1982).

Several nucleolar proteins of different eukaryotic species exhibit a similar tripartite structural organization (Table 1 and see legend of Fig. 1 for details). In this review, we will call these proteins 'nucleolin-like proteins'. This does not imply that they are all orthologs of nucleolin in these different species; however, based on what is known of nucleolin's properties, we can propose that all these proteins are multifunctional nucleolar proteins involved in different aspects of ribosome biogenesis.

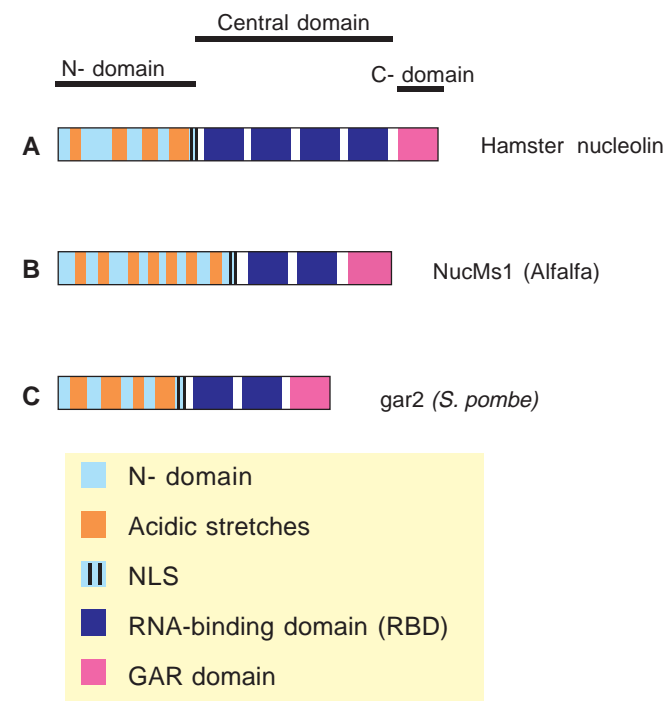
### N-terminal domain

The length of this domain is very variable among the different nucleolin-like proteins (Fig. 1). The presence of highly acidic regions separated from each other by basic sequences is one feature of this domain. Yeast *gar2* and Nsr1p N-terminal domains have long stretches of serine residues in the acidic regions. Acidic domains have been proposed to bind histone H1 (Erard et al., 1988), and could be responsible for a displacement of H1 from its interaction with linker DNA. This interaction with histone H1 would induce chromatin decondensation (Erard et al., 1988). These acidic stretches also determine the Ag-NOR stainability of nucleolin (Roussel et al., 1992). The presence of the basic and repeated octapeptide motifs (XTPXKKXX, X being a non-polar residue) bears strong similarity to an analogous sequence of histone H1 (Erard et al., 1990). These motifs could be responsible for the capacity of nucleolin to modulate DNA condensation in chromatin (Erard et al., 1990; Olson and Thompson, 1983). In addition to its interaction with chromatin and histone H1, the N-terminal domain of nucleolin has been involved in many protein-protein interaction, such as with the U3 snoRNP (Ginisty et al., 1998) and some ribosomal proteins (Bouvet et al., 1998; Sicard et al., 1998).

The N-terminal domain of nucleolin is highly phosphorylated (Bourbon et al., 1983; Mamrack et al., 1979; Rao et al., 1982). Nucleolin is a substrate for several kinases,

including casein kinase II (CK2) (Caizergues-Ferrer et al., 1987), p34<sup>cdc2</sup> (Belenguer et al., 1989; Peter et al., 1990) and protein kinase C- $\zeta$  (Zhou et al., 1997). CK2 co-purifies with nucleolin (Caizergues-Ferrer et al., 1987), and recent work indicates that  $\alpha$  or  $\alpha'$  subunits of CK2 interact directly with nucleolin (Li et al., 1996). CK2 phosphorylates nucleolin in vitro and in vivo at serine residues found predominantly in two highly acidic regions (Caizergues-Ferrer et al., 1987). p34<sup>cdc2</sup> phosphorylation occurs on threonine residues within the basic TPXKK repeat (Belenguer et al., 1989; Peter et al., 1990). All potential sites for p34<sup>cdc2</sup> kinase are not used with the same efficiency in vivo as in vitro (Belenguer et al., 1989). Phosphorylation of nucleolin by CK2 and p34<sup>cdc2</sup> is highly regulated during the cell cycle (Belenguer et al., 1989; Peter et al., 1990). Extensive phosphorylation by CK2 occurs in interphase and by p34<sup>cdc2</sup> in mitosis, and this regulated phosphorylation of nucleolin probably regulates nucleolin function during the cell cycle.

In plants and yeast, phosphorylation sites in the N-terminal domain have been diversely conserved. Nucleolin-like proteins from plants (Bogre et al., 1996; de Carcer et al., 1997) and yeast (Gulli et al., 1997) exhibit consensus CK2 phosphorylation sites, or are highly phosphorylated by this kinase. Plant nucleolin-like proteins are also phosphorylated by p34<sup>cdc2</sup>. Although *S. pombe* *gar2* is most likely phosphorylated by p34<sup>cdc2</sup> in mitosis on a single serine residue (Gulli et al., 1997), a *gar2* protein with this serine changed into a non-phosphorylated alanine is able to complement perfectly a *gar2*-



**Fig. 1.** Schematic representation of the organization of three nucleolin and 'nucleolin-like proteins'. RBDs are defined from the  $\beta 1$  and  $\beta 4$  strand (Kenan et al., 1991). (A) Organization of hamster nucleolin: mouse, rat chicken, human and *Xenopus laevis* nucleolin have the same organization. (B) Alfalfa and pea 'nucleolin-like protein' have the same organization. (C) Organization of *gar2*. Nsr1p possesses only 3 long acidic stretches. N-, N-terminal; C-, C-terminal.

**Table 1. Characteristics of nucleolin and ‘nucleolin-like proteins’ in several organisms**

Organism	Name	Molecular mass (kDa)*	Number of RBDs	pI	References	Remarks
Hamster	Nucleolin	77.0 (100)	4	4.49	Lapeyre et al. (1985)	
Mouse	Nucleolin	76.7 (105)	4	4.48	Bourbon et al. (1988)	
Human	Nucleolin	76.3 (100)	4	4.39	Srivastava et al. (1989)	
Rat	Nucleolin	77.1 (110)	4	4.46	Bourbon and Amalric (1990)	
Chicken	Nucleolin	75.6	4	4.68	Maridor et al. (1990)	
<i>Xenopus laevis</i>	Nucleolin	75.52 (95)	4	4.58	Rankin et al. (1993); Caizergues-Ferrer et al. (1989)	Two different forms. Difference essentially in the N-terminal domain
		70.20 (90)	4	4.64		
Alfalfa	NucMs1	67.1 (95)	2	4.62	Bogre et al. (1996)	Higher expression in root meristematic cells
Pea	Nucleolin	64.8 (90)	2	4.71	Tong et al. (1997)	Expression is induced by red light
Onion	Nop64A	(64)	2	ND	de Carcer et al. (1997)	Two different forms. Only Nop61A is phosphorylated. cDNA not characterized
	Nop61A	(61)	2			
<i>Arabidopsis thaliana</i>	FMV3bp	57.9	2	4.43	Didier and Klee (1992)	Very short C-terminal domain with only one RGG motif. N-terminal acidic stretches are very short
<i>Tetrahymena thermophila</i>	Nopp52	51.7 (52)	2	6.16	McGrath et al. (1997)	Only one RGG motif in C-terminal domain
<i>S. pombe</i>	gar2	53.0	2	4.82	Gulli et al. (1995)	gar2 <sup>-</sup> strain is cold-sensitive
<i>S. cerevisiae</i>	Nsr1p	44.5 (67)	2	4.65	Lee et al. (1992)	Overexpression of Nsr1p is lethal. Nsr1 <sup>-</sup> strain is cold-sensitive.

\*Apparent molecular masses are given in parentheses.  
ND, not determined.

*null* strain (Gulli et al., 1997). In *S. cerevisiae*, Nsr1p lacks consensus p34<sup>cdc2</sup> phosphorylation sites, so it was proposed that p34<sup>cdc2</sup> phosphorylation had become useless in cells where mitosis is not accompanied by nucleolar disassembly/reassembly.

### The RNA-binding domains

Nucleolins from hamster, mouse, rat, human, chicken and *Xenopus laevis* possess four RNA-binding domains called RBDs, also known as RRM (RNA recognition motif) (see Fig. 1 and Table 1). These domains, found in a large number of proteins implicated in various functions (Burd and Dreyfuss, 1994), are known to confer an RNA-binding specificity to the protein they belong to (Nagai, 1996). For example, one of the RBDs found in the U1A protein is responsible for the RNA-binding specificity of the U1A protein towards two structurally different RNA targets (Jovine et al., 1996). No structural data are yet available for nucleolin RBDs. One interesting feature of nucleolin RBDs is that they are less conserved within the same protein than between RBDs from divergent species (Table 2). For example, RBDs 1 and 4 are less than 10% identical within the same protein. According to their sequence identity, nucleolins and nucleolin-like proteins can be divided in three groups. In the first group is found nucleolin from hamster, mouse, rat, human, chicken, *Xenopus laevis* and fish. In the second group are the yeast proteins gar2 and Nsr1p, and in the third group the plant proteins NucMs1 and nucleolin from pea. Within each of these groups, the RBDs are very similar. Mouse and human nucleolin RNA-binding specificity is beginning to be well characterized, but there is very little information on the RNA-binding properties for the other nucleolin-like proteins.

Even before the identification of RBDs in nucleolin, it was known that nucleolin interacted with nucleic acids (Herrera and Olson, 1986; Olson et al., 1983). The high concentration of nucleolin in the dense fibrillar region of the nucleolus (Escande et al., 1985; Lischwe et al., 1981) suggested that nucleolin could be associated with rRNA. It was latter shown that nucleolin associated with nascent pre-ribosomal RNA (Herrera and Olson, 1986), and could even be detected on Christmas trees (Ghisolfi-Nieto et al., 1996). Nucleolin binds with high affinity and specificity with rRNA fragments from the 5' ETS (Ghisolfi et al., 1992b; Serin et al., 1996). A SELEX experiment performed with hamster nucleolin purified from CHO cells identified an 18-nt RNA consensus motif (Ghisolfi-Nieto et al., 1996). Sequence comparison between this RNA motif and sequences of the rRNA 5' ETS previously identified as nucleolin binding sites, in addition to extensive mutagenesis, allowed the detailed characterization of one nucleolin binding site (Ghisolfi-Nieto et al., 1996; Serin et al., 1996). A small stem-loop structure composed of a short stem (5 base pairs) and a 7-10 nt loop containing the motif U/G CCCGA forms a minimal RNA-binding site (NRE; Nucleolin Recognition Element). The binding affinity ( $K_d$ ) of nucleolin for this RNA target can vary from 5 nM for the SELEX sequence to 100 nM for a natural sequence found in rRNA (Ghisolfi-Nieto et al., 1996). The factors responsible for this binding affinity difference are not known.

Taken separately, none of the four individual RBDs interact significantly with this RNA target, but a peptide that contains the first two RBDs (RBD 12) is sufficient to account for nucleolin RNA-binding specificity and affinity towards this RNA target (Serin et al., 1997). A SELEX experiment

**Table 2. Percentage of sequence identity of the RDB domain of nucleolin from different species**

	RDB 1	RBD 2	RDB 3	RBD 4
Hamster	100	100	100	100
Mouse	96	99	93	100
Human	85	83	96	99
Chicken	62	64	70	89
<i>Xenopus laevis</i>	53	53	67	85
Carp*	ND	51	64	85
Nsr1p	100	100		
Gar2	58	61		
NucMs1	100	100		
Nucleolin of pea	86	86		
FMV3bp (Arabidopsis)	22	22		

According to their sequence identity, nucleolin and nucleolin-like proteins have been divided into three groups. All RBDs are compared to the corresponding domain of one member of each group (Hamster in group 1, Nsr1p in group 2 and NucMs1 in group 3).

These percentages have been calculated for each domain between the predicted  $\beta$ 1 and  $\beta$ 4 strand, excluding the linker between each domain.

RBD domains of FMV3bp present low sequence identity with each of the other three groups.

\*P. Ganot and J. P. Bachelierie, personal communication.

ND, sequence not determined.

performed with this recombinant RBD 12 protein selected exactly the same 18-nt motif as full-length nucleolin (G. Serin and P. Bouvet, unpublished result), demonstrating that these two domains were sufficient for this interaction. A genetic study of this RBD12-NRE interaction provided more details on this interaction (Bouvet et al., 1997). The constraints provided by these studies allowed us to propose a three-dimensional model of this interaction. In this model, both domains participate in a joint interaction with the NRE using a different surface to contact the RNA. The high-resolution determination of the structure of this RNA protein complex is now currently being investigated by NMR and X-ray crystallography.

Human, mouse and hamster nucleolin interact with the same binding affinity and specificity with a mouse 5' ETS RNA fragment that contains a NRE motif. A similar human 5' ETS RNA fragment is also efficiently recognized by mouse nucleolin (Serin et al., 1996). This conserved binding specificity for RBD 12 of human, mouse and hamster is in agreement with the high percentage of identity of this domain between these different species (Table 2). Interestingly, *Xenopus laevis* RBD 1 and 2, which are only 53% identical to the hamster protein, do not interact significantly with the NRE motif (G. Serin and P. Bouvet, unpublished data).

The presence of several RBDs in nucleolin suggests that nucleolin is potentially able to interact with multiple RNA targets. Indeed, studies on nucleolin function in pre-rRNA processing (Ginisty et al., 1998) identified an RNA target within the mouse 5' ETS, that was distinct from the NRE motif. The RNA sequences and nucleolin RBDs involved in this interaction remain to be determined. RNA sequences containing the telomeric repeat UUAG (Ishikawa et al., 1993), sequences in the 3' UTR of the amyloid protein precursor (Zaidi and Malter, 1995) and in the 3' noncoding region of poliovirus (Waggoner and Sarnow, 1998) have also been described as human nucleolin binding sites. However, little information on binding affinity, specificity or mechanism of nucleolin interaction with these RNA motifs, or the biological relevance of these interactions, is available.

### The C-terminal GAR/RGG domain

This domain is defined as spaced Arg-Gly-Gly (RGG) repeats interspersed with amino acids, which are often aromatics. It seems that the motif RGGF is particularly frequent in nucleolar proteins. The length of the GAR/RGG domain is variable among nucleolins, with its sequence and arrangement of the repeats not well conserved. For example, plant nucleolin-like proteins have a longer GAR domain than mammalian nucleolins (Bogre et al., 1996). The presence of this domain in a protein is associated with the presence of an RNA-binding domain (RBD or others) (Burd and Dreyfuss, 1994). Structural studies of this domain indicate that it can adopt repeated  $\beta$ -turns (Ghisolfi et al., 1992a). The GAR domain of hamster nucleolin interacts nonspecifically with RNA, leading to unstacking and unfolding of this RNA (Ghisolfi et al., 1992a). The presence of this GAR domain does not influence the binding affinity and specificity for the NRE sequence (Ghisolfi-Nieto et al., 1996; Serin et al., 1997), but one function of this domain could be to facilitate the interaction of nucleolin RBD domains with targets located within large and complex RNA, such as rRNA (Ghisolfi et al., 1992b; Heine et al., 1993). Recently it was shown that the GAR domain is also a protein-protein interaction domain. The hnRNP A1 GAR domain interacts in vitro with itself and with other hnRNP proteins (Cartegni et al., 1996). The GAR domain of mouse nucleolin, and probably also of the yeast gar2 protein, interacts with several ribosomal proteins (Bouvet et al., 1998; Sicard et al., 1998). It is not known how the GAR domain mediates protein-protein interaction, but since the GAR domain of nucleolin interacts with only a subset of ribosomal proteins, these interactions are likely to be specific.

Soon after its discovery, it was shown that nucleolin contained high levels of  $N^G, N^G$ -dimethylarginines (Lischwe et al., 1982). This post-translational modification is found on arginine located in the GAR domain (Lapeyre et al., 1986; Lischwe et al., 1985). This modification is not absolutely required for the non-specific interaction of the GAR domain with RNA (Serin et al., 1997), or for the interaction with other proteins (Bouvet et al., 1998). However, it could be an important signal for the regulation of these interactions, the stability of the protein, or its localization. It is not known if this post-translational modification is reversible.

### Localization of nucleolin

The intracellular localization of nucleolin has been extensively studied by electron microscopy analysis and/or immunofluorescence ultrastructural localization in vertebrates (Escande et al., 1985; Lischwe et al., 1981; Spector et al., 1984), plants (Martin et al., 1992; Minguez and Moreno Diaz de la Espina, 1996; Tong et al., 1997) or even *S. pombe* yeast cells (Leger-Silvestre et al., 1997). There is a general agreement that nucleolin is mainly found in the fibrillar component around the fibrillar centres. However, a small portion has also been detected in the granular component in higher eukaryotes (Biggiogera et al., 1990; Escande et al., 1985), but is rarely seen in the fibrillar centres (Escande et al., 1985; Martin et al., 1992).

Nucleolin relocation during mitosis is a specific feature of higher eukaryotes. A general agreement is that nucleolin is present at the periphery of metaphasic chromosomes (Medina et al., 1995), in the nucleolar remnant (NR) of CHO cells

(Azum-Gelade et al., 1994) and in cytoplasmic nucleolus-derived foci (NDFs) (Dundr et al., 1997). These NDFs disappear at telophase and are replaced by nucleolin-containing pre-nucleolar bodies (PNBs) (Gas et al., 1985; Medina et al., 1995; Ochs et al., 1983). The NR, NDFs and PNBs also contain other factors necessary for pre-rRNA processing, whereas the Nucleolar Organiser Regions (NORs) only gather factors responsible for rDNA transcription (Hernandez-Verdun and Gautier, 1994). In spite of some controversial observations (Gas et al., 1985; Lischwe et al., 1981; Ochs et al., 1983; Spector et al., 1984), the mitotic NORs are almost certainly devoid of nucleolin (Azum-Gelade et al., 1994; Scheer and Weisenberger, 1994; Weisenberger and Scheer, 1995). At the end of mitosis, during nuclear membrane formation, the NR, NDFs and PNBs gather around the NORs to form new nucleoli so that rRNA transcription and then processing of the newly transcribed pre-rRNA can start again very rapidly.

Structural domains necessary for the proper nuclear targeting and nucleolar localization of nucleolin in mammalian cells as well as Nsr1p in *S. cerevisiae* have also been extensively studied (Creancier et al., 1993; Meßmer and Dreyer, 1993; Schmidt-Zachmann and Nigg, 1993; Yan and Melese, 1993), but the results are somewhat puzzling. First, they clearly demonstrate the presence of a conserved functional bipartite NLS, which is necessary and sufficient for nuclear targeting. Nucleolin, Nsr1p and gar2 from *S. pombe* are also able to bind to NLS peptides in vitro, thanks to their amino-terminal domain (Lee et al., 1991; Xue et al., 1993). This has led to the proposal that these proteins may be implicated in nucleolar-cytoplasmic transport (Xue et al., 1993). However, the nucleolar accumulation of nucleolin and Nsr1p seems to be determined by a complex interplay between several domains and is therefore not well understood. Basically, the RNA-binding domains of nucleolin or Nsr1p/gar2 are crucial for their nucleolar localization (Creancier et al., 1993; Meßmer and Dreyer, 1993; Schmidt-Zachmann and Nigg, 1993; Yan and Melese, 1993), but are unable by themselves to target hybrid proteins to the nucleolus. Moreover, the recent finding that RNase treatment induces the release of nucleolin from nucleoli in A6 cells (Schwab and Dreyer, 1997) confirms that nucleolar accumulation of nucleolin requires the presence of RNA. The N-terminal domain of Nsr1p, fused to its NLS, is able to direct  $\beta$ -galactosidase to the nucleolus, but progressive deletions result in nucleoplasmic localization (Yan and Melese, 1993). The same domains (N-terminal + NLS) of nucleolin are able to target chloramphenicol acetyl transferase (CAT) only to the nucleus (Creancier et al., 1993), although Schmidt-Zachmann and Nigg (1993) find it in the nucleolus. The C-terminal GAR domain allows total nucleolar accumulation when it is associated with at least one RBD (Creancier et al., 1993). The nucleolar localization of nucleolin lacking only its RGG repeats is also controversial (Creancier et al., 1993; Schmidt-Zachmann and Nigg, 1993). In conclusion, although nuclear targeting of nucleolin-like proteins depends on a classical bipartite NLS, we assume that nucleolar accumulation is a consequence of the affinity of the various domains for nucleolar factors, possibly rRNA (Olson and Thompson, 1983; Schwab and Dreyer, 1997) or other nucleolar components with which nucleolin interacts (Li et al., 1996).

A relationship between the function of nucleolin NLS and the phosphorylation of this protein has also been pointed out in *Xenopus* egg extract (Schwab and Dreyer, 1997). The presence of p34<sup>cdc2</sup> phosphorylation sites improves the efficiency of nuclear translocation when they are dephosphorylated and enhances cytoplasmic localization when phosphorylated. These results therefore indicate that the changes in the phosphorylation state of nucleolin during the cell cycle might be a possible regulatory element of nucleolin localization.

In addition to the nucleolar localization of nucleolin, several reports indicate the presence of nucleolin at the cell surface (see Table 3). However, further experiments are needed to demonstrate unambiguously that the protein detected in the cell membrane is nucleolin.

### Functions of nucleolin in ribosome biogenesis

The almost exclusive localization of nucleolin within the nucleolus and its transient interaction with rRNA and pre-ribosomes strongly point to a role in ribosome biogenesis. Indeed, experimental data suggest that nucleolin is involved in multiple steps of this process. However, most of these data are based on in vitro studies and only correlate the presence or absence of nucleolin with a function. The expression of nucleolin (and nucleolin-like proteins) and rRNA seem well coordinated (Bogre et al., 1996; Meyuhas et al., 1990; Tong et al., 1997), and the accumulation of nucleolin is correlated to the proliferative activity of the cell (Derenzini et al., 1995; Mehes and Pajor, 1995; Sirri et al., 1995, 1997; Yokoyama et al., 1998). Moreover, during *Xenopus laevis* early development, the appearance of nucleolin precedes the transcription of rDNA and synthesis of ribosomal proteins, suggesting that nucleolin is involved in the early steps of ribosome biogenesis (Caizergues-Ferrer et al., 1989).

### Nucleolin and rRNA transcription

A small fraction of nucleolin tightly binds to chromatin (Olson et al., 1975; Olson and Thompson, 1983). Moreover, in vitro, nucleolin interacts with single-stranded DNA with higher affinity than with double-stranded DNA, and binds preferentially to a DNA fragment containing the region of the non-transcribed spacer of rDNA located upstream of the site of transcription initiation (Olson et al., 1983). Taken together with the ability of nucleolin to interact with histone H1 and to modulate chromatin structure (Erard et al., 1998, 1990), this suggested that nucleolin could be involved in the regulation of rRNA transcription. Indeed, following inhibition of rRNA transcription, a rapid release of nucleolin from the dense fibrillar zone of the nucleolus is observed (Escande-Geraud et al., 1985) demonstrating that its presence in this subnucleolar compartment was dependant upon rRNA transcription. In an in vitro transcription system, full-length nucleolin inhibits rRNA transcription from a plasmid template (Bouche et al., 1984). Furthermore, injection of anti-nucleolin antibodies in *Chironomus tentans* salivary gland cells stimulate 2- to 3.5-fold the synthesis of pre-rRNA (Egyhazi et al., 1988). Altogether, these data indicate that nucleolin could be involved in a control of rRNA transcription, probably without its having a direct role in transcription initiation since it is not found in the polymerase I initiation complex. The relevance of the interaction of nucleolin with rDNA sequences upstream of the initiation site

and with chromatin is therefore unclear for the regulation of rRNA transcription.

Nucleolin phosphorylation is induced by androgens and growth factors (Bonnet et al., 1996; Bouche et al., 1987; Issinger et al., 1988; Suzuki et al., 1985, 1991; Tawfic et al., 1994) and is accompanied by increased rRNA transcription and cell proliferation. Taken together with the cell-cycle regulation of mammalian nucleolin phosphorylation and the presence of highly phosphorylated nucleolin in actively growing cells (Belenguer et al., 1989) this suggests that rRNA transcription and nucleolin phosphorylation are intimately linked. Nucleolin is susceptible to limited proteolysis *in vivo* (Bourbon et al., 1983; Bugler et al., 1982; Chen et al., 1991) and a strong correlation between nucleolin phosphorylation (probably by CK2) and this proteolysis has been observed (Bourbon et al., 1983; Warrenner and Petryshyn, 1991). The mechanism of nucleolin proteolysis is still controversial. It is inhibited by the general protease inhibitor leupeptin (Bouche et al., 1984) and also by thiol enzyme inhibitors (Bourbon et al., 1983). The protease activity seems tightly associated with nucleolin (Bourbon et al., 1983), and it has even been suggested that nucleolin itself could possess a self-cleavage

activity (Chen et al., 1991; Fang and Yeh, 1993). Nucleolin cleavage products have not been fully characterised, but several proteolytic sites have been mapped within the N-terminal domain of nucleolin.

In run-off experiments, addition of nucleolin blocks rRNA transcription only in the presence of leupeptin, which inhibits nucleolin proteolysis (Bouche et al., 1984). This led to the proposal of an elegant, but still unproven model, where nucleolin is involved in the regulation of rRNA transcription elongation (Bouche et al., 1984) through the binding of its RNA-binding domains with the nascent rRNA transcript, whereas the N-terminal domain interacts with the polymerase I machinery, which would block transcription elongation. In this model, the phosphorylation of nucleolin by CK2 during interphase would be required to proteolyse nucleolin (between the N- and central domains) and to release the transcription complex (Bouche et al., 1984), while the central and C-terminal domains of nucleolin could remain bound to the rRNA and participate in the pre-ribosome assembly.

**Nucleolin and rRNA maturation and ribosome assembly**  
In yeast numerous factors involved in rRNA processing have

**Table 3. The multiple-functions of nucleolin**

Properties/interaction with:	Proposed functions:	References
3' UTR of APP	Cytoplasmic RNA stability	Zaidi and Malter (1995)
Telomeric DNA		Ishikawa et al. (1993)
3' splice site	Pre-mRNA metabolism	Ishikawa et al. (1993)
B-motif	Transcriptional repressor of AGP gene	Yang et al. (1994)
Immunoglobulin switch	Switch recombination	Borggreffe et al. (1998); Dempsey et al. (1998); Hanakahi et al. (1997); Miranda et al. (1995)
DNA repeats; SWAP complex, LR1		Miranda et al. (1995)
ATP-dATP binding		
MAR	Nuclear matrix	Caizergues-Ferrer et al. (1984); Dickinson et al. (1995); Martelli et al. (1995); Gotzmann et al. (1997); de Carcer et al. (1997); Minguéz and Moreno Diaz de la Espina (1996)
Topoisomerase I	Topo I localization	Bharti et al. (1996)
Human myeloid cell nuclear differentiation antigen (MND A)		Xie et al. (1995)
3' non-coding region of poliovirus	Viral genome amplification	Waggoner and Sarnow (1998)
Hepatitis delta antigen	Nucleolar targeting, HDV replication	Lee et al. (1998)
FKBP binding protein		Jin and Burakoff (1993)
Protein kinase C- $\zeta$ substrate	Transduction signal for NGF from cell surface to nucleus in PC12 cells	Zhou et al. (1997)
Helicase activity	DNA replication, recombination, repair, RNA unfolding	Tuteja et al. (1995)
Interaction with ssDNA parvovirus MVMp	Regulation of parvoviral life-cycle	Barrijal et al. (1992)
Interaction E47 helix-loop-helix		Dear et al. (1997)
Interaction with mRNP in <i>Xenopus</i> oocytes	mRNP formation	Yurkova and Murray (1997)
Binding to granzyme A	Apoptosis	Pasternack et al. (1991)
Presence of nucleolin at the cell surface:	-laminin-binding protein	Kleinman et al. (1991) Kibbey et al. (1995) Yu et al. (1998)
	-binding of lipoprotein in HepG2 cells	Semenkovich et al. (1990)
	-binding of V3 loop domain of HIV: receptor of HIV	Callebaut et al. (1998)
	-binding to coxsackie B viruses	de Verdugo et al. (1995)
	-binding to fructosyllysine	Krantz et al. (1995)
	-surface of Hep-2 cells	Deng et al. (1996)
	-substrate of ecto-protein kinase	Jordan et al. (1994)
	-receptor of Factor J. Cellular adhesion	Larrucea et al. (1998)
	-binding to MK and HB-GAM	Take et al. (1994)

been identified (Tollervey, 1996). The yeast nucleolin-like proteins Nsr1p and gar2 are required for correct processing of rRNA (Gulli et al., 1995; Lee et al., 1992). Gene disruption of these two proteins results in a deficit in the production of 18S rRNA and 40S ribosomal subunit. However, it has not been yet demonstrated that these proteins are involved in a specific step of rRNA maturation. The nucleolar function of gar2 requires the concerted action of its N-terminal domain and its RNA-binding domains (Sicard et al., 1998), suggesting that gar2 could act as an assembly factor allowing the proper formation of pre-rRNP particles, which are efficiently processed. The methylation of the 18S rRNA is severely reduced in the *nsr1*<sup>-</sup> mutant, indicating that Nsr1p may be directly or indirectly involved in this post-transcriptional modification (Kondo and Inouye, 1992). Despite the homology of the structural organization of Nsr1p and mammalian nucleolin, hamster nucleolin does not complement an *nsr1*<sup>-</sup> mutant (Xue et al., 1993), which could point to a difference in their RNA-binding specificity.

A long proposed role for vertebrate nucleolin in rRNA processing has only recently been demonstrated in the first step of mouse pre-rRNA processing (Ginisty et al., 1998). This processing step is well conserved during evolution and can occur at various position within the 5' ETS: +650/+657 in mouse, +414/419 in human, +105 in *Xenopus*, +1700 in *Physarum polycephalum* and +609 in *Saccharomyces cerevisiae* (Blum et al., 1986; Hughes and Ares, 1991; Kass et al., 1987; Miller and Sollner-Webb, 1981; Mougey et al., 1993). This processing event takes place during transcription of the pre-rRNA or just after its synthesis (Lazdins et al., 1997). The specific interaction of nucleolin with the pre-rRNA substrate is required for the processing reaction in vitro. The N-terminal domain together with the RBD region are required for nucleolin activity. It was proposed that nucleolin first interacts with the rRNA substrate, and then participates in the formation of the processing complex by recruiting different factors such as the U3 snoRNP (Ginisty et al., 1998). The injection of anti-nucleolin antibody in *Chironomus tentans* salivary gland cells had no drastic effect on pre-rRNA processing (measured by the relative amount of intermediate rRNA cleavage products) (Egyhazi et al., 1988). However, in these experiments, the antibody was injected after labeling of the precursor rRNA. If the assembly of the rRNP occurs co-transcriptionally, this pre-rRNA was already competent for the different processing steps when nucleolin was titrated with the antibody. The electroporation of anti-nucleolin antibodies in Hep-2 cells results in an increase of nucleolar size, and particularly of the dense fibrillar component of the nucleolus (Abadia-Molina et al., 1998), supporting a role for nucleolin in pre-rRNA processing.

The ability of nucleolin in vitro to promote the formation of secondary structures in complex RNA (Sipos and Olson, 1991), and the transient association of nucleolin with pre-ribosomal particles in vivo (Bourbon et al., 1983), suggest that nucleolin could be required for the correct assembly of these particles. Nucleolin could play a role as an assembly factor, bringing together a correctly folded rRNA and the other components necessary for rRNA maturation and/or assembly of the ribosome. The presence of nucleolin and nucleolin-like proteins in the dense fibrillar component of the nucleolus (Escande et al., 1985; Leger-Silvestre et al., 1997; Lischwe et

al., 1981; Martin et al., 1992; Minguez and Moreno Diaz de la Espina, 1996; Spector et al., 1984; Tong et al., 1997), and its detection on nascent pre-rRNA (Ghisolfi-Nieto et al., 1996; Herrera and Olson, 1986), suggest that this is one of the first proteins to interact with rRNA, and that it is possibly involved in a very early stage of ribosome assembly. It was recently shown that gar2 in *S. pombe* and human and mouse nucleolin interact with some ribosomal proteins (Bouvet et al., 1998; Sicard et al., 1998). The GAR domain of nucleolin is implicated in some of these interactions (Bouvet et al., 1998). Strikingly, all ribosomal proteins that interact with the GAR domain of mouse nucleolin are core ribosomal proteins, since these proteins are believed to be tightly associated with rRNA and to be among the first proteins assembled within the pre-ribosomal particles (Reboud et al., 1974; Welfle et al., 1976). The interaction of nucleolin with these ribosomal proteins further supports a role for nucleolin at an early step of ribosome assembly.

#### Nucleolin and nucleo-cytoplasmique transport

Although nucleolin is found almost exclusively within the nucleolus, assays based on interspecies heterokaryons have shown that nucleolin shuttles between nucleus and cytoplasm (Borer et al., 1989; Schmidt-Zachmann et al., 1993). This shuttling cannot be observed when proteins are injected directly within *Xenopus laevis* oocyte nuclei, suggesting that a very small amount of nucleolin is able to migrate to the cytoplasm or that nuclear export is compensated by efficient re-import of the protein (Schmidt-Zachmann et al., 1993). In the heterokaryon system, the N-terminal domain of nucleolin is required for efficient shuttling. However, it is unable to increase the nuclear export of an heterologous protein, indicating that nucleolin does not contain a positively acting export signal (Schmidt-Zachmann et al., 1993) as shown for other proteins (Izaurrealde and Adam, 1998; Nigg, 1997). Interestingly, during poliovirus infection, nucleolin was shown to relocate in the cytoplasm of the infected cells (Waggoner and Sarnow, 1998). This relocation is not a consequence of an inhibition of polymerase 1 transcription. Nucleolin was also found to be predominantly in cytoplasm of the rat epithelial cell IEC-6 when the cells are grown without laminin (Yu et al., 1998). These data prove that under some circumstances nucleolin is indeed able to shuttle between the nucleus and the cytoplasm. Several factors might affect the property of nucleolin to shuttle. During *Xenopus* oocyte maturation, cytoplasmic localization of nucleolin coincides with phosphorylation by p34<sup>cdc2</sup> (Schwab and Dreyer, 1997), and latter during development nuclear translocation is correlated with a dephosphorylation of nucleolin. These data show that phosphorylation of the N-terminal domain of nucleolin can regulate the function of nucleolin NLS (Schwab and Dreyer, 1997). A regulation of nuclear import by protein phosphorylation has been already described for several proteins, including transcription factors (Vandromme et al., 1996). In the heterokaryons system, it was shown that the GAR domain of nucleolin reduced slightly the export process (Schmidt-Zachmann et al., 1993). Recently, it was demonstrated that arginine methylation played a role in the rate of nuclear export of the hnRNPs Npl3p and Hrp1p in *S. cerevisiae* (Shen et al., 1998). Based on these results it would

be interesting to reinvestigate the role of the GAR domain, and of its post-translational modification on nucleolin nuclear-cytoplasmic shuttling.

The discovery that nucleolin is able to shuttle, even under specific conditions, has raised the possibility that it could be involved in the nuclear import of ribosomal components (like ribosomal proteins), or in the nuclear export of the ribosomal particles. The experimental systems required to study these processes are now available, and future experiments will determine if nucleolin is indeed involved in nucleolar-cytoplasmic transport of ribosomal components.

### Other functions of nucleolin

Nucleolin has been found to interact with several RNA/DNA/protein targets within the nucleus, cytoplasm and on the cell surface of several cell lines. From these different interactions a function for nucleolin in different processes was deduced (Table 3). These different functions are quite unexpected for a nucleolar protein, and it is doubtful that a single nucleolar protein is involved in so many different functions. It is, however, striking that several laboratories, using different approaches, have reached the same conclusions. For example, several reports suggest the presence of nucleolin on the surface of some eukaryotic cell types (Table 3). In most of these reports it is not absolutely clear if the detected protein is the nucleolar nucleolin or a closely related protein that shares some sequence homology with nucleolin. Expression of a tagged nucleolin (GFP-nucleolin for example) would be useful to determine if a fraction of nucleolin is really located on the cell surface.

Nucleolin has also been described to interact with or be a component of several transcription factor complexes, LR1, AGP and E47, and with other nuclear components potentially involved in the regulation of gene expression: MAR, switch DNA repeat, telomere DNA repeat, topoisomerase 1, etc... (see Table 3). These interactions with nucleolin, if real *in vivo*, might be important in understanding the plurifunctionality of the nucleolus (Pederson, 1998). The presence of nucleolin autoantibodies in patients with autoimmune disease (systemic lupus erythematosus, SLE), has also been reported (Minota et al., 1990, 1991). Autoantibodies in SLE are targeted predominantly to nucleoprotein particles (Elkon, 1995). The biological significance of the presence of antinucleolin antibodies remains to be determined.

Nucleolin was also identified as the human DNA helicase IV (Tuteja et al., 1995, 1991), which is able to unwind RNA-RNA/DNA-DNA and DNA-RNA duplexes in the 5' to 3' direction. This ATP-dependant duplex-unwinding activity was attributed to the GAR domain of nucleolin (Tuteja et al., 1995). Although it has been proposed that a binding site for ATP and dATP is present in nucleolin (probably in the RBD region) (Miranda et al., 1995), nucleolin and therefore the GAR domain do not seem to possess ATPase activity characteristic of a helicase. Furthermore, we have been unable to detect the helicase activity using different substrates with different nucleolin samples or recombinant GAR domain (P. Bouvet, unpublished results). The helicase activity described previously (Tuteja et al., 1995) might therefore result from a sequence specificity of the substrate used in this study, or from a protein contaminant in the nucleolin preparation. Further work is clearly needed to determine if nucleolin exhibits this

helicase activity or if this activity is tightly associated to and co-purifies with nucleolin.

### Conclusions

The implication of nucleolin in the different steps of ribosome biogenesis and the role of the different domains of nucleolin in this process are beginning to be unravelled. Numerous observations have been accumulated on nucleolin over the last 25 years. Together with recent data that demonstrate a direct role for this protein in ribosome biogenesis (Ginisty et al., 1998; Gulli et al., 1995; Kondo and Inouye, 1992; Sicard et al., 1998), an integrated view of nucleolin function in ribosome biogenesis can now be proposed.

Nucleolin has been implicated in many other functions than ribosome biogenesis. Its interaction with the 3' UTR of the APP and poliovirus RNA, 3' splice site, telomeric repeat, might simply reflect nucleolin's binding preference for some nucleic acid sequences, and not be biologically relevant. The same holds for the interaction of nucleolin with different proteins. The highly acidic and basic regions within the N-terminal domain of nucleolin could be responsible for these non-specific interactions. Conversely, recent evidence suggests that the nucleolus could be involved in other functions than ribosome biogenesis, including mRNA processing, snRNA modification, RNP assembly, tRNA processing and RNA export (Bond and Wold, 1993; Jacobson and Pederson, 1998; Pederson, 1998; Schreiner et al., 1995), opening a wide range of possibilities for additional nucleolin functions outside ribosome biogenesis. Finally, several viruses have been localised within the nucleoli of the infected cells. Nucleolin interacts with the genome of some of these viruses (parvovirus MVMp; Barrijal et al., 1992) and with the viral protein HDV (Lee et al., 1998). It is not known why these viruses localize preferentially within the nucleoli of infected cells, but this localization and the interaction with the cellular components of the host (including nucleolin and other nucleolar proteins) may provide the virus with the machinery required for propagation and infection of the cell.

Many proteins have been proposed, like nucleolin, to play a role in both transcriptional and post-transcriptional regulation of RNA. Several examples of proteins that have these properties can be found both for pol II and pol III transcripts. The most famous example is probably TFIIA, which has been extensively studied in the *Xenopus oocyte* system. This protein can interact with the promoter region of the 5S RNA gene and promotes the formation of competent transcription complexes (Shastri, 1996). TFIIA can also bind the transcribed RNA to form the 7S particles that are then exported out of the nucleus, and this interaction of TFIIA with the 5S RNA prevents this RNA from being re-imported into the nucleus (Rudt and Pieler, 1996). Therefore the same protein is required for the transcription, export and cytoplasmic storage of the 5S RNA. The Y-box proteins are another example of proteins involved in several functions. They have been implicated in the up- or down-regulation of the transcription of diverse genes (Ladomery and Sommerville, 1995; Wolffe et al., 1992). Later, it was shown that the Y-box protein FRGY2 is also a major mRNA-binding protein in *Xenopus oocytes* (Sommerville and Ladomery, 1996a,b), and plays an active role in masking mRNA from translation in somatic cells (Ranjan et al., 1993) and in *Xenopus oocytes* (Bouvet and Wolffe, 1994).



Immunostaining of *Xenopus* oocyte lampbrush chromosomes showed that the Y-box protein associates with nascent RNP (Sommerville and Ladomery, 1996a,b).

The different nucleolar functions of nucleolin and 'nucleolin-like proteins' must rely on the presence of several distinct structural domains. It is likely that the N- and C-terminal domains of nucleolin are involved in protein-protein interaction whereas the central domain, which contains the RBD motifs, is involved in specific interactions with nucleic acids. The different functions of nucleolin are also likely to be the result of the assemblies of nucleolin with other factors to form large complexes specialised in a specific function.

Analyzing the nucleolin roles in eukaryotic ribosome assembly is a major challenge because of the complexity of this highly integrated process, which cannot be efficiently reproduced in vitro. One major task now will be to determine if the different functions proposed for nucleolin in chromatin structure, rRNA transcription, rRNA maturation and assembly of the pre-ribosomes are linked to each other. If this is the case, nucleolin would appear to be a key player in the co-ordination of rRNA transcription, processing and protein assembly, reminiscent of the coupled transcription-polyadenylation-splicing of pol II transcripts (Corden and Patturajan, 1997; Neugebauer and Roth, 1997). Studies performed on nucleolin over the last 25 years have set the stage for the design of the molecular tools required to test these hypotheses.

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