Chapter 1

Introduction

The eukaryotic cell nucleus is a dynamic organelle responsible for storing, copying and utilizing virtually all the genetic information required for forming complex organisms. The nucleus is highly structured and contains several prominent sub-regions, the most prominent being the nucleolus. In the nucleus DNA is copied both as DNA for progeny cells, and as RNA for use in the manufacture of cellular components. Since the nucleus contains extremely high densities of nucleic acid and protein, it must be highly ordered to be able to cope with the demands of managing the eukaryotic cell. The nuclear matrix, a fibrous structure of nucleic acid and protein, presumably functions to organize nuclear components. This work addresses the composition of the nuclear matrix in plants, with the goal of understanding how the organization of plant chromatin might regulate gene expression. In this work, I have analyzed the protein composition of plant nuclei and matrices. I have identified several proteins that bind in vitro to DNA regions believed to organize chromatin into loops. I have cloned three related members of a nucleic acid binding protein family from the model plant Arabidopsis thaliana. These proteins have homologues in animal, insect, yeast and even in the Archaea, a phylum lacking true nuclei. The homologues are involved in vivo in processing of ribosomal RNA, and may have
multiple functions. I have demonstrated the binding of these proteins to specific DNA and RNA targets.

The majority of the work discussed in this introductory section pertains to mammalian and yeast cells, since we have much more data regarding nuclear organization from those systems. While many differences exist between plant and non-plant nuclear organization, such as total DNA content and the ability of plants to support polyploidy, a great deal of basic information regarding nuclear organization appears to be conserved in eukaryotes. The intranuclear relationships from non-plant organisms discussed below will be useful in understanding the plant nucleus.

The typical nucleus measures 3 to 10 µm across. Total nuclear DNA of length up to 1 meter or more must be compacted over 4 to 5 orders of magnitude to be contained within the nucleus (Pikaard, 1998). Competing with the DNA for limited space is twice the equivalent mass of proteins (van Holde, 1989), and transcribed RNA. How this densely composed mass can efficiently respond to disparate and often rapidly changing needs of various cell types within an organism remains a challenging question in biology. Nuclear DNA is organized into a number of long, single molecule chromosomes, which are complexed with protein to form chromatin. This DNA must be compacted, presumably in an orderly fashion. At the lowest level of organization, the 2 nm wide DNA coils first around a core octamer of histones H2A, H2B, H3 and H4 to form the core nucleosome, a structure of about 10 nm wide (Pruss et al., 1996). The nucleosomal core particle is the fundamental subunit of chromatin (Widom, 1997). Organization of DNA into core nucleosomes shortens the chromatin
approximately five-fold (van Holde, 1989). Beyond this level of compaction, we are less certain of chromatin structure. In many, but not all cases, histone H1 binds to linker DNA between nucleosomal DNA to further compact chromatin to form a 30 nanometer fiber (Finch and Klug, 1976). The exact structure of the 30 nanometer fiber is still being debated (Bednar et al., 1998; Leuba et al., 1994). Regardless of the underlying form of the 30 nanometer fiber, the DNA in this structure is now compacted to about 40 fold. The remaining compaction of chromatin appears to be due to formation of chromatin loops, as seen in electron micrographs of metaphase chromosomes stripped of histone proteins1 (Paulson and Laemmli, 1977). The loops seen in these micrographs attach to a non-histone nuclear structure, commonly called the nuclear matrix. The same type of loops can also be detected in interphase cells (Gerdes et al., 1994; Weipoltshammer et al., 1999). These loops are variable in size, both within a cell type, and among species (Marshall and Sedat, 1997; Paul and Ferl, 1998). For example, limited DNaseI digestion of nuclei from three plant species produced putative loops of median sizes ranging from approximately 25 to 45 kDa. These peaks were not seen in genomic DNA (non-chromatin) (Paul and Ferl, 1998). Similar loops have been seen in cells or matrices treated with nucleases, including micrococcal nuclease, mung bean nuclease or topoisomerase II (Gromova et al., 1993). The individual loops may also be dynamic in size and in the points of attachment. Some loops may be anchored by more permanent attachments, while other anchorage points could be more transient (Breyne et al., 1992; Hakes and Berezney, 1991; Paul and Ferl, 1998). The points of attachment between the matrix

1 These structures will be referred to in this work as chromosomal scaffolds. The protein composition of chromosomal scaffolds overlaps with the proteins of the nuclear matrix, but the composition of chromosomal scaffolds does differ significantly from that of the nuclear matrix (Saitoh et al., 1995).
and DNA occur at specific DNA elements, called Matrix Attachment Regions (MAR). Michalowski et al (1999) cloned several MARs from tobacco matrices and demonstrated that different MARs have different relative affinities for the nuclear matrix. Chromatin loops anchored to the matrix by MAR DNA have been hypothesized to represent functional domains, in which genes or groups of domains are coordinately regulated (van Driel and Otte, 1997; Paul and Ferl, 1998).

Evidence for existence of the nuclear matrix

There remain a few skeptics (Hancock, 2000; Pederson, 1998, 2000) regarding the existence of a nuclear matrix in vivo. Their ideas reflect the opinions of many scientists expressed during the emergence of the nuclear matrix hypothesis in the 1970’s. Much of the skepticism is based on evidence that certain nuclear proteins can form aggregates under the conditions used for nuclear isolation and nuclear matrix preparation. For example, the mitotic spindle apparatus protein NuMa has been reported to form multiarm oligomers via interactions at the C-terminus of monomeric NuMa (Harborth et al., 1999). Additionally, Tan et al. (2000) report that stable nuclear matrix-like filaments can be induced to form from hnRNPs by removal of RNA or by incubation of hnRNPs with 150mM NaCl and magnesium. It may be true that the isolation procedures employed to extract the nuclear matrix generate some artifactual associations. However, considerable evidence supports the idea that the nuclear matrix does exist in vivo, and that the matrix functions to organize nuclear activities. This evidence is reviewed below.
Nuclear matrices have been isolated by a variety of methods, and precipitation of the same proteins into the same complex under such a range of conditions is unlikely. Associations seen in matrix preparations have been confirmed by methods not expected to generate artifacts possible in nuclear matrix extractions. Crosslinking by irradiation of intact cells significantly reduces the ability of exogenous labeled MAR DNA to bind to matrices prepared from the crosslinked cells in proportion to the level of irradiation, presumably by blocking available binding sites. Crosslinking of labeled MAR DNA to isolated matrices significantly increases the amount of bound MAR DNA in proportion to the level of irradiation. Similar effects were not seen for a non-MAR control. These results support the idea that the MAR DNA–matrix association seen in vitro represents an in vivo association (Balasubramaniam and Oleinick, 1995). Three separate MAR DNAs that bind to the MAR binding protein SATB1 in vivo were identified by crosslinking. These MARs were shown by in situ hybridization to localize to the bases of DNA loops in nuclear halos (nuclei stripped of nucleosomes), and to nuclear matrices (deBelle et al., 1998). Several nuclear proteins have been isolated and are required for specific steps in the splicing of mRNA. These proteins are enriched in nuclear speckles in living cells, and this localization is preserved in the nuclear matrix (Blencowe et al., 1994, 2000). These experiments independently support the concept of the nuclear matrix as an in vivo structure involved in the organization of the nucleus.

As illustrated in Figure 1.1, matrices are prepared by stripping nuclei of membranes, chromatin, and soluble nuclear proteins (Berezney and Coffey, 1974). Most matrix preparation protocols involve the use of an endonuclease, such as DNaseI or
restriction enzymes, along with extraction of soluble material. Nuclear matrices have been isolated from a number of cell types and under a variety of extraction procedures, each revealing structures with similar morphology and protein composition (Belgrader et al., 1991; Dwarakanath et al., 1991; Neri et al., 1997; Stuurman et al., 1990; Wan et al., 1999). While variations in the extraction procedures can create residual nuclear structures with increasing or decreasing amounts of protein, nucleic acid and nuclear membrane, similar nuclear matrices can be obtained by each of several distinctly different isolation procedures. The extracted nuclear structures visualized by microscopy are spherical, and contain a network of fibrous and granular components. Residual structures resembling nucleoli and nuclear pores can be seen (Berezney et al., 1995).

Among the most common methods of matrix preparation are the high salt extractions, usually with sodium chloride (Berezney and Coffey, 1974, 1977). Ammonium sulfate (He et al., 1990; Brancolini and Schneider, 1991), potassium chloride (Hendzel et al., 1991) and combinations of high salt extractions (He et al., 1990; Stuurman et al., 1990) have also been used to prepare matrices. Matrices also can be prepared using the chaotropic reagent lithium diiodosalicylate (LIS) (Mirkovitch et al., 1984; Hall et al., 1991).

A number of diverse methods have been employed to prepare matrices. The similarity of these matrices confirms that isolation procedures are not responsible for artifactual creation of a nuclear matrix structure. Jackson and Cook (1985) used nuclei encapsulated in agarose to extract chromatin electrophoretically in the absence of chaotropic agents, and found a structure they termed the nucleoskeleton, which
appears to be the same as the nuclear matrix. Nickerson et al. (1997) crosslinked nuclear proteins with formaldehyde before digesting and eluting chromatin, again finding a nuclear matrix structure. Wan et al (1999) compared high salt matrices to those prepared with sulfo-NHS, a reagent believed to interfere with electrostatic interactions between chromatin and the matrix by modifying protein amino groups. They found nuclear matrix structures similar to those seen with the high salt matrices (Figure 1.3).

Some basic facts of nuclear composition are conserved in all eukaryotes. Light microscopy, fluorescence microscopy, and electron microscopy have identified structural features in the nucleus (Figure 1.2). The nucleus is bound by a double membrane nuclear envelope and the outer membrane is continuous with the endoplasmic reticulum. The nuclear envelope bilayers are joined only at nuclear pore complexes (Ellenberg et al., 1997), providing a means of import from and export to the cytosol for large molecules. The inner envelope anchors a proteinaceous meshwork, or lamina. In animal nuclei, the lamina is composed of lamin B, which binds to a receptor in the inner membrane, and lamins A and C, which bind to lamin B and to each other (McNulty and Sanders, 1992). Plants also have a structure morphologically similar to the lamina, though the presence of nuclear lamins has only been demonstrated immunologically (Beven et al., 1991; McNulty and Sanders, 1992; Minguez and Moreno Diaz de la Espina, 1993). In plants, lamin-like proteins are detected both at the periphery and in the internal matrix structure (Beven et al., 1991; Minguez and Moreno Diaz de la Espina, 1993). This distribution is similar to that of animal lamins, which are seen primarily at the nuclear rim, but can also be detected in
the nuclear interior as nucleoplasmic foci (Moir et al., 1995). Transmission electron microscopy of the nucleus shows dark staining regions of condensed heterochromatin, often positioned at the periphery, but also present throughout the nucleoplasm (Berezney and Coffey, 1974). Additional nuclear structures have been reported, including coiled bodies, also known as Cajal bodies (Gall et al., 1999; Boudounck et al., 1998) and nucleolus associated bodies (Moreno Diaz de la Espina et al., 1992); interchromatin granules, which correspond to spliceosomal speckles seen in fluorescence microscopy (Spector, 1994; Lamond and Earnshaw, 1998); perichromatin granules; and perichromatin fibrils (Lamond and Earnshaw, 1998). Each of these morphological features have been seen in both animal and plant nuclei. The roles of each of these morphological features remain to be resolved, but their presence supports the idea of a functional ultrastructure in the nucleus.

Nucleic acids of the nuclear matrix

The foremost nucleic acid components of the nuclear matrix are matrix associated region DNA sequences (MARs). MARs are defined principally by their ability to bind to extracted nuclear matrices in vitro, and this property leads to their theoretical role as attachment points of chromatin loops to the nuclear matrix. While the in vitro interactions demonstrated between MAR DNA and nuclear matrices have not yet been conclusively shown to reflect in vivo or in situ associations, a large body of evidence supports the idea that loops detected in situ are anchored by MAR DNA. The spacing of MAR DNA in the genome correlates with the size of loops predicted from microscopy and from binding site titration estimates (reviewed in van Driel and Otte,
MARs typically have a size of approximately 1 kb (Michalowski et al., 1999), though artificial fragments as small as 175 bp (Dickinson et al., 1992) have been shown to bind to the matrix protein SATB1. MARs are generally AT rich (greater than 65% AT), but AT-richness alone does not make a DNA fragment a MAR (Michalowski et al., 1999). Some fragments with >65% AT did not bind to the matrix in the in vitro exogenous binding assays (for example, See SCS fragments, Appendix A, figure A3). Numerous MARs have been isolated and sequenced from a variety of organisms, including plant (Hall et al., 1991), animal (Mirkovitch et al., 1984), and fungi (Gasser et al., 1989), but no consensus sequence has been deduced from these sequences. Rules and algorithms for predicting MARs from sequence information have been attempted (Benham et al., 1997; Boulikas, 1993; Kramer et al., 1996; Singh et al., 1997) but with only limited success. Several motifs have been suggested to be important for MAR activity (reviewed in Benham et al., 1997; Michalowski et al., 1999), the most predictive being stretches of 20 base pairs or more of 90% AT. The propensity for MAR DNA to unwind, and perhaps to form non B-DNA structures may be an essential feature for MAR activity (Kay and Bode, 1994; Benham et al., 1997).

MAR DNA sequences have generated a great deal of interest because of their ability to affect gene expression in transgenic organisms. In animal cell culture systems, the first transgenic experiments used the chicken lysozyme “A element,” which contains the 5’ MAR as well as additional regulatory sequence from the lysozyme locus (Stief...
et al., 1989; Phi-Van et al., 1990; Phi-Van and Strätling, 1996). These experiments showed approximately ten-fold increases in transgene expression levels in constructs flanked by the A element, compared to controls without these flanking sequences. Initial results suggested that this element also conferred reduction in the variability of transgene expression, but some later experiments do not demonstrate such a reduction (Poljak et al., 1994). Some of the decrease in variation seen in the initial experiments may be due to the small populations used and to possible confounding effects of the additional regulatory sequence present in the lysozyme A element used in those experiments (Bonifer et al., 1994). Additional experiments in animal systems using flanking MARs have shown similar increases in transgene expression. In these experiments a variety of MAR containing elements as well as a variety of cell types were used (reviewed in Spiker and Thompson, 1996). Experiments with smaller pieces of the chicken lysozyme MAR showed that matrix binding activity can be separated from the increases in expression (Phi-Van and Strätling, 1996).

Experiments in plants show similar increases in expression in MAR flanked constructs compared to constructs without flanking MAR DNA. In one of the most dramatic instances, the MAR isolated from the region 3’ to the tobacco RB7 gene has been shown to elevate transgene expression in tobacco cell lines by 60 fold (Allen et al., 1996). Comparable, but less dramatic effects on transgene expression have been seen in numerous plant experiments using MARs of different origins, using various cell types and using several different transformation methodologies (reviewed in Allen et al., 2000).
In addition to the DNA component of nuclear matrices, RNA is also present in the nuclear matrix. The RNA components of the nucleus are not well studied, but may be important to overall matrix structure and function. The most prominent RNA containing component of the nuclear matrix is the nucleolus. This important subregion will be discussed in detail below. Additionally, ribonucleoproteins are thought to comprise a significant portion of the fibrogranular structure of the matrix (Mattern et al., 1997; Berezney et al., 1995; Moreno Diaz de la Espina, 1995).

Berezney et al. (1995) estimate that close to thirty percent of nuclear RNA is retained in rat liver matrices treated with DNase I and extracted with both 0.25 M ammonium sulfate and 2M NaCl, but not treated with RNase. It has been suggested that the contribution of RNA to the matrix is an important area of difference between plants and animals, since RNase treatment of matrices had little morphological effect on plant matrices (Moreno Diaz de la Espina, 1995). However, in many cases similar extractions using RNases reveal similar structures in both plant and animal nuclear matrices. No controlled comparisons have been done, so differences seen in some reports may be due to differences in technique or cell types. Treatment of HeLa cell nuclei with RNase A reduces the total amount of protein remaining in the matrix, but both a nucleolar structure and a matrix structure still remain, based on transmission electron microscopy (Belgrader et al., 1991; Nickerson et al., 1995). Since RNase treatment did not completely eliminate RNA from the matrix in these experiments, it may be that undigested RNA provided structure to the matrices prepared in these experiments. Other researchers have found that much of the matrix protein is removed when extracts are treated with RNases, though a residual structure containing the lamina, a residual nucleolar structure, and an internal structure remains (Fey et al.,
1986). In onion cells similarly extracted, total protein is reduced after RNase treatment, as seen on SDS polyacrylamide gels, but the architectural organization is preserved. In addition, specific protein bands are lost after RNase treatment (Moreno Diaz de la Espina, 1995). Similarly, Avramova and Bennetzen (1993) report that maize nuclei that are RNase treated and extracted with either the LIS or NaCl extraction methods each produce matrices that are functionally equivalent in an exogenous MAR DNA binding assay. At this time, the most we can conclude is that significant differences in protein content and matrix morphology have been seen between the RNA components of plant and animal matrices in some cases. The differences may be due to differences in extraction conditions or other unknown experimental variables.

Proteins of the nuclear matrix

The composition of the nuclear matrix and the interactions of matrix components remain poorly understood, despite many reports of proteins and nucleic acids that associate with the nuclear matrix. The composition of the relatively well studied mammalian nuclear matrix varies between cell type, organism, and extraction technique. It is estimated that thousands of matrix proteins exist, but few of the individual proteins have been well characterized (Boulikas, 1995). The number of and possible mixtures of matrix proteins are quite complex and variable. In matrices prepared from single cultured human cell lines, the number of proteins seen on an two dimensional gel was estimated at more than 200 (Fey and Penman, 1988), while Gerner et al. (1998) counted approximately 300 spots on two dimensional separations
of cells from human tissue and cell lines. Many proteins appear to be common to different types of cells, based on the electrophoretic mobility of matrix proteins in two dimensions (Fey and Penman, 1988; Mattern et al., 1997; Stuurman et al., 1990).

Some of the more interesting reports are of proteins involved in transcriptional regulation that may become tethered to the nuclear matrix in a developmentally regulated manner. These include the phosphorylated form of RNA polymerase II in mammalian cells (Bissoto et al., 1995; Nayler et al., 1998; Patturajan et al., 1998), histone acetyl transferase and histone deacetylase (Davie and Hendzel, 1994), components of the human SWI/SNF chromatin remodeling factors (Reyes et al., 1997), and numerous transcription factors (reviewed in Boulikas, 1995). Experiments by Stein and colleagues have focused particularly on several matrix associated transcription factors involved in differentiation of osteoblast cells in culture (Guo et al., 1995; Merriman et al., 1995; Oesterreich et al., 1997; Zeng et al., 1998). Kim et al. (1996) showed that the Oct-1 transcription factor binds to an AT rich silencer element upstream of a homeodomain binding site. Oct-1 is a general transcription factor known to associate with numerous nuclear factors and binding sites. They found that both Oct-1 and the silencer region are enriched in the nuclear matrix in a human cell line. Newberry et al (1999) describe MINT, a matrix protein that binds to Msx2, a homeodomain transcriptional repressor that exerts tissue-specific actions during craniofacial skeletal and neural development in mouse cells. Matakatsu et al. (1999) report that the nuclear matrix protein plexus regulates wing development in Drosophila, perhaps by tethering transcription factors to the matrix. Trubiani et al (2000) report that the transcription factor NF kappa B, a protein that translocates from
the cytoplasm to the nucleus in response to extracellular signals, associates with the nuclear matrix in DMSO stimulated human cells, but not in unstimulated cells. Samuel et al. (1998) show that a number of transcription factors and cofactors can be crosslinked to DNA bound to the nuclear matrix. Boulikas (1997) predicted numerous transcription factor binding sites available in a study of numerous MAR sequences. Taken as a group, these reports point to the nuclear matrix as a potential anchorage point for regulators of the transcription machinery.

Evidence has also been presented that mRNA processing machinery is tethered to the nuclear matrix. Newly transcribed hnRNA is capped, spliced and polyadenylated before being transported out of the nucleus. Xing and Lawrence (1991) showed that RNA tracks observed in situ (Lawrence et al., 1989) were preserved even after nuclear matrix extraction procedures. RNA processing factors, including splicing factors (Blencowe et al., 1994, 2000; Nayler et al., 1998; Eggert et al., 1997; Meissner et al., 2000), and heterogeneous nuclear ribonucleoproteins (Fackelmayer and Richter, 1994a; Mattern et al., 1996, 1997, 1999; Gerner et al., 1998) have been identified in nuclear matrix preparations.

The heterogeneous ribonuclear proteins (hnRNPs) are an extremely complex and interesting group of matrix proteins. HnRNPs are a group of more than thirty proteins identified due to their formation of complexes with hnRNA. HnRNPs have been reported to be the major protein present in the matrix of HeLa cells (Mattern et al., 1996). They are involved in numerous nuclear and non-nuclear processes ranging from transcription and pre-mRNA processing in the nucleus to cytoplasmic mRNA
translation and turnover. HnRNPs are even thought to be involved in maintenance of telomeres (Eversole and Maizels, 2000; reviewed in Krecic et al., 1999). Their association with large hnRNA processing complexes defines hnRNPs, but proteins encoded by that have been identified are not highly similar to each other. Many hnRNPs contain both RNA binding motifs and protein-protein interaction motifs. Some contain multiple RNA binding motifs. Another interesting characteristic of hnRNPs is the number of isoforms generated by alternative pre-mRNA splicing and post-translational modification. This variability creates a very large number of combinatorial possibilities. Individual hnRNPs have preference for different RNA targets (Krecic et al., 1999). Some also show DNA binding specificity (e.g., hnRNP K, which binds ssDNA).

In a study of four of the human hnRNPs identified as matrix proteins, Mattern et al. (1999) showed that these proteins have different spatial distributions and are approximately 50% retained in the matrix. The proteins retain their nuclear localization after undergoing the harsh conditions of matrix preparation. HnRNP U has been identified by at least two groups as a MAR binding protein (Romig et al., 1992; Tsutsui et al., 1993; von Kries et al., 1994).

As mentioned earlier in this section, nuclear speckles seen by fluorescent labeling of splicing proteins such as SC35, have been correlated with the interchromatin granules, one of the nuclear features that remains as part of the matrix after extraction (Spector, 1994; Lamond and Earnshaw, 1998). This type of association has led to the idea that speckles are possible storage or assembly sites for splicing factors, including some
hnRNPs. Nayler et al (1998) report that the matrix protein SAF-B interacts with a number of transcription proteins and splicing factors. SAF-B has also been reported to interact with the AUF1/hnRNP D class of hnRNPs (Krecic et al., 1999).

A particularly notable chain of relationships involves hnRNP K. This example demonstrates linkages between regulation of transcriptional, translational, and nucleolar processes. HnRNP K can be found in complexes with hnRNA. This protein has also been reported to be involved in both activating or repressing transcription factor activation of gene expression (Du et al., 1998; Miau et al., 1998). To activate the c-myc promoter, hnRNP-K interacts with single stranded polypyrimidine stretch called the CT element as well as with components of the RNA polymerase II holoenzyme (Michelloti et al., 1996). HnRNP K has been reported to bind to translationally silenced mRNA (reviewed in Krecic, 1999). In addition, hnRNP K is thought to interfere with the formation of an activation complex between Nopp140 and C/EBP-β. Nopp140 is a mammalian protein that localizes predominantly to the nucleolus but also shuttles between the nucleus and cytoplasm. Nopp140 also interacts with known snoRNA associated proteins. Two major classes of small nucleolar RNAs (snoRNAs) are involved in ribosomal RNA processing. Box C/D snoRNA is involved in 2’O-methylation, and H/ACA snoRNAs are involved in pseudouridylation. Nopp140 has been shown to co-immunoprecipitate with the C/D box associated nucleolar proteins fibrillarin, NAP65 (a NOP58 snoRNP homologue) as well as H/ACA associated protein NAP57 (Yang et al., 2000). Thus, a nucleolar protein (Nopp140) with multiple nucleolar interactions is also involved in regulation of gene expression, most likely via its interaction with a DNA binding protein (hnRNP
K) and in processing of hnRNA transcripts. This series of interactions illustrates the complex interrelationships possible between nuclear functions often considered quite distinct from each other.

The involvement of hnRNPs in multiple nuclear processes makes them an intriguing group of proteins. The hnRNPs are a functionally diverse group of proteins that recognize a number of RNA and DNA motifs to form nucleoprotein complexes that mediate gene expression at transcriptional and translational levels. A considerable amount has been revealed about these proteins recently, but the number of proteins, their variability and the multifunctional capabilities of many of these proteins leaves many questions regarding their nature to be answered. The identities of hnRNPs present in the matrix may help us understand more about the interactions of DNA with the matrix. These interactions may be a critical factor in understanding the effects of MAR elements in transgenic experiments.

Numerous other RNA binding proteins and nucleolar proteins (Dickinson et al., 1992; Grondin et al., 1996; Hatton and Gray, 1999; Sanvito et al., 1999) have been reported as components of the matrix, including several identified in vitro as having MAR DNA binding activity (discussed below). Additional proteins reported to be associated with the nuclear matrix include actin, and actin-binding proteins (Krauss et al., 1997; reviewed in Rando et al., 2000), mitotic apparatus proteins (Kallajoki et al., 1991; Zhelev et al., 1990; Saitoh et al., 1995), DNA replication proteins (Vishwanatha et al., 1992), and structural nuclear proteins (Belgrader et al., 1991; Luderus et al.,
1992). In many cases, multiple functions have been assigned to these proteins (Holzmann et al., 1998; Gohring and Fackelmayer, 1997).

**MAR DNA binding nuclear matrix proteins**

While our knowledge of the proteins that comprise the nuclear matrix is incomplete, our knowledge of the functions of these proteins remains even more of a mystery. Due to the insoluble nature of the matrix, it is difficult to isolate and identify proteins, and more difficult to maintain the natural activity of these proteins. This problem has hindered the identification of proteins of the nuclear matrix that bind specifically to MAR DNA. However, several proteins have been identified, many of which have turned out to have specific functions apart from general MAR binding.

One of the best characterized MAR binding matrix proteins is SATB1. SATB1 is a nuclear protein that was originally cloned by virtue of its ability to bind to a core unwinding element within the MAR located 3' of the immunoglobulin \( \mu \) heavy chain gene enhancer (Dickinson et al., 1992). It is expressed predominantly in thymocytes, but is also found expressed at lower levels in other tissues. SATB1 has been shown to act as a transcriptional repressor of a reporter construct (Kohwi-Shigematsu et al., 1997) and as part of a repressor complex with NPB and with UBP (Liu et al., 1997). In tissue from SATB1 knockout mice, SATB1 was shown to have effects on 11 of the 597 genes tested by microarray hybridization (hybridization results confirmed by RT-PCR). Ten of the eleven genes were downregulated by SATB1, and one was upregulated. The SATB1 matrix protein is therefore a regulator of multiple genes, and
that regulation is likely accomplished by affecting formation of complexes between regulatory MAR elements and additional proteins. In a similar interaction at a different MAR in the same locus in B lymphocytes, the nuclear matrix-bound protein Cux/CDP inhibits the transcriptional activator Bright. Bright is a protein that regulates the immunoglobulin (Eµ) intronic enhancer activity by binding to MARs flanking the heavy chain Eµ intronic enhancer (Herrscher et al., 1995). Chattopadhyay et al (1998) report that Cux/CDP and SATB1 can bind simultaneously to a MAR located 5’ to the enhancer of the T cell receptor β locus. More recently, the same group has identified another protein, SMAR1, that binds to the same MAR (Chattopadhyay et al., 2000). Banan et al. (1997) also demonstrate an interaction between Cux/CDP and SATB1 at the CD8a locus. The in vivo effects of these interactions have not yet been elucidated, but probably involve regulation of this receptor locus. These findings as a group indicate that a similar regulatory scheme involving MAR binding proteins appears to be associated with the each of these enhancers. Together these reports suggest that differential regulation of nuclear matrix attachment may be a common mechanism of gene regulation.

Several MAR binding proteins have been identified by Southwestern blotting. In this type of experiment, matrix proteins are solubulized with denaturants such as SDS, separated electrophoretically and transferred to a membrane. The membranes are probed with labeled MAR DNA, and proteins with specific binding to MAR DNA can be identified. Several matrix proteins with general DNA binding activity were identified using this technique but with labeled genomic DNA as probe (Hakes and Berezney, 1991). The first MAR binding protein to be identified this way was ARBP
(von Kries et al., 1991). ARBP was isolated as an abundant (100,000 copies per nucleus) protein present in chicken oviduct nuclei. This protein was shown to bind specifically to MARs from various organisms, including chicken, Drosophila, and human. Later this protein was shown to be the chicken homolog of a rat protein MeCP2. The rat protein, as well as the chicken homolog, binds to methylated CpG islands and is targeted specifically to heterochromatin (Nan et al., 1996). MECP2 protein selectively binds methylated CpG dinucleotides in the mammalian genome and mediates transcriptional repression through interaction with histone deacetylases and the corepressor SIN3A. Immunoprecipitation studies show that MeCP2/ARBP binds to the histone deacetylase via the SIN3A protein (Nan et al., 1998). MeCP2/ARBP has a much lower affinity for non-methylated CpG. The protein region responsible for MeCP2/ARBP binding activity to both MAR DNA and to methylated CpG has been identified as a 125 amino acid region that is 96.8% conserved between chicken and rat. The MAR DNA used to identify the MAR binding activity of this protein does not contain CpG motifs, so the binding to these two substrates appears to be independent (Weitzel et al., 1997). It may be that there is some structural similarity that is conserved between methylated DNA and MAR DNA, or the binding of the two substrates may be independent of each other. It has been suggested that the protein operates by both histone deacetylase dependent and independent mechanisms in silencing transcription. These two silencing mechanisms operate via the same domain in in vitro experiments (Yu et al., 2000). Surprisingly, several studies have identified MeCP2-like proteins encoded by genes of Drosophila melanogaster, an invertebrate species supposed to be devoid of detectable methylation at CpG (Roder et al., 2000).
It seems likely that the activity of this type protein in vivo is variable, and depends on its associations with other proteins and nucleic acids.

Using the southwestern blotting approach with HeLa cell matrix protein, Romig et al. (1992) identified 4 nuclear proteins, termed scaffold attachment factors (SAFs) A, B, C, and D, that specifically interact with MAR DNA elements. The first of these proteins to be characterized was SAF-A. Several other groups have identified this protein by its MAR binding activity. The chicken homolog was identified by southwestern blotting with MAR DNA (von Kries et al., 1994), as was the rat homolog (Tsutsui et al., 1993). SAF-A was found to be an abundant nuclear matrix protein expressed in all tissues tested. Cloning of the cDNA encoding SAF-A revealed that this protein was identical to a protein previously identified as hnRNPU, an RNA binding protein. The protein contains multiple “RGG” RNA binding domains, and binds to both RNA and to ssDNA (Kiledjian and Dreyfuss, 1992). The protein has a novel scaffold-associated region (SAR)-specific bipartite DNA-binding domain. This domain is separated by 400 amino acids from the previously identified RNA-binding domain, the RGG box (Gohring et al., 1997). SAF-A can be directly cross-linked in vivo to chromatin by formaldehyde, a “zero-length” crosslinker (Gohring and Fackelmayer, 1997). Given the in vivo association of SAF–A, and its multiple substrate binding capacity, it is likely that that this protein is multifunctional, and that least part of the in vivo function of SAF-A is to bind to chromatin as a structural protein.

2 In older literature, the term “scaffold” had been used, usually to discriminate between nuclei extracted with LIS from those extracted with NaCl. Since these extraction methods produce a similar structure, most researchers now use the term matrix to describe the structure revealed by any of the extraction methods.
SAF-B has also been purified from HeLa cells and its gene cloned. The RNA for this protein is ubiquitously expressed in all cell lines and tissues tested (Renz and Fackelmayer, 1996). It seems likely that this protein also has multiple functions, since it too has been characterized by separate functions. This protein has been independently identified as HET, an HSP27 promoter-binding protein isolated from a breast cancer cell line. Overexpression of this protein in breast cancer cell lines caused a repression in the activity of the HSP27 gene (Oesterreich et al., 1997).

Weighardt et al. (1999) also isolated this protein from HeLa cells by immunoprecipitation with hnRNP A1, indicating that SAF-B protein is an hnRNP. They further showed that antibodies to SAF-A (aka HAP and HET) immunoprecipitate the complete set of proteins normally found in the hnRNP complex. Therefore, like SAF-A, SAF-B associates with MAR DNA, binds to RNA, and appears to be involved in the regulation of gene expression.

SAF-C and SAF-D, which had the weakest binding of the four MAR binding proteins identified in the initial study (Romig et al., 1992) have not yet been further characterized.

A novel yeast screen was used to identify two proteins putatively involved in MAR binding in vivo (Fishel et al., 1993). Taking advantage of the fact that a MAR sequence inserted into the promoter region of a reporter construct inhibits the expression of the reporter, the researchers screened for mutants with normal induction of the reporter construct. They uncovered two proteins that (when mutated) allowed
induction of the reporter gene. One of the proteins found was a mutated version of calmodulin, a calcium binding regulatory protein. It was suggested that this protein could be involved in regulation of MAR-matrix attachments. The second mutant, Smi1, encoded a protein that was nuclear localized in immunofluorescence analysis. However, this same protein has also been cloned due to its involvement in cell wall synthesis (Hong et al., 1994), so the complete function of this gene product is not resolved.

Several ubiquitous and well known nuclear proteins have been described as having MAR binding activity in addition to their more commonly known functions. These proteins include histone H1, high mobility group proteins (HMGs), topoisomerases I and II, lamins, and nucleolin.

The linker histone H1 is present in the nuclear matrix, and binds to both MAR DNA and non-MAR DNA (this work). It has been described as a MAR binding protein (Izaurralde et al., 1989), but the MAR affinity shown by H1 may be due primarily to its affinity for AT rich DNA. An artificial double stranded AT polymer showed H1 affinity when the polymer reached a critical length of approximately 130 bp. Similarly, the AT-hook containing high mobility group proteins have been suggested as possible MAR binding proteins due to their affinity for AT rich DNA (Pederson et al., 1991; Ivanchenko and Avramova, 1992; Boulikas, 1995). However, these low molecular weight proteins do not appear to be matrix proteins (this work and Ingram, 2000).
Topoisomerases, enzymes that can interconvert different topological isomers of DNA, have also been reported to be nuclear matrix proteins (Boulikas, 1995). Topoisomerase II is an abundant enzyme present in metaphase chromosomal scaffolds (Earnshaw et al., 1985). Topoisomerase II cleaves and rejoins DNA via a double stranded break, whereas topoisomerase I cleaves only a single strand. Topoisomerase II consensus sites are present in many MAR sequences, (Gasser and Laemmli, 1987), and at least one isoform of the protein is present in the nuclear matrix (Berrios et al., 1985). Similar cleavage patterns are generated in both cells and matrices treated with drugs that block topoisomerase II at the cleavage intermediate (Gromova et al., 1995b). This suggests that at least some specific DNA is tightly bound to the matrix and associated with topoisomerase II in vivo. Topoisomerase I, though present in the matrix, is present only at greatly reduced amounts relative to the amounts in whole nuclei, based on immunological detection. Topoisomerase I has not been reported to be associated with MAR DNA (Zini et al., 1994).

Lamin B binds specifically to MAR DNA, based on southwestern blotting experiments (Luderus et al., 1992). In these experiments, lamin preparations from rat liver cells, from calf thymus and from Drosophila all bound to MAR DNA. Comparison of MAR binding between matrix preparations and lamin “shell” preparations suggested that about half of the binding sites available per nucleus are found in the lamina. Further research from this group indicated that MAR DNA also binds to lamin A, and probably to the very similar protein lamin C (Luderus et al., 1994). They showed that the binding is due to interactions with AT rich DNA, via the
minor groove of DNA. Competition experiments suggest that DNA binding to lamins occurs at unpaired regions of the MAR.

Nucleolin, a major protein component of the nucleolus, was identified as the primary MAR binding protein in human erythroleukemia cells. It was purified by affinity chromatography using a synthetic MAR element, and the purified protein was shown to bind specifically to MAR DNA (Dickinson and Kowhi-Shigematsu, 1992). Nucleolin is a multifunctional protein primarily involved in ribosome synthesis, and as its name implies, it is localized primarily to the nucleolus. However, nucleolin has been implicated in many other metabolic processes including cytokinesis, nucleogenesis, cell proliferation and growth, cytoplasmic-nucleolar transport of ribosomal components, transcriptional repression, replication, signal transduction and chromatin decondensation (reviewed in Ginisty et al., 1999). Nucleolin has three different domains that appear to be involved in different functions. In plants it is developmentally, cell-cycle, and light regulated (Tuteja and Tuteja, 1999). The diverse functions of nucleolin continue to be investigated, but its involvement in so many cellular activities is a good example of the interdependence of nuclear functions.

The first novel plant MAR binding protein to be reported is MFP1 (MAR-binding Filament-like Protein 1), isolated from tomato (Meier et al., 1996). MFP1 was cloned from an expression cDNA library using an oligomerized regulatory element. The similarity of predicted protein structure to lamins led to experiments showing that this protein is present in the nuclear matrix and binds MAR DNA with high specificity. Later experiments demonstrate that MFP1 is localized near the nuclear envelope, but
unlike lamins, MFP1 is distributed in a speckled pattern, with approximately 100 speckles per nucleus. Interestingly, the pattern in nuclear matrices is different, the speckle-like structures can be detected in the interior of the nuclear matrix. The authors suggest that this redistribution indicates that a structure containing MFP1 associated with the nuclear envelope collapses during nuclear matrix isolation. A similar redistribution is seen with MAF1, a matrix protein that is believed to associate with MFP1 in vivo, based on yeast two hybrid experiments and by co-localization seen in fluorescence microscopy (Gindullis et al., 1999; Gindullis and Meier, 1999). Antibodies to the C-terminus of this protein, containing the DNA binding domain recognize similarly sized protein from all of six different plant species, both monocot and dicot, suggesting that this protein region is highly conserved. Antibodies directed to the central region of the protein recognized only a tobacco homolog in a similar immunoblot (Harder et al., 2000).

Concurrent to the work reported in this dissertation, very similar experiments conducted with pea nuclei resulted in the cloning of two MAR binding proteins. Using southwestern blotting, the authors report four protein bands that bind to MAR DNA. Using amino acid sequence from microsequencing of the fastest migrating protein, PCR primers were made. The PCR product generated with these primers and a pea leaf cDNA template was used to screen a cDNA library. Two cDNA clones selected from the screen contained the coding sequence for two very similar (85% amino acid identity) cDNAs (Hatton and Gray, 1999). These cDNAs code for proteins homologous to a nucleolar protein NOP5/58, which was cloned from yeast (Gautier et al., 1997). Similarly sized proteins in both pea and White bush (Cucurbita pepo)
nuclear matrices were reported earlier, (Rzepecki et al., 1995a, 1995b), but the proteins were not characterized beyond their identification as MAR binding proteins by southwestern blotting.

Morisawa et al., (2000) have published a cDNA sequence for a MAR binding protein from wheat, that has no significant homology to other sequences found in Genbank. This protein has a predicted size (58 kDa) and pI (9.9) consistent with the MAR binding proteins from pea and Arabidopsis. This protein could be related to one or more of the proteins seen on southwestern blots in this work, but further experimentation will be required to investigate that possibility.

The nucleolus and ribosomal RNA processing

The nucleolus has long been recognized as the site of synthesis and processing of pre-ribosomal RNA, and of assembly of ribosomal proteins and RNA into ribosomal subunits (Verheijen et al., 1988). However, recent evidence suggests that the nucleolus may have additional roles. These include telomere maintenance, cell cycle regulation, tRNA processing, processing of spliceosomal U6 RNA, and maturation of the signal recognition particle (Johnson et al., 1998; Olson et al., 2000; Visintin and Amon, 2000). Figure 1.4 illustrates many of the complex processes involving the nucleolus.

The basic activity of the nucleolus, ribosome biogenesis, is a highly complex process. In a rapidly dividing cell, thousands of ribosomal subunits must be synthesized per
minute. This process involves hundreds of proteins, transcription of hundreds of separate RNA molecules by RNA polymerase I, II and III, post-transcriptional modifications of the RNAs, assembly of the ribosomal subunits, and transport of all these molecules.

The organization of ribosomal genes and transcription of these genes is well conserved in eukaryotes. The basic organization of the rRNA transcripts is similar in all species (Venema and Tollervey, 1999). The yeast operon, which is well understood, will be used as the eukaryotic archetype. In the yeast *Saccharomyces cerevisiae*, the large ribosomal RNA precursor, containing three of the four rRNAs, is transcribed as a single transcript by RNA polymerase I. This transcript is first cleaved at the 3’ external transcribed spacer, then modified by 2’O ribose methylation and pseudouridylation by a large number of small ribonucleoprotein particles (snoRNPs). The transcript is then cleaved in a multi-step process to generate three of the four ribosomal RNA species: 5.8S, 18S, and 25-28S (Venema and Tollervey, 1999). The fourth ribosomal RNA, the 5S rRNA, is transcribed by RNA Polymerase III, and requires only cleavage of a few nucleotides at the 3’ end to form the mature molecule.

Small nucleolar RNAs (snoRNAs) are involved in various stages of ribosome biogenesis. A few snoRNAs appear to be involved in cleavage of rRNA, but most appear to serve as guides for base specific modification of rRNA. The two major classes of snoRNAs appear to be involved in the two most common forms of rRNA modification. Most box H/ACA snoRNAs are required for pseudouridylation, the conversion of uridine to pseudouridine. H/ACA snoRNAs are characterized by two
regions predicted to form hairpin loops, separated by a variable hinge (H) region, and a 3’ “ACA” sequence. Most C/D box snoRNAs appear to be involved in 2’O ribose methylation. C/D box snoRNAs are characterized by conserved box C (UGAUGA) and D (CUGA) elements (Smith and Steitz, 1997).

The function of each of these rRNA modifications has yet to be elucidated. Deletion of modification guide snoRNAs in yeast has shown no discernible defects in growth or ribosome synthesis, even in multiple mutants (Venema and Tollervey, 1999). Both the box C/D and the H/ACA snoRNAs direct the modification of rRNA by specifically base pairing with the pre-rRNA (Newman et al., 2000). It is estimated that \textit{S. cerevisiae} has 55 rRNA methylation sites, each requiring a specific C/D box snoRNA, although some C/D box snoRNAs may function at more than one site. A computational screen using the known methylation target sites in rRNA to screen the yeast genome has assigned 51 of 55 known rRNA methylation sites to 41 different snoRNAs (Lowe and Eddy, 1999). It is expected that similar numbers of H/ACA snoRNAs are present in yeast. In vertebrates and plants, it is estimated that about 100 of each class of snoRNA are present (Brown and Shaw, 1998).

In yeast and in vertebrates, the majority of snoRNAs are often found within introns, often in the introns of genes encoding proteins involved in nucleolar or ribosomal function (Leader et al., 1999). Some highly expressed snoRNAs are expressed from their own promoters. However, processing of the yeast and vertebrate snoRNAs appears to be splicing-dependent (Brown and Shaw, 1998). In plants, the organization of snoRNA genes may be different. It appears that plant snoRNA genes are non-
intronic. Instead, the plant snoRNAs analyzed are transcribed polycistronically. It has been shown that plant snoRNAs of both classes can be processed from intronic or non-intronic precursor RNAs. These results indicate that plant snoRNAs can be processed by a splicing-independent mechanism (Leader et al., 1997, 1999). However, very few snoRNA genes have been isolated from plants. It remains possible that the differences seen so far are due to the small number of genes studied, and that similar processing mechanisms are used by all eukaryotes (Brown and Shaw, 1998).

Each of the two classes of snoRNAs associate with a separate base set of proteins, along with proteins more specific for individual snoRNA species. H/ACA snoRNAs precipitate in snoRNP complexes with the glycine/arginine rich protein Gar1p. Gar1p has been shown to form a complex with the putative pseudouridine synthase Cbf5. Gar1p affinity experiments revealed two additional proteins, Nhp2p and Nop10p, associated with Gar1p. These proteins are present in the nucleolus and are essential for pseudouridylation (Henras et al., 1998; Lafontaine et al., 1998). Interestingly, telomerase RNA shows homology to the second hairpin loop of H/ACA snoRNA U17, and Gar1p is complexed with telomerase RNA in vivo (Dragon et al., 2000). This suggests that nucleolar proteins also function outside the nucleolus.

C/D box snoRNAs form complexes with Nop1p, the yeast homolog of mammalian fibrillarin, an essential protein. In mouse, C/D box snoRNA was used to affinity purify two additional pairs of proteins. These proteins are conserved in eukaryotes and even in Archaea (Newman et al., 2000; Hatton and Gray, 1999). The first pair of proteins, designated p50 and p55, are related to each other, 42% identical and 68%
similar at the amino acid level. The proteins are evolutionarily conserved, nearly 90% similar to homologs from yeast. These proteins were found to localize predominantly in the nucleoplasm. The nucleoplasmic location of these proteins led the authors to speculate that their association with snoRNA may be transient (Newman et al., 2000). A rat homolog of p55 has been described (Tip49) as a TBP interacting protein. Additional homologs include the human nuclear matrix protein NMP238 (Holzmann et al., 1998).

The second set of proteins is known in yeast as Nop56p and Nop58p (also called Nop5). Nop56p was first identified as SIK1 (suppresor of Iκβ) by Morin et al (1995). Iκβ inhibits certain transcription factors by binding them, and thus prevents their interaction with other members of the transcription complex. In these experiments, overexpression of Nop56p relieved the growth inhibition caused by a fusion of GAL4 DNA binding domain and Iκβ. The mode of action of the overexpressed Nop56p remains unclear.

Nop56p and Nop58p were separately identified in yeast by a synthetic lethal screen for mutations interacting with temperature sensitive mutants of Nop1p, the fibrillarin homolog. In yeast, Nop56 and 58 are 45% identical and 63% similar to each other at the amino acid level. Co-precipitation experiments with C termini-truncated epitope tagged versions of these proteins showed that these proteins form complexes in vivo. Interestingly, in these experiments, the authors report that snoRNA was not coprecipitated, indicating that the complex forms without snoRNA (Gautier et al., 1997). Similar coprecipitation of Nop58p, with and without the highly charged lysine
rich C-terminal region showed that Nop58p does form a complex with all tested C/D box snoRNAs. H/ACA snoRNAs showed a small level of association as well (Lafontaine and Tollervey, 1999). Similar experiments with Nop56p showed that this protein is also complexed with C/D box snoRNAs in vivo. Further coprecipitation experiments suggest that Nop56p association is dependent on the presence of Nop1p (fibrillarin), while Nop58p can associate with snoRNAs independently. Similar to Nop58p, Nop56p does not require the lysine rich C-terminus to associate with snoRNA (Lafontaine and Tollervey, 2000).

While most reports describe the two classes of snoRNA binding proteins as completely separate sets, there has been a protein reported to link the C/D and H/ACA snoRNAs. The mammalian Nopp140, discussed earlier due to its involvement in a transcription activation complex, has been shown to interact in vivo with proteins from each group of snoRNA associated proteins. Anti-Nopp140 was used to immunoprecipitate proteins from rat liver nuclei. Microsequencing of the proteins specifically complexed with Nopp140 included NAP57, the rat homologs of yeast Cfb5 (the putative pseudouridine synthase) and NAP65, the rat homolog of Nop58. Immunoblotting of anti-Nopp140 immunoprecipitated protein revealed both Gar1p and fibrillarin in the immunoprecipitate. This report suggests that Nopp140 serves as a chaperone to shuttle the snoRNAs between the nucleus, coiled bodies, and the nucleolus (Yang et al., 2000).

This dissertation describes the identification, cloning and expression of the Arabidopsis homologs of Nop56 and Nop58. These proteins were selected based on
their ability to bind to MAR DNA in vitro. In Arabidopsis, I have identified two members of the Nop58 family, and a single Nop56 homolog. Based on EST data available to date, these three clones represent the complete set of Nop56/58 proteins expressed in this plant. I have analyzed the DNA and RNA binding characteristics of these proteins in vitro, and found that all three proteins bind MAR DNA and C/D box snoRNA. Competition studies surprisingly show a cooperative effect when both MAR DNA and C/D box snoRNA are present in the binding reaction. These results suggest that the plant Nop56 and Nop58 homologs are involved in complex interactions involving nucleolar RNA, protein and MAR DNA.
Figure 1.1. Matrix isolation protocol. A generalized view of matrix preparation includes isolation of nuclei from cytoplasmic material, removal of soluble or loosely bound nuclear material with chaotropic agents, and removal of most nuclear DNA with endonucleases. The resulting nuclear fraction is the nuclear matrix.
Figure 1.2 Diagrammatic representation of the nucleus. This generalized diagram presents most of the major features seen in eukaryotic nuclei. (based on Moreno Diaz de la Espina, 1995).
Figure 1.3 Transmission electron micrograph of a nuclear matrix. (Wan et al, 1999) (a) human cell culture line SAOS-2 cells were extracted by amine modification and digested with restriction enzymes to reveal the nuclear matrix. (b) inset is a view of the nucleolus, demonstrating the continuity of the nuclear matrix with the nucleolar matrix. Nu - nucleolus, L - lamina, IF - cytoskeletal intermediate filaments.
Figure 1.4 RNA processing in the nucleolus (from Lewis and Tollervey, 2000). Within the nucleolus, rRNA is transcribed at high levels, modified via interactions with RNA-protein complexes, and assembled into ribosomal subunits for export to the cytoplasm. Additional nuclear processes have also been localized to the nucleolus, including processing of tRNA, SRP and U6 processing.