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# Nucleolin: A Multifunctional Major Nucleolar Phosphoprotein*

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* This paper is dedicated to Prof. Francois Amalric, Toulouse, France

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**ABSTRACT**: Nucleolin is a major protein of exponentially growing eukaryotic cells where it is present in abundance at the heart of the nucleolus. It is highly conserved during evolution. Nucleolin contains a specific bipartite nuclear localization signal sequence and possesses a number of unusual structural features. It has unique tripartite structure and each domain performs a specific function by interacting with DNA or RNA or proteins. Nucleolin exhibits intrinsic self-cleaving, DNA helicase, RNA helicase and DNA-dependent ATPase activities. Nucleolin also acts as a sequence-specific RNA binding protein, an autoantigen, and as the component of a B cell specific transcription factor. Its phosphorylation by cdc2, CK2, and PKC-zeta modulate some of its activities. This multifunctional protein has been implicated to be involved directly or indirectly in many metabolic processes such as ribosome biogenesis (which includes rDNA transcription, pre-rRNA synthesis, rRNA processing, ribosomal assembly and maturation), cytokinesis, nucleogenesis, cell proliferation and growth, cytoplasmic-nucleolar transport of ribosomal components, transcriptional repression, replication, signal transduction, inducing chromatin decondensation and many more (see text). In plants it is developmentally, cell-cycle, and light regulated. The regulation of all these functions of a single protein seems to be a challenging puzzle.

**KEY WORDS:** nucleolin, nucleolus, RNA processing, ribosome biogenesis, helicase, rDNA transcription, nucleolar proteins.

## INTRODUCTION

Nucleolin (~100 kDa) is an important protein of the eukaryotic cell and is known to be involved in many metabolic processes besides potential role in ribosome biogenesis, which is still largely unknown. In eukaryotic cells ribosome biogenesis is needed throughout the life of a cell and it takes place within the nucleolus (Eichler and Craig, 1994). Nucleolous contains proteins of the pre-ribosomes and those with specific nucleolar functions such as RNA poly-

merase I, protein kinases, phosphatases, methylases and nucleases. The various proteins present in the nucleolus are nucleolin, B23 (numatrin, N038, nucleophosmin), NSR1 (yeast nucleolin-like protein) fibrillarin, (B36, NOP1), GAR 1, SSB1, NOP3, NOP4 (NOP77), NP146, DRS 1, P120, Nopp140, S1, ribocharin, 180 kDa, 145 kDa, etc. (Sommerville, 1986; Shaw and Jordan, 1995). Nucleolin is one of the best studied of all the nucleolar proteins.

Ribosome biogenesis in eukaryotes is a complex process that involves the coordinate expression of a large number of genes. The main steps of ribosome biogenesis occur in the nucleolus (Sommerville, 1986) where ribosomal genes are actively transcribed by RNA polymerase I. The intranuclear location of the nucleolus around the chromosomal regions that code for ribosomal RNAs (5.8S, 18S, and 28S) facilitates the active transcription of these genes by RNA polymerase I (Hadjiolov, 1985; Shaw and Jordan 1995; Shaw, 1996). The nascent transcripts are associated with two types of proteins: ribosomal proteins, found in the mature cytoplasmic ribosomes and a group of proteins that are transiently bound to preribosomes in the nucleolus. These latter proteins play a role in the transcription process, in the packaging of pre-RNA or in its maturation. Nucleolin is among one of them that is highly conserved during evolution and was originally called C23 (Orrick et al., 1973). It is a well-characterized major nucleolar phosphoprotein that represents up to 5% of the nucleolar proteins in exponentially growing cells (Sapp et al., 1986; Lapeyre et al., 1987). This protein was first identified in Chinese hamster ovary (CHO) cells and Novikoff hepatoma cells (Orrick et al., 1973; Bugler et al., 1982). It is a highly phosphorylated multifunctional nonribosomal acidic protein and is present at the heart of the nucleolus (Jordan, 1987). Because of its specific nucleolar localization it is called nucleolin. Antibodies against CHO nucleolin recognize the homologus protein in different species (Pfeifle and Anderer, 1983; Caizergues-Ferrer et al., 1989).

The name nucleolin was originally given to the CHO, human and rat proteins and later to frog and chicken proteins, which are not only structurally very similar but also their primary sequence are highly homologus. However, the proteins from yeast, pea, alfalfa and *Arabidopsis*, which are only structurally related to nucleolin without having a very similar sequence are called nucleolin-like proteins.

To date, nucleolin or nucleolin-like proteins have been reported in multiple species, including hamsters (Bouche et al., 1984; Lapeyre et al., 1987), human (Srivastava et al., 1989; Tuteja et al., 1995), mouse (Bourbon et al., 1988), rat (Ohmori et al., 1990), chicken (Maridor and Nigg, 1990), *Xenopus laevis* (Caizergues-Ferrer et al., 1989), insects (Olson, 1990), yeast (Lee et al., 1991, 1992; Kondo and Inouye, 1992; Gulli et al., 1995; Leger-Silvestre et al., 1997) and in plants (Didier and Klee, 1992; Martine et al., 1992; Bogre et al., 1996; Tong et al., 1997; deCarcer et al., 1997).

The mouse nucleolin gene is the first eukaryotic gene known to encode a protein that is both an RNA binding protein involved in RNA processing and a specific nucleolar protein (Bourbon et al., 1988). In mouse, the nucleolin gene extends over 9000 base pairs and is split into 14 exons that encode the 706 amino acid residues of the protein (Bourbon et al., 1988). It has been found that intron 11 of the nucleolin gene in humans and rodents encodes a novel small nucleolar RNA, termed U20 (Nicoloso et al., 1994). Southern hybridization analysis shows that nucleolin is encoded by a single copy gene in mouse and pea (Bourbon et al., 1988; Tong et al., 1997). This single protein, ubiquitously present in eukaryotic cells,

has been attributed with a remarkable number of diverse functions. There is at present no comprehensive review on the available information on nucleolin and its role in various functions in eukaryotes. The aim of this manuscript therefore is to focus on the structural and functional aspects of nucleolin.

## II. LOCALIZATION, TRANSPORT, AND SHUTTLING OF NUCLEOLIN

### A. Nucleolus

The nucleolus is the most prominent non-membrane subnuclear structure seen by phase contrast microscopy in most eukaryotic cells. It was first described in the early 19th century and was discovered in the 1960s to be the seat of ribosome synthesis. The discovery of the chromosomal nucleolar organizer locus established the nucleolus as a genetically determined element (Heitz, 1931; McClintock, 1934). Subsequently, the nucleolus was found to be the site of rRNA synthesis (Perry, 1960, 1962; Brown and Gordon, 1964). Nucleolus is the plurifunctional suborganelle and is a major site of many nuclear functions including rRNA transcription, rRNA processing and ribosome assembly, import and export of RNA and proteins (Goessens, 1984; Hadjiolov, 1985; Jordan, 1991; Scheer and Weisenberger, 1994, Pederson, 1998). In addition, new results indicate that biosynthesis of signal recognition particle RNA and telomerase RNA involve a nucleolar stage and that the nucleolus is also involved in processing of U6 RNA, one of the spliceosomal small nuclear RNAs (Pederson, 1998).

Morphologically, nucleoli consist of three distinguishable components: the fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC), which are the sites for different steps of ribosome biogenesis (Jordan, 1984; Goessens, 1984; Shaw and Jordan, 1995; Thompson et al., 1997). However, the nucleolus is a dynamic and variable structure that varies in size and number and it disappears and reappears with metabolic state of the cells (Montgomery, 1898; Heitz, 1931; Shaw and Jordan, 1995). Recently, Derenzini et al. (1998) have shown that quantitative distribution of nucleolar structures within the cell represented a cytohistological parameter of the rapidity of cell proliferation. The majority of the nucleolus is accounted for by the GC (~75%) with the DFC accounting for only ~17% and the FCs only ~2% (Jordan and McGovern, 1981). A typical higher plant nucleolus has much higher proportion of DFC (~50%), with FCs nearer 1% (Shaw and Jordan, 1995). RNA polymerase I is mainly present in FCs (Scheer and Rose, 1984) while rDNA is known to be present in FCs (Derenzini et al., 1983; Vandelae et al., 1993) as well as in DFC (Wachtler et al., 1989; Hozak et al., 1993).

### B. Localization of Nucleolin

There has been some difference of opinion regarding the exact localization of nucleolin. An earlier report showed that it is absent in the fibrillar center of nucleolus (Biggiogera et al., 1991). However, Martin et al. (1992) reported that nucleolin is also present, to a lesser extent, in the interior of fibrillar centers. The various reasons why it was undetected earlier could be due to the nature of the particular epitopes recognized by the antibody, to the concentrations of the nucleolin in fibrillar centers or even to a particular hindrance for the accessibility of the antibody to these nucleolar domains (Martin et al., 1992). Later it was shown

that although it is not evenly distributed but is preferentially localized in the dense fibrillar and granular component regions of nucleolus (Shaw and Jordan, 1995). The localization of nucleolin is well studied in onion root meristematic cells where it is localized in the proximal zone of the dense fibrillar component with respect to fibrillar centers while it is absent in the distal part, which strongly suggests that the processing of ribosomal precursors occurs in a topologically organized way in this nucleolar component (Martin et al., 1992).

Recently, *in vitro* cellular localization of nucleolin in rat intestinal epithelial IEC-6 cells has been shown to be influenced by laminin which is a major component of extracellular matrix (Yu et al., 1998). When the IEC-6 cells were cultured on laminin-coated plate the nucleolin was found to be translocated to the nucleus, while in the cells cultured without laminin the nucleolin was observed in cytoplasm (Yu et al., 1998). The presence of nucleolin in other organelles has not been well studied. Our preliminary findings show that antibodies against human nucleolin cross-react with chloroplast protein(s) of below 85 kDa molecular mass (Tuteja, N., unpublished data), suggesting that it may be present in the chloroplast also.

The abundance of nucleolin in the cell is correlated directly with nucleolar transcriptional activity (Escande-Geraud et al., 1985; Bouche et al., 1987). Immunocytologically and biochemically it has been shown that nucleolin is associated with chromatin (Olson and Thompson, 1983; Erard et al., 1988), preribosomes (Bugler et al., 1982; Herrera and Olson, 1986), pre-rRNA (Bugler et al., 1987), nucleolar matrix and ribosomal DNA (Caizergues-Ferrer et al., 1984). In resting cells, nucleolin is present at low levels and is preferentially associated with chromatin. The amount of nucleolin is cell-cycle dependent. Expression of nucleolin is low in serum-deprived cells and increases mostly in S phase or G1-S phase during cell cycle stimulation (Sirri et al., 1997). In plants, its expression is highest in root meristematic cells, but it is also found in other meristematic cells (Bogre et al., 1996).

## C. Transport of Nucleolin

The protein import into the cell nucleus occurs through large multiprotein structures, termed nuclear pore complexes (NPCs) (Schmidt-Zachmann and Nigg, 1993). NPCs mediate bidirectional transport between the cytoplasm and the nucleus (Gorlich and Mattaj, 1996; Nigg, 1997; Merkle and Nagy, 1997). In order to enter the nucleus, proteins larger than about 60 kDa generally require a specific nuclear localization signal (NLS), characterized by the presence of basic residues in either one or two clusters. Accordingly, these NLSs are referred to as mono- or bipartite. Nucleolin uses a bipartite NLS to enter the nucleus and then accumulates within the nucleolus by virtue of binding to other nucleolar components and is later transported through the rest of the nucleus and the cytoplasm (Martin et al., 1992; Schmidt-Zachmann and Nigg, 1993). The nucleolar accumulation of nucleolin requires at least two of its RNA-binding domains in addition to the NLS (Creancier et al., 1993).

Phosphorylation sites of cdc2 and CK2, which are clustered in the N-terminal domain of nucleolin, also regulate its function of the bipartite NLS (Schwab and Dreyer et al., 1997). In *Xenopus laevis*, a maternal store of nucleolin was known to be accumulated in the multiple nucleoli generated during oogenesis. This maternal nucleolin was reported to be distributed throughout the cytoplasm of the egg during oocyte maturation and after fertilization it was found to be

accumulated in the nuclei of the embryo. Schwab and Dreyer (1997) reported that cytoplasmic localization of nucleolin coincided with massive phosphorylation by cdc2 or CK2 kinases and nuclear translocation was accompanied by net dephosphorylation.

## D. Shuttling of Nucleolin

Nucleolin is also known as a shuttling protein that migrates constantly back and forth between nucleus and cytoplasm (Borer et al., 1989), similar to hnRNP proteins (Dreyfuss et al., 1993). However, hnRNP protein A1 shuttles rapidly and contains both import and export signals, whereas nucleolin shuttles slowly and contains only an import signal (Nigg, 1997). The existence of shuttling proteins was first inferred from nuclear transplantation studies carried out on amoebae (Goldstein and Ko, 1981).

Ribosome biogenesis is one of the most fundamental cellular process that requires the extensive transport of proteins and ribonucleoprotein particles across the nuclear envelope (Hadjiolov, 1985; Sommerville, 1986; Nigg, 1988). Ribosomal proteins are synthesized in the cytoplasm and imported into the nucleus where they are assembled with rRNAs to form pre-ribosomal particles. The nonribosomal nucleolar proteins that are required in processing and packaging of these particles are generally believed to dissociate from the maturing ribosomal subunits before these are exported to the cytoplasm (Borer et al., 1989). Biochemical and genetic studies have shown that the basic mechanisms of nucleocytoplasmic transport are highly conserved during evolution (Gorlich and Mattaj, 1996; Koepp and Silver, 1996). Nucleolin, through its nucleocytoplasmic shuttling property, may help in transporting ribosomal components during ribosome biogenesis.

# III. STRUCTURE OF NUCLEOLIN

Biophysical and biochemical studies have shown that nucleolin has a unique multidomain structure that is tripartite and conserved. It possesses a number of unusual basic sequences, a putative globular region, and a Glycine-rich segment (Lapeyre et al., 1987; Bugler et al., 1987; Ghisolfi et al., 1992a). The structures of nucleolin and nucleolin-like proteins from different species are shown in Figure 1. Nucleolin is a monomer with a sedimentation coefficient of ~5S, an isoelectric point in the range of 5.5 to 6.1 (Gotzmann et al., 1997) and has an elongated shape. Nucleolin has few potential Asn-glycosylation sites (Srivastava et al., 1989; Tong et al., 1997). It contains three distinct domains which are as follows

## A. N-Terminal Domain

The amino-terminal domain contains several highly charged acidic sequence repeats interspersed with basic segments (Figure 1). The number of acidic stretches differ in different species, for example, mouse, human, and chicken nucleolin contain four, whereas the nucleolin-like protein from pea and *Arabidopsis* contain seven and alfalfa contains nine (Bourbon et al., 1988; Srivastava et al., 1989; Maridor and Nigg, 1990; Bogre et al., 1996; Tong et al., 1997). The plant nucleolin-like proteins contain considerably more but shorter acidic repeats as compared with others. The acidic stretches are about 10 to 20 amino acids long. The highly basic repeats contain more lysine and proline residues (Bogre et al., 1996). Due to the presence of a high content of negatively charged amino acids in the N-terminal end of the protein there is a large difference between the actual (derived from

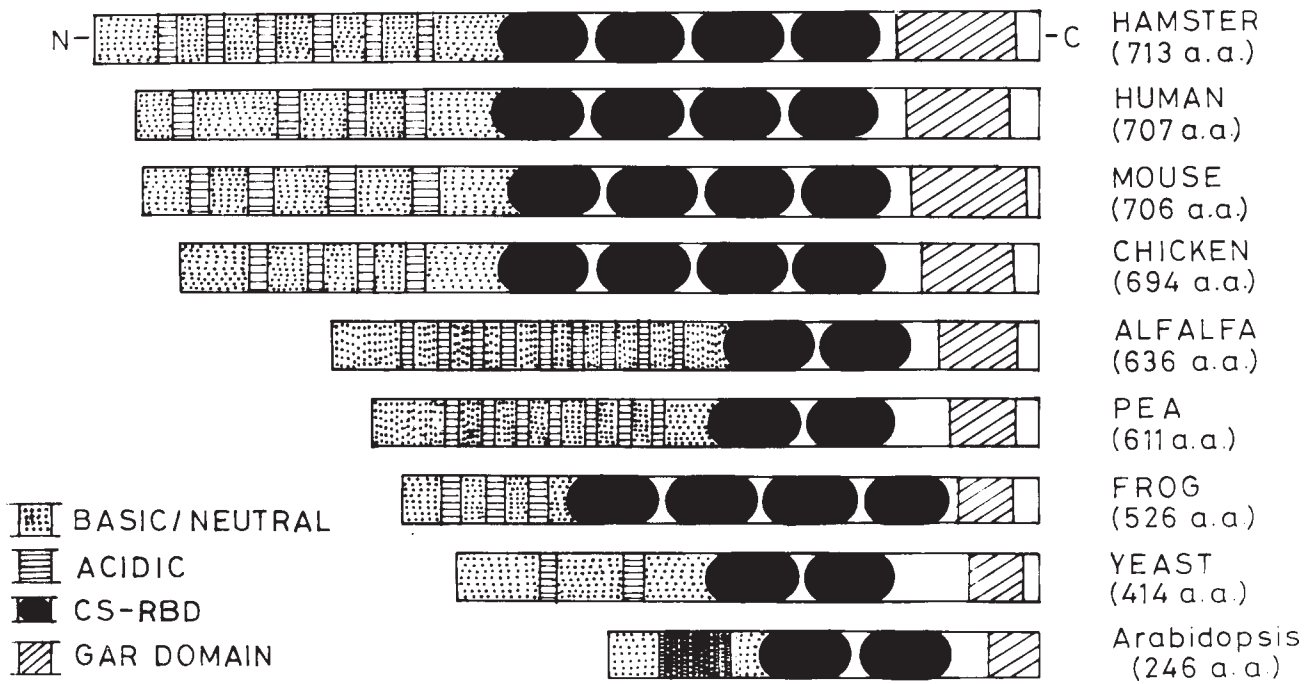


**FIGURE 1.** Schematic representation of domain diagram of structural features of the primary sequence of nucleolin and nucleolin-like proteins from different species. The number of amino acids (a.a.) is mentioned in parenthesis. (CS-RBD: consensus RNA binding domain, GAR: Glycine Arginine domain).

sequence) and the apparent (after SDS-PAGE) molecular mass ($M_r$) of nucleolin. In these cases the electrophoretic mobility on SDS-PAGE is slow and gives a higher apparent $M_r$ of the proteins. For example, the difference in the $M_r$ as determined by sequence and SDS-PAGE is 77 and 100 kDa for hamster (Lapeyre et al., 1987), 44 and 67 kDa for yeast (Lee et al., 1991), 65 and 90 kDa for pea (Tong et al., 1997). Thus, a large increase in the apparent $M_r$ determined by SDS-PAGE over $M_r$ determined by compositional analysis seems to be a common feature of all nucleolins (Tong et al., 1997).

Recently, Ginisty et al. (1998) showed that the N-terminal domain of CHO nucleolin is required for an interaction with U3 snoRNP during ribosome biogenesis. The N-terminal domain shows sequence homology to the high-mobility group (HMG) of proteins and interacts with nucleolar chromatin (Erard et al., 1988). This domain also contains several target sites for phosphorylation by cdc2 (S/TPXKK) and casein kinase 2 (CK2) (XS/TXXE/D) protein kinases (Belenguer et al., 1990; Peter et al., 1990; Caizergues-Ferrer et al., 1987). Phosphorylation plays an important role in modulating various activities of nucleolin, which is discussed later. Downstream part of the N-terminal domain contains bipartite NLS motifs and recognizes SV-40 type monopartite NLS motifs (Xue et al., 1993; Xue and Melese, 1994). Of the several structural domains present in nucleolin, only the N-terminal was found to be dispensable for nucleolar accumulation. The functional bipartite NLS in the chicken nucleolin KRKKEMANKSAPEAKKKK was shown to be responsible for targeting nucleolin to the nucleus (Schmidt-Zachmann and Nigg, 1993). The potential bipartite NLS sequences of human, KRKKEMAKQAAPEAKKQK, (Srivastava et al., 1989), yeast NSR1, KKRKSEDAEEEEDEESSNKKQK (Lee et al., 1991) and pea nucleolin-like protein, KKGKRQAEEEIKKVSAKKQK (Tong et al., 1997) affirms a consensus motif for nuclear targeting. However, there is no consensus signal sequence for targeting nucleolin to the nucleolus. Instead, it is proposed that the accumulation of nucleolin in the nucleolus results from specific binding of nucleolin to other nucleolar components, particularly rDNA, rRNA, and also protein constituents of nucleolar matrix structure (Schmidt-Zachmann and Nigg, 1993).

## B. Central Domain

This domain of nucleolin is globular and contains four RNA recognition motifs (RRM) also called consensus RNA-binding domain (CS-RBD) that are conserved among different species (Serin et al., 1997). However, nucleolin-like proteins from yeast, pea, alfalfa and *Arabidopsis* contain only two CS-RBD (Figure 1). The CS-RBD is found in proteins implicated in heterogeneous RNA packaging (Dreyfuss et al., 1993), pre-mRNA splicing (Amrein et al., 1988), as components of pre-ribosomes (Bourbon et al., 1983), in poly(A) tail synthesis and maturation (Adam et al., 1986), in translational control (Naranda et al., 1994), and in mRNA stability (Zhang et al., 1993). A typical CS-RBD contains 80 to 90 amino acids residues with two highly conserved sequences, the RNP-1 octapeptide (R/K)G(F/Y)(G/A)(F/Y)VX(F/Y) and the RNP-2 (L/I)(F/Y)(V/I)(G/K)(G/N)L hexapeptide motifs (Query et al., 1989). Computer analysis of murine nucleolin revealed that the putative ATP binding domains may be present within two of the phylogenetically conserved RNA binding domain (Miranda et al., 1995).

The central domain interacts and binds specifically with short RNA stem-loop structures of 18S and 28S ribosomal RNA

(Bugler et al., 1987; Ghisolfi et al., 1992b; Serin et al., 1997; Bouvet et al., 1997). This domain has alternating hydrophilic and hydrophobic segments and has some similarities to the proteins of spliceosomes (Jordan, 1987). These properties probably account for nucleolin's demonstrated association with early transcribed RNA in the nucleolus (Herrera and Olson, 1986). The fourth CS-RBD of human nucleolin has the highest sequence conservation between species as reported in the comparison of the rodent and *Xenopus* sequences (Caizergues-Ferrer et al., 1989). The second CS-RBD of pea and alfalfa lack an RNP-2 motif, which is less conserved than RNP-1 motif. The two CS-RBD in pea nucleolin are separated by 100 amino acids, while in animal nucleolin they have a distance of 90 amino acids (Bandzialis et al., 1989; Tong et al., 1997). Ghisolfi et al. (1996) reported that mouse and human nucleolin interact specifically with pre-rRNA and with *in vitro*-selected RNAs that contain a hexanucleotide motif U/GCCCGA within a short stem-loop structure. Recently, Serin et al. (1997) determined the minimal domain of nucleolin responsible for interacting with RNA. Out of four only two CS-RBD (CS-RBDs 1 and 2) are necessary and sufficient to account for the specific interaction of animal nucleolin with its RNA target. The full integrity of these two domains is required, because N- or C-terminal deletion abolishes the specific interaction with the RNA. It is also reported that mutation of conserved amino acids within the RNP-1 sequence of CS-RBD 1 or 2 significantly reduces the interaction with the RNA, whereas mutation of the analogous residues in CS-RBDs 3 and 4 has no effect. Serin et al. (1997) suggested that RNA binding specificity of nucleolin arises from a cooperation between two CS-RBDs. Bouvet et al. (1997) have shown that both CS-RBDs 1 and 2 participate in a joint interaction with NRE (nucleolin recognition element) and that each domain uses a different surface to contact the RNA. Bouvet et al. (1997) have proposed and described a three-dimensional model of nucleolin CS-RBD 1 and 2 bound to the NRE stem-loop.

## C. C-Terminal Domain

The COOH-terminal proximal portion of nucleolin consists of Glycine- and arginine-rich (GAR) repeat segments also called RGG domain (Bouvet et al., 1998) with regularly interspersed phenylalanine and $N^G$,$N^G$-dimethylarginine residues (Lapeyre et al., 1986, 1987; Ghisolfi et al., 1992a). The proteins containing GAR are efficiently recognized by methyltransferase(s) modifying arginine (Najbauer et al., 1993). This domain is in an extended conformation and has no hydrophobic regions (Jordan 1987; Lapeyre et al., 1987). It is capable of unstacking bases in RNA secondary structure (Ghisofli et al., 1992a). The overall composition is almost conserved except for the presence of two glutamine residues in *Xenopus* (Caizerguas-Ferrer et al., 1989). Also, this domain is little longer in *Xenopus* (61 amino acid, a.a.) when compared with hamster (53 a.a.), mouse (49 a.a.), human (50 a.a.), pea (53 a.a.), and alfalfa (55 a.a.). The C-terminal 10-kDa domain of animal nucleolin is shown to be essential for efficient binding of nucleolin to RNA but does not itself contribute to the specificity of the interaction (Ghisolfi et al., 1992b, Heine et al., 1993). Circular dichroism spectroscopic probing of the RNA component shows that the C-terminal domain significantly modifies the RNA-binding properties of the central CS-RBD core (Ghisolfi et al., 1992a). Infrared spectroscopic studies revealed that the central 40 kDa domain is structured in $\alpha$ helices and $\beta$ sheets and the interaction with the specific pre-rRNA site induces subtle

changes in the β sheet conformation (Ghisolfi et al., 1992b).

The GAR region of nucleolin is strikingly similar to sequence in a region of the hnRNP A1 protein, which is about 40 residues downstream from the carboxy-terminal consensus sequence (Dreyfuss et al., 1993). This GAR domain appears likely to function primarily in protein-protein interactions (Bandziulis et al., 1989). Recently, Bouvet et al. (1998) showed that nucleolin interacts with several ribosomal proteins through its RGG domain. However, this GAR domain may also influence the polynucleotide binding properties of CS-RBD. This domain is known to destabilize rRNA/rRNA helical regions so that rRNA regions can be recognized by the central domain of the nucleolin (Ghisolfi et al., 1992a). We have expressed this domain (10 kDa) in an *E. coli* expression vector and showed that it contains RNA and DNA unwinding activities (Tuteja et al., 1995), which will be discussed later.

## IV. STABILITY OF NUCLEOLIN

Nucleolin is more stable in actively dividing cells when compared with resting cells where it auto-catalyzes its own degradation (Chen et al., 1991). This shows that the stability of the nucleolin molecule is cell proliferation-dependent. The self-cleaving activity of nucleolin was inhibited by nuclear extract prepared from proliferating cells that showed that the putative proteolytic inhibitor, present in nuclei of actively dividing cells, controls the stability of nucleolin molecule (Chen et al., 1991). It seems that functions of nucleolin are regulated by the appearance of this inhibitor. However, in contrast to this it was previously proposed that intact nucleolin inhibited rRNA transcription and processing unless it was cleaved by protease (Bouche et al., 1984). This is to note that the fragmentation of nucleolin, usually observed during extraction, may not be caused by digestion with other cellular proteases, but by itself. Triton X-100 extraction immediately activates the self-cleavage, indicating that *in vivo* nucleolin is associated with certain nuclear components, that is, DNA, RNA, nuclear matrix, etc. to maintain its integrity (Chen et al., 1991).

Warrener and Petryshyn (1991) observed that the phosphorylation (probably by CK2) of nucleolin enhances its degradation by protease. Tawfic et al. (1994) also observed that phosphorylation and degradation of nucleolin appear to be concordant, suggesting that the stability of nucleolin is dependent on phosphorylation. The intrinsic protease activity of nucleolin for autodegradation has been mapped to C-terminal two-thirds part of nucleolin, which also determines its molecular dynamics in relation to cell proliferation (Fang and Yeh, 1993). Proteins with self-cleaving activity are not common in eukaryotic cells, but they are quite common among viral encoded polyproteins (Krausslich and Wimmer, 1988). Nucleolin was found to be up-regulated in its DNA and ATP binding properties on the mitogenic stimulation of murine splenocytes with bacterial lipopolysaccharide. This induction was found to be mediated by both an increased stability and synthesis of the nucleolin (Miranda et al., 1995).

## V. POSSIBLE FUNCTIONS OF NUCLEOLIN AND ITS INTERACTION WITH OTHER MOLECULES

The most important feature of nucleolin is its multifunctionality. It interacts with DNA, RNA, and many proteins (Olson et

al., 1983; Herrera and Olson, 1986; Jordan, 1987; Bugler et al., 1987; Erard et al., 1988; Kondo and Inouye, 1992; Martin et al., 1992; Schmidt-Zachmann and Nigg, 1993; Bogre et al., 1996; Tong et al., 1997; Hanakahi et al., 1997; Schwab et al., 1998). The elucidation of amino acid sequence of nucleolin provides many fascinating clues to its function (Jordan, 1987; Lapeyre et al., 1987). However, most of the nucleolin's functions are based on hypothesis and speculation. Through direct or indirect evidences it has been implicated that nucleolin or nucleolin-like proteins may be involved in at least following activities of the cell:

- Regulation of rDNA transcription or pre-rRNA synthesis (Bouche et al., 1984, 1987, Jordan, 1987, Egyhazi et al., 1988).
- Processing of pre-rRNA (Ginisty et al., 1998). [In yeast, the deletion of the NSR1 gene impairs the processing of pre-rRNA and the production of mature 18S rRNA (Hadjiolov, 1985, Sommerville, 1986; Kondo and Inouye, 1992; Lee et al., 1992)].
- Assembly and maturation of ribosomes (Herrera and Olson, 1986; Bugler et al., 1987).
- Cytoplasmic-nucleolar transportation of ribonuclear proteins and pre-ribosomal particles (Borer et al., 1989; Schmidt-Zachmann and Nigg, 1993).
- Inducing chromatin decondensation (Erard et al., 1988) as well as in condensing certain forms of DNA during mitosis (Kharrat et al., 1991).
- As a sequence-specific (UCCCGA) RNA-binding protein (Ghisolfi-Nieto et al., 1996).
- Destabilization of rRNA/rRNA helices (Ghisolfi et al., 1992a).
- As an RNA helicase, DNA helicase, and DNA dependent ATPase (Tuteja et al., 1991, 1995; Tuteja and Tuteja, 1996).
- As a transcriptional repressor in negatively regulating the expression of the alpha-1 acid glycoprotein gene (Yang et al., 1994).
- Cell proliferation and growth (Hoffman and Schwock, 1989; Ohmori et al., 1990; Lee et al., 1991; Fang and Yeh, 1993; Bogre et al., 1996; Derenzini et al., 1995; deCarcer et al., 1997; Yokoyama et al., 1998).
- A role in oogenesis, embryogenesis and in nucleolar reformation (nucleogenesis) in *Xenopus laevis* (Caizergues-Ferrer et al., 1989).
- In activation of rat hepatocytes and during early stage of liver regeneration, (the induction of nucleolin is functionally linked with heat shock protein 70) (Ohmori et al., 1990; Konishi et al., 1995).
- Differentiation and maintenance of neural tissue (Kibbey et al., 1995).
- It acts as an autoantigen in patients with systemic lupus erythematosus and systemic autoimmune disorders (Minota et al., 1990; Valdez et al., 1995).
- As a switch region targeting factor in a B cell specific recombination complex by binding specifically to switch region DNA (Hanakahi et al., 1997; Borggrefe et al., 1998).
- It is involved in the regulation of hepatitis delta virus (HDV) replication (Lee et al., 1998).
- A nucleolin-like protein gar2, from fission yeast *Schizosaccharomyces pombe*, has a role in cytokinesis and nuclear division (Leger-Silvestra et al., 1997).
- Indirect evidence shows that it may play a role in DNA replication because it is found in a human cell DNA synthesome that is a multiprotein DNA replication complex (Applegren et al., 1998).

## A. Nucleolins Role in Ribosome Biogenesis

Nucleolin might play a key role in ribosome biogenesis that includes transcription and processing of rRNA as well as ribosome assembly and maturation as described in Figure 2. It is suggested that nucleolin induces chromatin decondensation by displacing the chromatin binding domain of

histone H1 and the phosphorylation of nucleolin may play a role in this interaction. In this way, nucleolin is capable of modifying the basic structure of chromatin for transcription (Erard et al., 1988). Nucleolin is also known to bind to the amino terminus (166 to 210 a.a.) of human topoisomerase I (topo I), which may relate to the cellular localization of topo I or to the known role of this protein in transcription (Bharti et al., 1996). The involvement of nucleolin at various steps of ribosome biogenesis as well as its interaction with different components of this machinery has been documented by using diverse experimental systems.

In nucleolus each nucleolar organizing region contains a cluster of tandemly repeated rRNA genes that are separated from each other by non transcribed spacer (NTS) DNA. In Navikoff hepatoma cells, nucleolin has been shown to bind strongly with AT-rich DNA of the NTS regions between the genes for preribosomal 45S RNA, suggesting that it may serve as a bridge between chromatin and ribonucleoprotein (Olson et al., 1983, Lapeyre et al., 1986). Eukaryotic ribosomes contain four types of rRNAs (5S, 5.8S, 18S, and 28S). The genes for 5.8S, 18S, and 28S rRNAs are very actively transcribed as a single unit within the nucleolus by RNA polymerase I, yielding a 45S ribosomal precursor RNA (Figure 2). Nucleolin is also known to regulate transcription by RNA polymerase I (Jordan, 1987). Nucleolin molecules are found to be associated with nascent pre-rRNAs (Ghisolfi-Nieto et al., 1996; Schwab et al., 1998). In mouse it has been shown that nucleolin binds with high affinity specifically to an 18-nucleotide long stem-loop structure of RNA sequence (5′-CCGAAA(U/G)CCCGAAGUAGG-3′) that shares a common UCCCGA motif with the characterized pre-rRNA binding sites (Ghisolfi-Nieto et al., 1996).

The 45S pre-rRNA also contains two external transcribed spacers 5′ and 3′ ETS and two internal transcribed spacers (ITS1 and ITS2) that lie between the 18S, 5.8S, and 28S rRNA sequence (Figure 2). Interestingly, nucleolin of mouse origin is also shown to recognize the analogous sequences in the 5′ ETS of human pre-rRNA (Ghisolfi-Nieto et al., 1996). RNA binding studies have shown that nucleolin interacts specifically with a short stem loop structure known as NRE. Serin et al. (1996) have shown that human, hamster, and mouse nucleolin interacts with the same specificity and affinity to a mouse 5′ ETS RNA fragment that contains a NRE motif. It is also reported that putative NRE are present in the 3′ ETS, ITS, and in the 18S and 28S RNA sequences (Serin et al., 1996). These data suggest that these nucleolin binding sites might be functionally important, in particular for ribosome biogenesis.

Several small nucleolar RNAs (snoRNAs) have been shown to be essential for processing steps that led to production of 18S rRNA (U3, U14, and U22 in vertebrates; U3, U14, snR10, and snR30 in yeast) and for generation of 5.8S and 28S rRNAs) (Eichler and Craig, 1994; Lafontaine and Tollerrey, 1995; Maxwell and Fournier, 1995; Sollner-Webb et al., 1995; Venema and Tollervey, 1995; Tollervey and Kiss, 1997). snoRNA U3 is required for initial cleavage of pre-rRNA within the 5′ ETS and may also be involved in subsequent steps of pre-rRNA processing (Kass et al., 1990; Azum-Gelade et al., 1994). Some snoRNAs are reported to base pair with pre-rRNA, suggesting that they are directly involved in the processing reaction (Beltrame and Tollervey, 1992). The snoRNAs are complexed with a subset of nucleolar proteins and depletion of these proteins leads to defect in pre-rRNA processing (Girard et al., 1992). Yeast nucleolin-like protein, NSR1, apparently does not bind snoRNAs but binds to pre-rRNA and ribosomal proteins, thereby affecting pre-rRNA process-

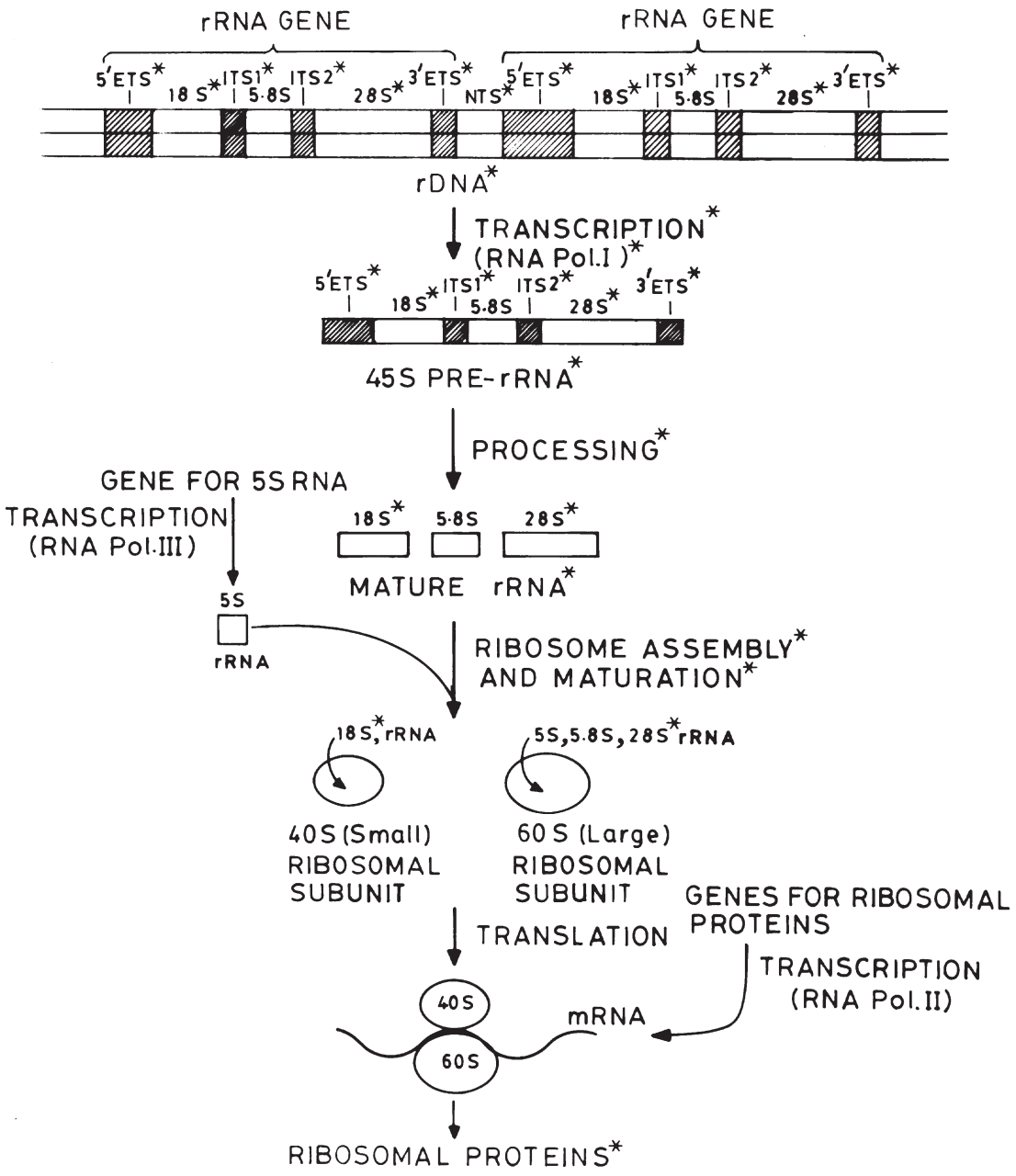


**FIGURE 2.** Organization of ribosomal genes and their transcription, processing, ribosome assembly and maturation. Each rRNA gene is a single transcription unit containing the 18S, 5.8S, and 28S rRNAs, which are organized in tandem repeats in which sequences coding for the 45S rRNA precursor are interspersed by untranscribed spacers. After the formation of ribosomes the ribosomal proteins are synthesized through translation in cytoplasm. Nucleolin is known to interact or associate (as shown by star) with rDNA, nascent 45S pre-rRNAs, NTS, 5′ ETS, 3′ ETS, ITS, and RNA polymerase I, 18S, and 28S rRNAs and ribosomal proteins. It is also directly or indirectly involved in transcription, pre-rRNA processing, ribosome assembly and maturation. Transcription of rDNA occurs in the nucleolus, while the transcriptions of 5S rDNA and genes for ribosomal proteins occur outside the nucleolus. (ETS: external transcribed spacer, ITS: internal transcribed spacer; NTS: non-transcribed spacer.)

ing (Sun and Woolford, 1994; Lee et al., 1992). For example, disruption of the NSR1 gene from *Saccharyomyces cerevisiae* in yeast causes a defect in both pre-rRNA processing and the production of mature 18S rRNA and leads to a severe growth defect (Kondo and Inouye, 1992; Lee et al., 1992; Sun and Woolford, 1994).

Recently, a direct role of CHO nucleolin has been reported in the first step of pre-rRNA processing (Ginisty et al., 1998). UV cross-linking and a single point mutation in the RNA region (RNA 541/1250) confirmed the high specificity of the interaction between nucleolin and NRE RNA that further stimulate the pre-RNA processing (Ginisty et al., 1998). This is the first demonstration that a metazoan proteinaceous factor (nucleolin) interacts directly with the rRNA substrate and is required for the processing reaction. The final step of processing is the conversion of the 45S pre-rRNA to the 18S rRNA of the 40S (small) ribosomal subunit and to the 5.8 and 28S rRNAs of the 60S (large) ribosomal subunit (Figure 2).

Transcription of the 5S rRNA, which is also found in the 60S ribosomal subunit, takes place outside the nucleolus and is catalyzed by RNA polymerase III. The *in vivo* transcription of 5S rRNA in *Xenopus* is regulated by histone H1 (Bouvet et al., 1994) and because nucleolin is known for its interaction with histone H1, it is possible that nucleolin is playing indirect role in 5S rRNA transcription. The genes for ribosomal proteins are transcribed outside the nucleolus by RNA polymerase II, yielding mRNAs that are translated on cytoplasmic ribosomes. The ribosomal proteins are then transported from the cytoplasm to the nucleolus, where they are assembled with rRNA to form pre-ribosomal particles. Nucleolin interacts only transiently with rRNA and pre-ribosomal particles (Ghisolfi et al., 1996, Bourbon et al., 1983) and is not detectable in mature cytoplasmic ribosomes. It has been shown in a recent report that nucleolin directly interacts with a subset of ribosomal proteins through its C-terminal domain (Bouvet et al., 1998). These findings suggest that nucleolin may have a direct role in the assembly of the ribosomal subunits by bringing together ribosomal proteins and RNA. Using its characteristic shuttling property, nucleolin plays an important role as a carrier, either during the import of ribosomal proteins to the nucleus or during the export of ribosomal subunits to the cytoplasm (Borer et al., 1989; Schmidt-Zachmann and Nigg, 1993; Xue and Melese, 1994).

A nucleolin-like protein gar2 from *S. pombe* is shown to be required for 18S rRNA and 40S ribosomal subunit accumulation (Gulli et al., 1995). gar2 is able to rescue a *S. cerevisiae* mutant lacking another nucleolin-like protein NSR1, thus establishing gar2 as a functional homolog of NSR1. It is proposed that gar2 helps in the assembly of pre-ribosomal particles containing 18S rRNA (Gulli et al., 1995). Recently, it has been shown that gar2 helps the assembly on rRNA of factors necessary for 40S subunit synthesis by providing a physical link between them. This function depends on the concerted action of its highly charged N terminus and its RNA-binding domains (Sicard et al., 1998).

## B. Nucleolin's Other Functions

Nucleolin plays a role not only in ribosome assembly but also in nucleogenesis as reported in *Xenopus laevis* by following its expression throughout oogenesis and embryogenesis (Caizergues-Ferrer et al., 1989; Schwab and Dreyer, 1997). The maximal accumulation of nucleolin was observed at gastrulation which coincides with nucleolar

reformation. It was observed that nucleolin started appearing and accumulating when ribosomal synthesis was activated during oogenesis and embryogeneis (Caizergues-Ferrer et al., 1989).

Nucleolin also functions as a transcriptional repressor for alpha-1 acid glycoprotein (AGP) gene. It has been shown that purified as well as recombinant nucleolin recognize the negative cis element (i.e., B motif) in the AGP promoter region in a sequence specific manner (Yang et al., 1994). Nucleolin is also known to be one of the components (106 kDa) of the B cell-specific transcription factor LR1, which is also a switch region binding protein (Hanakahi et al., 1997) and plays an important role in the regulation of the transcription in activated B cells. Homology between nucleolin and histone H1 suggested that nucleolin might alter the DNA organization in response to cell cycle controls, and the nucleolin component of LR1 therefore might function to organize switch regions before, during, or after switch recombination (Hanakahi et al., 1997). Recently, nucleolin has been reported as a component of a B-cell-specific DNA recombination complex known as SWAP that contains a recombination activity that is specific for switching B-cells (Borggrefe et al., 1998). Because nucleolin specifically binds to switch region DNA (Hanakahi et al., 1997), it might act as a switch region targeting factor in the SWAP complex (Borggrefe et al., 1998).

Nucleolin acts as an autoantigen because autoantibodies (IgM class) against it have been found in sera of patients with systemic lupus erythematosus, some other systemic autoimmune diseases and also in some patients with acute hepatitis A infections and infectious mononucleosis (Minota et al., 1990). The function of nucleolin as a helicase (Tuteja et al., 1991, 1995; Tuteja and Tuteja, 1996) will be discussed later. Similar to nucleolin, the functions of a protein as an autoantigen as well as a helicase were also reported for human DNA helicase (HDH) II or Ku autoantigen (Tuteja et al., 1994). Deng et al. (1996) reported that anti-nucleolin antibody reacts with Hep-2 cell membrane and subsequently gains access into cells in a process related to pinocytosis. Recently, human nucleolin has been shown to interact with hepatitis delta antigens (HDAgs) and modulate the hepatitis delta virus (HDV) replication (Lee et al., 1998). HDV, a satellite virus of hepatitis B virus, is a human pathogen associated with fulminant hepatitis and progressive chronic liver cirrhosis. Lee et al. (1998) have demonstrated that the amino-terminal domain of HDAg was essential for its binding to nucleolin.

Hoffmann and Schwach (1989) suggested that nucleolin is a nuclear target proteins of cyclic AMP in the cyclic AMP-influenced regulation of the transition of cells from the G1 to the S phase. The expression of nucleolin was reported as a proliferation marker (Sirri et al., 1995). The quantity of nucleolin in human cancer cells is related to the rapidity of cell proliferation; the faster the rapidity of cell proliferation, the greater the interphase Ag-NOR quantity (Derenzini and Trere, 1994; Derenzini et al., 1995). Trere et al. (1996) have shown that in hepatocellular carcinoma Ag-NOR protein expression correlated with tumor mass doubling time that could be a reliable parameter for predicting the tumor growth rate. In diseases such as meningioma and histological malignancy, the antibody against nucleolin has been used immunohistochemically for assessing cell proliferation (Ohkoudo et al., 1996). The expression was found to be low in serum-deprived cells and high in S-phase during cell-cycle stimulation (Sirri et al., 1997). Recently, a sex

steroid progestin has been shown to substantially increase the nucleolin protein in the rabbit uterus that is found to be associated with the proliferative potential of the cells (Yokoyama et al., 1998). It could thus be possible that nucleolin plays a role in DNA replication. Recently, HDH IV/nucleolin has been found in a human cell DNA synthesome that is a multiprotein DNA replication complex and is known to be involved in DNA replication (Applegren et al., 1998). This complex also contains DNA helicase activity similar to that of nucleolin. These indirect evidences also suggested that nucleolin might be involved in DNA replication.

Nucleolin is reported to bind to the neurite-promoting IKVAV site of laminin-1, a basement membrane protein that has been found to promote the differentiation of primary neurons and a variety of neural cell lines (Kibbey et al., 1995). Significant levels of nucleolin in mature brain and in differentiating neural cells were found, which suggested that it also functions in the differentiation and maintenance of neural tissue. Their identification of cytoplasmic and cell-surface nucleolin and IKVAV-binding protein suggested that this protein may function in signaling the extracellular matrix. Nucleolin interacts with B23 (nucleophosmin), which is a putative nucleolar-localization-signal-binding protein (Li, Y.-P. et al., 1996). Such binding of two proteins requires specific amino acid motifs (194 to 239 of B23 and 540 to 628 of nucleolin) that may be important for the nucleolar localization of nucleolin.

Recently, it is reported that the disruption of the *gar2+* gene from *Schizosaccharomyces pombe*, which encodes a nucleolin-like protein, results in a mutant that is defective in cytokinesis and nuclear division (Leger-Silvestra et al., 1997), suggesting its role in these processes also.

## VI. PHOSPHORYLATION OF NUCLEOLIN

Nucleolin is subject to several post-translational modifications, including glycosylation (Srivastava et al., 1989), methylation (Lischwe et al., 1985), and phosphorylation (Olson et al., 1974; Bourbon et al., 1983). Issinger et al. (1988) reported that hyperphosphorylation of nucleolin fragment (N-60) in primary human fibroblasts was induced by tumor promoter okadaic acid and suppressed by antitumor promoter retinoic acid. However, the significance of these observation was not clear. The phosphorylation of nucleolin is coupled to growth control as supported by the observation that active rRNA transcription is correlated to highly phosphorylated nucleolin (Schneider et al., 1986; Suzuki et al., 1987). It has been proposed that phosphorylation of nucleolin regulates the maturation of protein into defined subfragments (Bourbon et al., 1983). Kharrat et al., 1991 suggested that phosphorylated nucleolin and histone H1 interact through their homologous domain structured in beta-spirals in order to condense certain forms of DNA during mitosis.

Nucleolin is a good substrate for CK2 and cdc2 protein kinases. Nucleolin is associated with $\alpha$ or $\alpha^1$ subunits of CK2 that may be important for regulating rDNA transcription (Li et al., 1996). Fibroblast growth factor-2 binds to the regulatory beta subunit of CK2 and nucleolin that stimulates CK2 activity toward nucleolin (Bouche et al., 1994; Bonnet et al., 1996). In growing cells, CK2 phosphorylates nucleolin on serine during interphase, while in confluent cells nucleolin is dephosphorylated and rRNA synthesis goes down to 5% of the growing cells (Caizergues-Ferrer et al., 1987; Belenguer et al., 1990). In mitosis, nucleolin is phosphorylated at threonine residues by

M-phase H1 kinase and cyclin-dependent kinase cdc2 (Belenguer et al., 1990). It is speculated that successive cdc2 and CK2 phosphorylation could modulate nucleolin function in controlling cell cycle-dependent nucleolar function and organization. As discussed earlier, cdc2 sites on nucleolin also play a dual role by enhancing nuclear translocation exclusively in their dephosphorylated state and in promoting cytoplasmic localization when phosphorylated, thereby they provide a powerful cell cycle-dependent regulatory element of the nuclear localization signal (Schwab and Dreyer, 1997). It is also suggested that while serine phosphorylation is related to nucleolin function in the control of rDNA transcription, threonine phosphorylation is linked to mitotic reorganization of nucleolar chromatin (Belenguer et al., 1990).

*In vivo* and *in vitro* nucleolin is phosphorylated by these kinases at the same sites. Peter et al. (1990) suggested that cdc2 phosphorylation of nucleolin is related not only to mitotic chromosome condensation, spindle formation, and nuclear envelope breakdown but also to the control of the mitotic fate of nucleoli and cytoskeletal rearrangement. CK2 phosphorylation of nucleolin appears to be important for the regulation of cell growth (Jin and Burakoff, 1993). Insulin is reported to regulate the phosphorylation/dephosphorylation of nucleolin, possibly via stimulation of CK2, and this may play a role in regulation of the RNA efflux from nuclei (Csermely et al., 1993). gar2 from *S. pombe* contains several potential CK2 phosphorylation sites and a single putative p34 (cdc2) phosphorylation site (Gulli et al., 1997). It is phosphorylated *in vitro* by a p13 (Suc1)-Sepharose-bound kinase from *S. pombe* extracts that displays cell cycle-regulated activity similar to that of the p34 (cdc2) kinase. However, this posttranslational modification of the gar2 protein does not appear to be essential for normal production of 18S rRNA (Gulli et al., 1997).

The phosphorylation of nucleolin is important for its interaction with histone H1 as discussed earlier as dephosphorylation of nucleolin reduces its binding to H1 (Erard et al., 1988). Furthermore, the 29-kDa nucleolin CNBr peptide, which has a strong affinity for H1, also contains the phosphorylation sites (Caizergues-Ferrer et al., 1987). Phosphorylation of nucleolin by a cyclic AMP-independent protein kinase NII is important for its role in the pre-rRNA transcription. *In vitro*, the unphosphorylated nucleolin acts as an inhibitor of transcription, whereas the phosphorylated form is cleaved during transcription process (Bouche et al., 1984; Bourbon et al., 1983). *In vivo*, in resting cells with a low level of pre-rRNA transcription, the residual nucleolin is recovered associated with chromatin in its unphosphorylated form (Lapeyre et al., 1987).

Recently, nucleolin has been shown to be a specific substrate of protein kinase C-zeta (PKC-zeta), which is activated and required for nerve growth factor (NGF)-induced differentiation of rat pheochromocytoma PC12 cells (Zhou et al., 1997). It is suggested that nucleolin is a target of PKC-zeta that serves to relay NGF signals from cell surface to nucleus in PC12 cells. It has been observed that only the nucleolin localized within the nucleus is phosphorylated by this kinase (Zhou et al., 1997). It is possible that nucleolin assumes an alternate conformation after exit from the nucleus that masks the phosphorylation sites. Phosphorylation of nucleolin by PKC-zeta as well as by other kinases may regulate its functional abilities in chromatin organization, rRNA packaging, rDNA transcription, or ribosome assembly. Pea nucleolin has at least 48 possible CK2 phosphorylation sites,

2 cAMP-dependent protein kinase phosphorylation sites, 12 protein kinase C phosphorylation sites, and one tyrosine phosphorylation site (Tong et al., 1997). However, the effect of the phosphorylations of pea nucleolin on various activities has not been studied.

## VII. NUCLEOLIN AS A NUCLEIC ACID HELICASE

Tuteja et al. (1995) have discovered the nucleolin as a nucleic acid helicase. Helicases are the ubiquitous enzymes that catalyze the unwinding of energetically stable DNA duplexes (DNA helicases) or intrastrand RNA hybrids (RNA helicases). These nucleic acid unwindings are needed transiently and are known to be essential for DNA replication, repair, recombination, transcription, translation initiation, RNA splicing, ribosome assembly, and mRNA stabilization, turnover, and export (Dalbadie-McFarland and Abelson, 1991; Pause and Sonenberg, 1992; Lavoie et al., 1993; Matson et al., 1994; Tuteja and Tuteja, 1996; Tuteja, 1997; Venema et al., 1997). These enzymes actually destabilize the hydrogen bonds between the complementary base pairs of two strands in a reaction that is coupled to the binding and hydrolysis of nucleoside 5′-triphosphates (Matson et al., 1994; Tuteja and Tuteja, 1996; Tuteja, 1997). All helicases contain intrinsic DNA-dependent ATPase activity that provides the energy to the protein in translocating unidirectionally along the bound strand in either 3′ to 5′ or 5′ to 3′ direction. Helicases generally need free one strand of the partial duplex DNA or ss-/ds-DNA junctions as a loading zone in order to bind and translocate. Mostly helicases are either DNA helicase or RNA helicase and play important roles in the processing of DNA and/or RNA (Matson et al., 1994). Very few helicases are known for unwinding both the DNA and RNA duplexes (Tuteja, 1997). Human nucleolin is one of the rare helicase that can function as a DNA helicase and RNA helicase as well as being able to unwind hetero duplexes (DNA-RNA or RNA-DNA hybrids) (Tuteja et al., 1995; Tuteja and Tuteja, 1996). DNA-RNA is a short RNA stretch hybridized to a long DNA strand and RNA-DNA is the opposite (Tuteja et al., 1992).

Nucleolin is classified as a human DNA helicase IV (HDH IV) that was purified from HeLa cells by ammonium sulfate precipitation and subsequent conventional column chromatography on DEAE-sephacel, heparin sepharose, and single-stranded DNA sepharose (Tuteja et al., 1991; Tuteja et al., 1995). The polyclonal antibody against HDH IV was used to clone the helicase gene from human cDNA expression library. The nucleotide sequence as well as derived amino acids sequence of HDH IV cDNA was found to be completely identical to the human nucleolin cDNA (Srivastava et al., 1989). It contains a 2121 bp coding region with 114 bp and 332 bp untranslated region at the 5′ and 3′ ends, respectively (Srivastava et al., 1989). A northern blot analysis showed the same transcript size of 3 kb as reported for nucleolin-mRNA (Srivastava et al., 1989).

Prompted by the above observations, Tuteja et al. (1995) tested a purified human nucleolin (Belenguer et al., 1990) for DNA and RNA helicase activities and found that it contained both DNA and RNA unwinding activities similar to HDH IV (Figure 3A). In addition, it was shown that the antibody against HDH IV cross-reacted with nucleolin protein and the antibody against nucleolin cross reacted with the HDH IV protein (Figure 3B) (Tuteja et al., 1995). These findings confirmed that both the HDH IV and nucleolin are the same

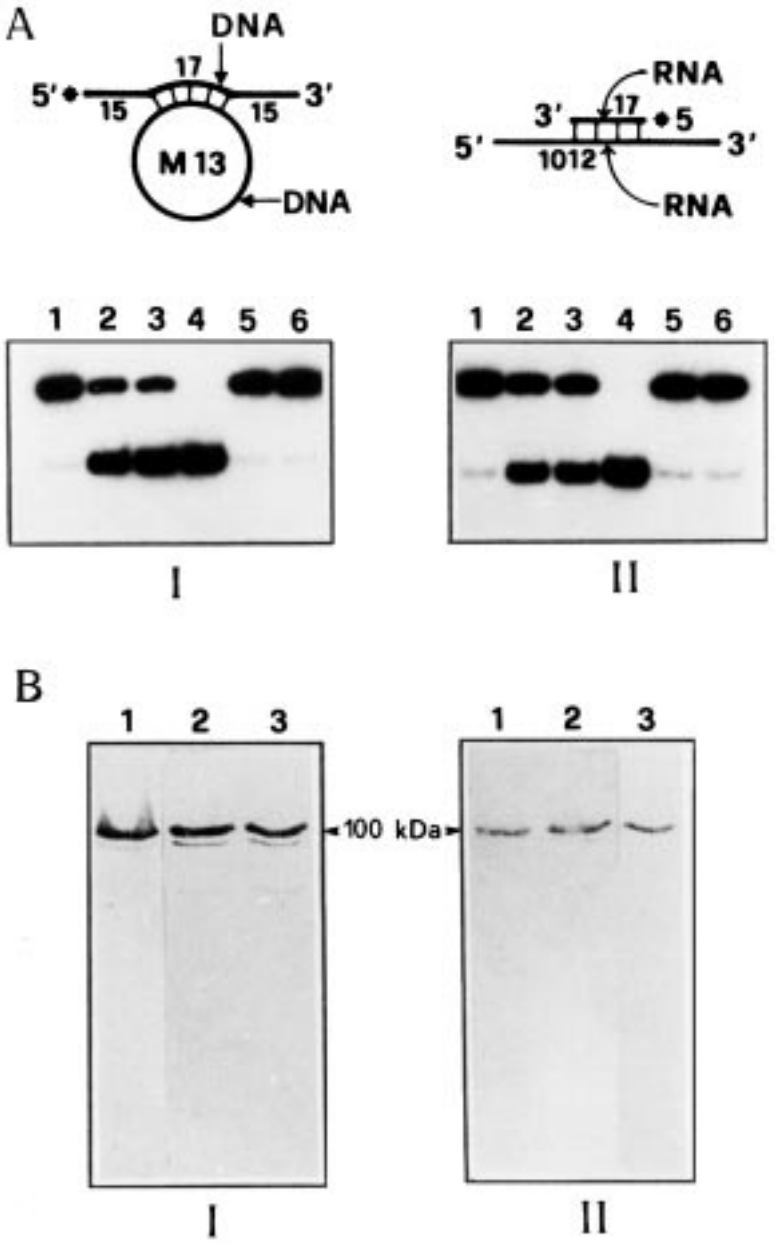


**FIGURE 3.** Catalytic and immunologic identity of HDH IV and nucleolin **(A)** DNA helicase (panel I) and RNA helicase (panel II) activities of nucleolin (lane 2) and HDH IV (lane 3). Lanes 1 and 4 in both panels are controls without enzyme and heat-denatured substrate, respectively. Lanes 5 and 6 in both panels are assays without ATP of nucleolin or HDH IV, respectively. The structure of the substrate used is shown at the top of each autoradiogram. The DNA and RNA substrates were prepared as described previously (Tuteja et al., 1991, 1994). For the helicase assays, 100 ng of each enzyme were used in the presence of 1 ng of substrate and the products were separated by native 12% PAGE. Asterisks denote the $^{32}$P-labeled end. In the RNA helicase assay, 1 unit of RNAase block was also included. **(B)** Western blotting with anti-HDH IV (panel I) and anti-nucleolin (panel II) Ab. In both panels lane 1 is nuclear extract (30 μg), lane 2 is HDH IV (0.5 μg) and lane 3 is nucleolin (0.4 μg). The polyclonal Ab against HDH IV and nucleolin were raised in rabbit. The antisera were used at 1:2000 dilution. Prior to blotting, proteins were separated on 0.1% SDS-10% PAGE. (From Tuteja et al., 1995, *Gene,* 160, 143–148.)

molecule (Tuteja et al., 1995). In another study it was shown that murine nucleolin contained ATP binding domain and binds strongly to ATP as well as to dATP, GTP, and dGTP (Miranda et al., 1995). This ATP binding is important for its ATPase activity.

Nucleolin/HDH IV is a monomer of 100 kDa in molecular mass and requires ATP or dATP and divalent cations ($Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$) for its unwinding activity. It is unique in showing this activity in the presence of zinc ions. Nucleolin/HDH IV unwinds DNA by moving in the 5′ to 3′ direction along the bound strand (Tuteja et al., 1991), a polarity opposite to most other human helicases (Tuteja et al., 1993; Tuteja and Tuteja, 1996). The unwinding activity of nucleolin resides in the C-terminal GAR domain (10 kDa) of the molecule (Figure 4) (Tuteja et al., 1995). This 10-kDa GAR domain was bacterially expressed and purified and tested for unwinding activity with various different DNA and RNA helicase substrates (Figure 4). Similar to HDH IV (Tuteja et al., 1991), the 10-kDa polypeptide of nucleolin did not require a fork-like structure of the substrate and showed similar activity whether the substrate contained no hanging tails (Figure 4A) or 5′ or 3′ hanging tails (Figure 4B and C) or both the hanging tails (Figure 4D). However, it could not unwound the longer duplexes (Figure 4E). Similar to HDH IV (Tuteja et al 1991) it was able to unwind RNA duplex (Figure 4F) and DNA-RNA duplex (Figure 4G) (Tuteja et al., 1995). Interestingly, to the best of our knowledge, this 10-kDa polypeptide of nucleolin is the smallest polypeptide that has been shown to exhibit helicase activity.

We have also shown that HDH IV/nucleolin is a substrate for cdc2 and CK2 protein kinases, and its unwinding activity is stimulated after *in vitro* phosphorylation by these kinases, presumably through long-range intramolecular interactions (Tuteja et al., 1995). The DNA unwinding and DNA-dependent ATPase activities of human nucleolin are found to be inhibited by DNA interacting ligands nogalamycin and daunorubicin (Tuteja, N., unpublished observations). These ligands bind to the major groove of the DNA and generate a complex that impedes the translocation of nucleolin. Similar results of inhibition were reported for Ku autoantigen (Tuteja et al., 1997). These studies could be useful for understanding the mechanism of nucleolin mediated unwinding and also the mechanism by which these DNA-interacting ligands inhibit cellular function.

It seems that the RNA helicase activity of nucleolin is contributing to pre rRNA processing during ribosome biogenesis because RNA unwinding reaction also takes place during these processes (Venema et al., 1997).

## VIII. PLANT NUCLEOLIN

The presence of plant nucleolin protein was first reported from onion root meristematic cells where it is associated with chromatin and helps in decondensation of chromatin, in rDNA transcription, and in the early steps of pre-rRNA processing (Martin et al., 1992). In onion root cells it has also been shown that nucleolin together with other components of the nucleolar processing complex co-localize during mitosis and later segregated to daughter cell nucleoli (Medina et al., 1995). It is also suggested that ribosome biogenesis restarts not only after mitosis at the level of transcription but also at the intermediate levels of pre-rRNA processing. The *Arabidopsis* nucleolin-like

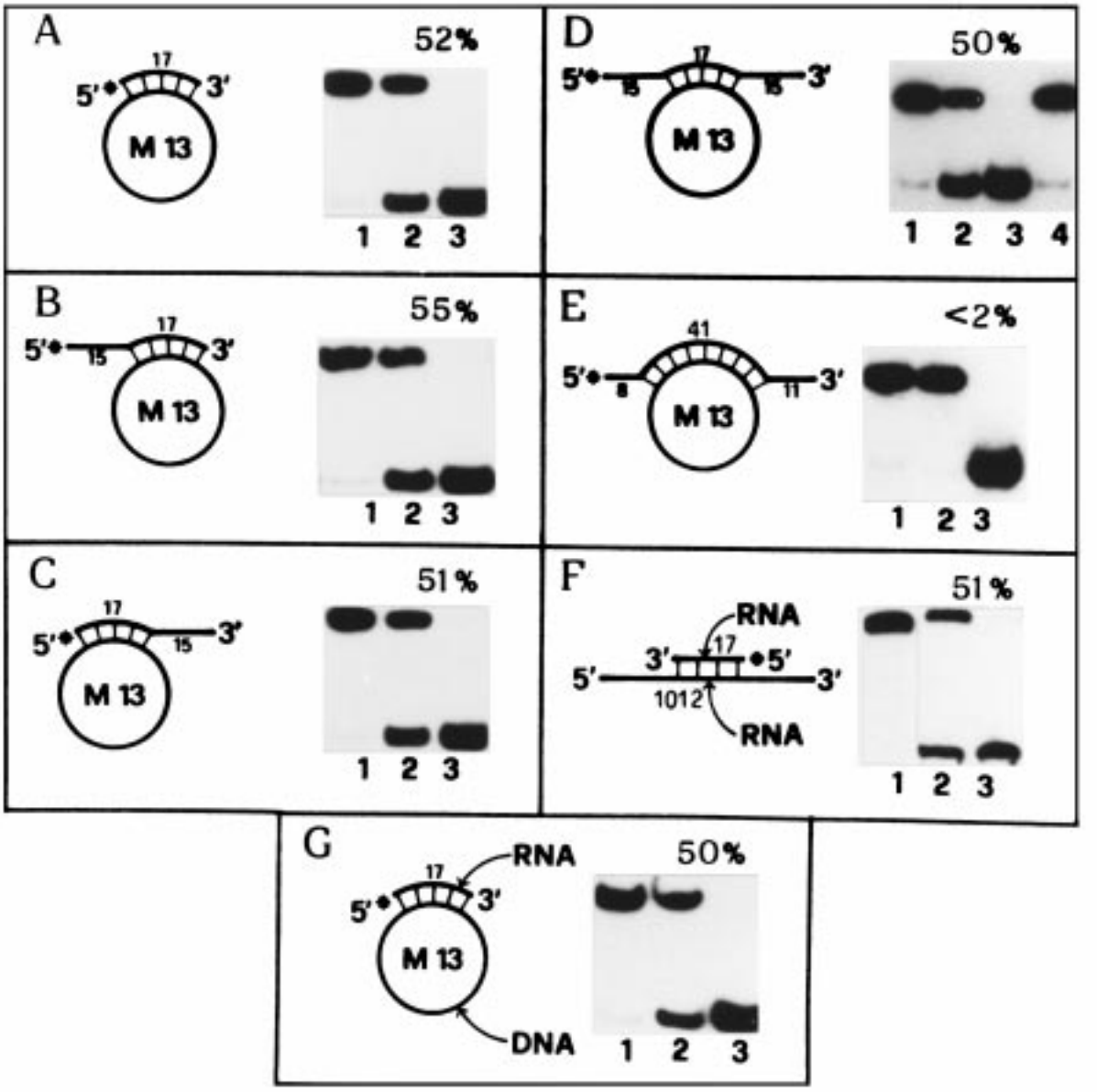


**FIGURE 4.** DNA and RNA helicase activities of expressed and purified C-terminal domain of the HDH IV/nucleolin protein with different substrates. For each assay, 200 ng of the polypeptide (p10) and 1 ng of the substrate were used. P10, corresponding to the Gly-rich C-terminal domain of HDH IV/nucleolin, has been synthesized by an *E. coli* expression vector system and purified to homogeneity, as described by Ghisolfi et al. (1992b). All the substrates were prepared and helicase assays performed as described previously (Tuteja et al., 1991, 1994). The asterisks denote the $^{32}$P-labeled end. Each panel shows the structure of the substrate used, an autoradiogram of the gel (native 12% PAGE) and the percentage unwound. In each panel, lane 1 is the control without enzyme, lane 2 is the reaction with enzyme, and lane 3 is the heat-denature substrate. In panel D, lane 4 is the helicase reaction of p10 without ATP. (From Tuteja et al., 1995, *Gene,* **160,** 143–148.)

protein has the same gene organization as three ribonucleoproteins of tobacco chloroplast (Li and Sugiura, 1990), which are suggested to be involved in splicing and/or processing of chloroplast RNAs (Didier and Klee, 1992).

The highest level of transcript was observed in the floral tissue of *Arabidopsis*

(Didier and Klee, 1992) and in the root meristematic cells of alfalfa (Bogre et al., 1996). In alfalfa, nucleolin is reported to be developmentally and cell cycle regulated. The transcript and protein levels of nucleolin in alfalfa correlate with cell proliferation, and nucleolin gene expression is induced in the G1 phase of cell cycle after mitogenic stimulation of G0-arrested leaf cells, similar to the D-type cyclin gene. In proliferating cells of alfalfa, nucleolin's transcript level is not changed in a cell-cycle phase-specific manner but disappears when cells exit the cell cycle and undergo differentiation or polar growth, indicating the role of nucleolin in cell proliferation (Bogre et al., 1996). Nucleolin gene expression is also known as a marker for proliferation events during flower development. In pea, nucleolin is shown to be light regulated (Tong et al., 1997). Light is also known to increase the rate of nuclear rRNA gene transcription in several plants and the light receptor for this response is the photoreversible pigment phytochrome (Thien and Schopfer, 1982). As we have described earlier, rRNA transcription is a crucial step in ribosome assembly and nucleolin plays an important role in this event as well as in later processing steps (Olson, 1990).

It is interesting to know whether light via phytochrome up-regulates the gene for nucleolin while stimulating the transcription of rRNA as a part of the overall process of promoting ribosome assembly. This question was answered by group of Stanley J. Roux at Austin (Tong et al., 1997). They observed that after irradiation of etiolated pea seedlings by red light, the transcript level of nucleolin in plumules decreased during 1st hour (h) and then increased six times to reach 0 to h level at 12 h. Far-red light reversed this effect of red light and the mRNA accumulation from red followed by far-red light irradiation was the same when compared with the dark control. These findings indicated the role of phytochrome in regulating the expression of nucleolin (Tong et al., 1997).

## CONCLUSIONS AND FUTURE PROSPECTS

The involvement of nucleolin protein in many metabolic processes has several implications of general interest. The high level of evolutionary conservation observed suggests that nucleolin performs many essential structural and functional roles including potential key role in ribosome biogenesis. The involvement of nucleolin in both the transcriptional regulation and processing of the rRNA suggests that these two events coordinate to each other and might be linked. This possible coordination between rRNA processing and transcription could be an efficient way for the cell to regulate the production of the large amount of ribosomes needed during the cell's life as suggested by Ginisty et al. (1998). Nucleolin is a complex protein that has been shown to interact with a variety of cell components. The multifunctionality of nucleolin could be due to its unusual multidomain structure. The different functions of nucleolin are performed by specialized domains within the large protein. However, how these multifunctions are regulated appears to be a major question facing nucleolin research as well as much of biology. A great deal of work is still needed to address this question. Nucleolin is phosphorylated by cdc2, CK2, and PKC-zeta protein kinases and only some of the functions of nucleolin are known to be controlled by its phosphorylation/dephosphorylation. Further insights are expected from three dimensional structural studies of the individual domains and their complexes with the respective ligands such as DNA or RNA. Electron microscopy and other more sophisticated approaches such as mutational

analysis, transient expression studies, and *in vivo* crosslinking are likely to reveal the nature of the macromolecular assembly of nucleolin with other cell components.

In cancer the relationship between the expression of nucleolar Ag-NOR proteins (including nucleolin) and cell proliferation represents a reliable parameter for predicting the tumor growth rate. Multifunctionality of nucleolin also has implications for evolutionary mechanisms. The ability of a cell to utilize the single protein for more than one function is obviously energetically favorable for the cell. As most of the functions of nucleolin are not obviously related to each other, the evolutionary history of this protein is truly challenging. Understanding the basis of multifunctionality of nucleolin will have to await the complete elucidation of how the protein functions in terms of its interaction with other cellular macromolecules. The future also lies in understanding the nature and function of nucleolin in other systems, especially plants and its role in both shoot and root meristematic tissues.



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