Epitope-tagged *Xenopus* nucleolin was expressed in *Escherichia coli* cells and in *Xenopus* oocytes either as a full-length wild-type protein or as a truncation that lacked the distinctive carboxy glycine/arginine-rich (GAR) domain. Both full-length and truncated versions of nucleolin were tagged at their amino termini with five tandem human c-myc epitopes. Whether produced in *E. coli* or in *Xenopus*, epitope-tagged full-length nucleolin bound nucleic acid probes in in vitro filter binding assays. Conversely, the *E. coli*–expressed GAR truncation failed to bind the nucleic acid probes, whereas the *Xenopus*-expressed truncation maintained slight binding activity. Indirect immunofluorescence staining showed that *myc*-tagged full-length nucleolin properly localized to the dense fibrillar regions within the multiple nucleoli of *Xenopus* oocyte nuclei. The epitope-tagged GAR truncation also translocated to the oocyte nuclei, but it failed to efficiently localize to the nucleoli. Our results show that the carboxy GAR domain must be present for nucleolin to efficiently bind nucleic acids in vitro and to associate with nucleoli in vivo.

INTRODUCTION

Vertebrate nucleolin is a nucleolar specific phosphoprotein of 90–110 kDa that is believed to quickly associate with nascent preribosomal RNA within the dense fibrillar regions of nucleoli (reviewed by Olson, 1990; Escande et al., 1985; Herrera and Olson, 1986). Nucleolin is abundant in rapidly dividing somatic cells (Orrick et al., 1973) and amphibian oocytes (Caizergues-Ferrer et al., 1989; DiMario and Gall, 1990) where rates of ribosome production are maximal. Herrera and Olson (1986) demonstrated that the majority of nucleolin rapidly associates with newly synthesized pre-ribosomal RNA, and recent studies on the yeast nucleolin-like NSR1 protein showed that 35S preribosomal RNA processing and ribosome biogenesis were impaired when the NSR1 protein was eliminated in a *nsr1* deletion strain (Kondo et al., 1992a,b). This combined evidence suggests that nucleolin plays an early role in the processing of preribosomal RNA or in the preassembly of ribosomes (Olson, 1990).

Nucleolin is a modular protein (Olson, 1990). Its amino terminal third consists of alternating basic and acidic domains, and the carboxy terminal two-thirds consists of four RNA-binding domains followed by a glycine- and N\(^2\),N\(^\text{G}\) dimethylarginine-rich (GAR) domain (Lischwe et al., 1985; Lapeyre et al., 1986). The amino terminal acidic domains have been implicated in binding histone H1 to decondense rDNA for transcription (Erard et al., 1988, 1990) or in binding basic ribosomal proteins to facilitate ribosome assembly (Olson, 1990). On the other hand, the amino terminal basic domains have been implicated in binding upstream rDNA sequences to regulate rRNA gene expression (Olson et al., 1983). The four RNA-binding domains are similar to those found in several other RNA-binding proteins such as the heterogeneous nuclear ribonucleoproteins (hnRNPs), poly(A)-binding proteins, and ribonucleoproteins specifically associated with U1 and U2 small nuclear (sn) RNAs (reviewed by Dreyfuss et al., 1993). For example, the RNA-binding domain of the U1 snRNP A protein can bind RNA in vitro (Query et al., 1989), and its crystal structure provides evidence for likely interactions with stem-loop II of U1 snRNA (Nagai et al., 1990). On the basis of what is already known about these RNA-binding domains in other proteins, we can assume that some or all of nucleolin’s four RNA-binding domains are important for observed in vitro RNA and DNA interactions (Olson et al., 1983; Bugler et al., 1987; Sapp et al., 1989). However, specific in vivo interactions between nucleolin’s four RNA-
binding domains and pre-rRNA have yet to be determined.

Just downstream of the fourth RNA-binding domain is the GAR domain that is referred to as the Arg-Gly-Gly (RGG) box by Dreyfuss et al. (1993) for other RNA-binding proteins. Ghisolfi et al. (1992) have shown that this domain consists of repeated β-turns that confer an overall helical structure on this domain (i.e. a β-spiral), and they have shown that this basic domain binds RNA or DNA independently of the four upstream RNA-binding domains. The GAR domain may nonspecifically bind preribosomal RNA to perhaps unwind secondary structures thereby facilitating more specific interactions between nucleolin and preribosomal RNA (Ghisolfi et al., 1992). Despite these predictions, the in vivo functions and associations of this GAR domain also remain unknown.

To gain more information about individual domains of nucleolin, specifically the carboxy GAR domain, we have epitope-tagged *Xenopus* nucleolin at its amino terminus and truncated the protein just before the GAR domain. Here we show that the tagged truncation produced in *Escherichia coli* no longer binds radiolabeled nucleic acids in vitro and that the tagged truncation produced in *Xenopus* oocytes fails to efficiently localize to the multiple nucleoli in vivo.

**MATERIALS AND METHODS**

**Recovery of Xenopus Nucleolin cDNAs**

The polymerase chain reaction (PCR) was used as described (Frohman et al., 1988) to recover full length *Xenopus* nucleolin cDNAs. Briefly, *Xenopus* ovaRNA was purified as described (Epstein et al., 1986), and poly(A)+ mRNA was further enriched by oligo(dT) chromatography (Kingston, 1993). To synthesize negative cDNA strands, 1 µg of poly(A)+ RNA was reverse transcribed using a cDNA Synthesis System Plus kit (Amersham, Arlington Heights, IL) according to the manufacturer’s recommended protocol. A *Xenopus* nucleolin-specific oligonucleotide was used in the PCR as the 5′ primer for the synthesis of positive nucleolin cDNA strands. This primer was homologous to nucleotides 28–57 of an incomplete *Xenopus* nucleolin cDNA (Cai, Zergues-Ferrer et al., 1989) except that nucleotides 34 (G) and 35 (C) were changed to C and T, respectively to provide a HindIII site for cloning purposes. The 3′ oligonucleotide primer contained poly(dT) at its 5′ end and XhoI, SalI, and ClaI restriction sites at its 3′ end for subsequent cloning. The PCR cycles were carried out essentially as described (Frohman et al., 1988) using a Perkin-Elmer-Cetus DNA Thermal Cycler (Norwalk, CT) and TaqI polymerase. A predicted PCR product of ~2000 base pairs (bp) was gel purified, cut with HindIII and XhoI to generate sticky ends, and ligated into a pBluescript vector (Stratagene, La Jolla, CA) at the appropriate polyclinker sites. The PCR product was determined to encode nucleolin by sequence analysis using a dyeoxy Sequenase kit (United States Biochemicals, Cleveland, OH).

A 645-bp HindIII/EcoRI fragment from the 5′ end of the PCR product was used to screen a *Xenopus* ovary cDNA lambda-ZAP library (Stratagene). Several positive cDNA clones were selected on the basis of size and three previously identified internal EcoRI sites (Cai et al., 1989). Single- and double-stranded sequencing techniques were used to further characterize several of these cDNAs. Two cDNAs, XIC23-92 and XIC23-56, were completely sequenced as described. Their sequences overlap and a full-length nucleolin cDNA was constructed by ligating the two cDNAs at a common XhoI site shown in Figure 1. The EMBL accession number for this full length nucleolin cDNA is X63091.

**Computer Sequence Analysis**

Analyses of the *Xenopus* nucleolin cDNA sequence and the deduced protein sequence were performed using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package, Version 7.1 (Devereux et al., 1984).

**cDNA Subcloning and Mutagenesis**

A 13–amino acid segment of the human *c-myc* protein has been used successfully as an epitope to tag cellular proteins (Monro and Pelham, 1986). Six tandem repeats of the DNA encoding the *myc* epitope were subsequently constructed and cloned downstream of the T3 promoter of pBluescript by Roth et al. (1991). For our studies the 5′ end of the nucleolin coding sequence was ligated to the 3′ end of the fifth tandem *myc* DNA repeat by using common NcoI sites, one shortly downstream of the fifth *myc* tag and the other at the predicted ATG translation start codon of the nucleolin cDNA. Ligation between the *myc* and nucleolin cDNA sequences placed 5 tandem *myc* repeats upstream and in frame with the nucleolin ATG start codon. The fifth *myc* repeat unit was followed by an additional eight nonnucleolin amino acids (MESLGDLT) and then the initial methionine of nucleolin. Although the nucleolin ATG start codon was left intact, each *myc* DNA repeat begins with its own ATG translation start codon. The β-gal promoter of pBluescript was upstream of the T5 promoter, and it permitted bacterial expression of the *mrc*-tagged fusion protein. The resulting *mrc*-nucleolin construct is referred to as *pmyc-XIC23*.

The XIC23-56 cDNA that was used to construct *pmyc-XIC23* contains the 3′ untranslated region, but it has no poly(A) sequence. To ensure stability of oocyte-injected mRNA, the 3′ untranslated region of *Xenopus* N038 cDNA with a poly(A) tail of 59 residues was used to replace most of the nucleolin 3′ untranslated region of *pmyc-XIC23*.

Specifically, the 3′ untranslated region of the N038 cDNA was excised by cutting at an EcoRV site that had been engineered within the 3′ untranslated region (construct C11 of Peculis and Gall, 1992) and at a downstream SacI polylinker site. The resulting 400-bp N038 fragment was ligated to *pmyc-XIC23* that had been first cut with SpeI; its ends made flush with the Klenow polymerase fragment and then cut again at a downstream SacI polylinker site. The resulting plasmid is called *pmyc-XIC23-N038*. *E. coli* and *Xenopus* oocyte translation products, derived from *pmyc-XIC23-N038*, are depicted in Figure 1, A and B, respectively.

The carboxy terminus of nucleolin was truncated upstream of the GAR domain by cutting *pmyc-XIC23-N038* at the unique XhoI site (Figure 1), filling in the overhangs with the Klenow polymerase fragment and then religating the plasmid. This procedure introduced four extra base pairs that shifted the reading frame such that the original nucleolin sequence of -L273DFAKPGSGS- (where GS is 4 residues upstream of the GAR domain) was changed to -L273ARLCKT-. The (+) strand becomes a newly created stop codon that is positioned less than four codons upstream of the GAR-coding DNA sequence. Sequence analysis verified the predicted mutagenesis, and the resulting plasmid is referred to as *pmyc-XIC235GAR*. *E. coli* and *Xenopus* oocyte translation products, derived from *pmyc-XIC235GAR*, are depicted in Figure 1, C and D, respectively.

**Bacterial Expression**

The protease deficient *E. coli* strain, BL21 (Sturdier et al., 1990), and the recombinant deficient *E. coli* strain, XL1-Blue, were transformed with cesium chloride-banded *pmyc-XIC23-N038* or *pmyc-XIC235GAR*. The BL21 was cotransformed with PUB5520 (from Dr. R. Mattes, Institut fur Industrielle Genetik, Universitat Stuttgart, Stuttgart). PUB5520 is a derivative of pACYC 177 that was modified to contain the *E. coli lac* gene that encodes the lac repressor to prevent weak constitutive expression from lactose inducible promoters.
pUBS520 also carries the argU (formerly called dnaY) gene of E. coli (Brinkman et al., 1989) for high level fusion protein expression. Fusion protein expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) once cultures had reached an OD600 of 0.5 at 37°C. Cells were allowed to express fusion protein for 3 h at 37°C with vigorous shaking after which the cells were pelleted, resuspended in 0.5 ml of sodium dodecyl sulfate (SDS)-sample buffer (Laemmli, 1970), and lysed by sonication. Samples were boiled for 5 min and then clarified by centrifugation. E. coli-expressed translation products derived from pmyc-XIC23-NO38 or pmyc-XIC234GAR are depicted in Figure 1, A and C, respectively.

**Oocyte Injections**

An unique BamHI site downstream of the poly(A) sequence of pmyc-XIC23-NO38 and pmyc-XIC234GAR permitted linearization the plasmid for in vitro runoff transcription. RNA transcripts were synthesized from the T3 promoter positioned upstream of the myc tags. The reaction mixture contained 2 μg of linearized template DNA, 0.75 mM each of the ribonucleotides CTP, UTP, and ATP, and 0.5 mM GTP. The diguanylate triphosphate cap analogue (New England Biolabs, Beverly, MA) was included at 0.75 mM. One microliter of undiluted T3 polymerase (Stratagene) was used for transcription along with 0.1% paraformaldehyde, 5 mM MgCl2, and 1.0 mM CaCl2, 1.0 mM Tris pH 7.2. A nucleus was cleaned of cytoplasm with 0.1% paraformaldehyde, resuspended in 0.5 ml of sodium dodecyl sulfate (SDS)-sample buffer (Laemmli, 1970), and lysed by sonication. Samples were boiled for 5 min and then clarified by centrifugation. E. coli-expressed translation products derived from pmyc-XIC23-NO38 or pmyc-XIC234GAR are depicted in Figure 1, A and C, respectively.

**Total Nuclear Proteins, Gel Electrophoresis, and Southwestern Analysis**

Nuclear proteins were prepared from hand isolated oocyte nuclei that were stripped of clinging cytoplasm by pipetting up and down (Roth and Gall, 1987). SDS-polyacrylamide gel electrophoresis was achieved according to Laemmli (1970). Two-dimensional (2-D) isoelectric focusing of oocyte nuclear proteins was as described (DiMario and Gall, 1990). Western blots were probed with labeled, heat denatured DNA (“Southwesterns”) as previously described (DiMario et al., 1989).

**Nucleolin Purification and Antiserum Production**

Nucleolin was purified from S100 extracts of Xenopus kidney cells (X1K2) by anion exchange chromatography and poly[G]-agarose chromatography. The extract was prepared according to the procedures of McStay and Reeder (1986, 1990). In the purification of nucleolin, an aliquot (17 ml) of cell extract was applied to DEAE-Sephadrose (DCL-6B-100, Sigma, St. Louis, MO) packed in a 20 x 1.5 cm I.D. BioRad Econo column Richmond, CA. The beads had been previously equilibrated in column buffer (20 mM Tris · HCl pH 6.2, 6 M urea, 1 mM diithiothreitol, and 1 mM EDTA). A linear gradient of NaCl (0.0-0.75 M, 350 ml total volume) passed through the column at 4°C. Each fraction (5.6 ml) was monitored by A223 and A280 to establish a column elution profile. Fraction aliquots (0.25 ml) were precipitated with 1 ml of acetone, and the pellets were resuspended in 50 μl of SDS sample buffer (Laemmli, 1970). Fractions were resolved on a 10% polyacrylamide SDS gel, transferred to nitrocellulose, and probed with monoclonal antibody (mAb) G1C7 and secondary reagents from the Vectastain detection kit (Vector Laboratories, Burlingame, CA). Nucleolin typically eluted from the cellulose diethylaminoethyl-cellulose (DEAE) column at ~0.35 M NaCl. Those fractions enriched for nucleolin were dialyzed overnight in poly[G] column buffer (50 mM Tris base pH 7.9, 5 mM MgCl2, and 1 mM EDTA) at 4°C. Dialyzed fractions were loaded onto a 5 ml poly[G]-agarose resin (P1908, Sigma) that had been packed in a 9 x 1.5 cm I.D. BioRad Econo column and equilibrated with poly[G] column buffer. A linear gradient of KCl (0.0-1.0 M, total volume of 140 ml) was passed through the column at 4°C. The flow rate was slowed to 0.35 ml/min using a peristaltic pump. Fractions of 3 ml were collected and the A223 and A280 were read again for each fraction to obtain a column elution profile. Aliquots (0.5 ml) of the fractions were precipitated with 2 ml of 100% acetone and pelleted. Pellets were resuspended in 50 μl of SDS sample buffer, and individual fractions were resolved on a 10% polyacrylamide SDS gel. Polyacrylamide gels were silver stained to determine which fractions contained only nucleolin. Nucleolin typically eluted from the poly[G]-agarose column at ~0.75 M KCl.

Purified nucleolin was next transferred to sterile PBS and condensed using an Amicon condenser (Darnestown, MA) and Millipore spin columns (Bedford, MA) to a final volume of 0.2 ml. The protein sample was then emulsified with 0.2 ml of Freund's complete adjuvant (F-4258, Sigma) and injected subcutaneously into a female New Zealand white rabbit. The rabbit was boosted twice; each boost used Freund's incomplete adjuvant (F-5506, Sigma), and each boost was administered 3 weeks after the previous injection. The resulting polyclonal serum (R2D2) was characterized for anti-nucleolin specificity by probing Western blots that contained a complex mixture of cellular proteins (Figure 5B).
Other Antibodies
Mouse mAb 9E10 was originally developed by Evan et al. (1985); it labels the myc repeat peptide used to tag nucleolin. Mouse mAb G1C7 was developed by Rabia Yuma and Mark Roth at the Fred Hutchinson Cancer Research Center, Seattle, WA; it labels both versions of Xenopus nucleolin and a larger unidentified protein of approximately 180 kDa on Western blots. A Vectastain ABC kit (Vector Laboratories) was used to immunochemically detect mAbs 9E10 and G1C7 on western blots. Anti-fibrillarin mouse mAb 72B9 was originally developed by K. Michael Pollard, and it was characterized by Reimer et al. (1987). For fluorescence microscopy, affinity-purified, fluorescein-coupled goat anti-mouse IgG (Cappel, West Chester, PA) and affinity-purified, rhodamine-coupled mouse anti-rabbit IgG (Pierce, Rockford, IL) were used to detect the respective primary antibodies within Xenopus nuclear preparations.

RESULTS
Two partial but overlapping Xenopus nucleolin cDNAs (XIC23-92 and XIC23-56) were ligated together at their common XbaI sites (bp 1779–1784) to provide a complete coding sequence of 2424 bp (EMBL accession number X63091). This sequence includes 63 bp of 5' untranslated DNA and 407 bp of 3' untranslated DNA. The translation start codon is nucleotides 64–66, and it is well defined by Kozak's criteria for translation initiation (Kozak, 1987). An upstream TAG stop codon at nucleotides 19–21 is in frame with the ATG start codon. This supports the probability that the entire protein coding sequence is present. The translation stop codon is at nucleotides 2017–2019, and the deduced protein is 651 amino acids in length (Figure 1A). Its calculated molecular weight is 70 110 Da. Two Xenopus nucleolin proteins display apparent molecular weights of 90 and 95 kDa on SDS-gels (Caizergues-Ferrer et al., 1989; DiMario and Gall, 1990). Their migrations are anomalous because of the overall acidic charge of Xenopus nucleolin (calculated pI = 4.65; observed pI = 5.0–5.2) (DiMario and Gall, 1990). Other proteins that show anomalous migrations on SDS-gels include nucleoplasmin (Dingwall et al., 1987), the RNA-binding hnRNP proteins C1/C2 (Swanson et al., 1987), and the RNA-binding protein of the fragile X gene, FMR1 (Siomi et al., 1993). All have acidic stretches like nucleolin.

The amino terminal third of Xenopus nucleolin is a modular composition of alternating basic and acidic domains (Figure 1), as in chicken (Maridor and Nigg, 1990) and Chinese hamster ovary (CHO) nucleolin (Lapeyre et al., 1987). Although shorter in overall length, the amino terminal region of Xenopus nucleolin contains five acidic domains and six basic domains, rather than four acidic and five basic domains as found in chicken and CHO nucleolin. As in other vertebrate versions of nucleolin, the carboxy two-thirds of Xenopus nucleolin consists of four RNA-binding domains, a GAR domain, and finally a short tail of seven amino acids (Figure 1A).

Wild-Type Xenopus Nucleolin Expression in E. coli
A 13–amino acid fragment of human c-myc (MEQKLISEEDLNE) has been used successfully to epitope tag several different proteins and then to localize these proteins within cell compartments (Monro and Pelham, 1986, 1987; Pelham et al., 1988). Six DNA repeats encoding the myc tag were cloned into pBlueScript downstream of the β-galactosidase and T3 promoters by Roth et al. (1991). Induction of the β-galactosidase promoter in E. coli with IPTG should produce a fusion protein of 751 amino acids that consists of a small lacZ peptide, five myc-tags, the eight amino acid linker, and then nucleolin. Figure 1A depicts this entire E. coli fusion protein.

Endogenous nucleolin from vertebrate cells can be observed simply by probing Western blots with radiolabeled, single-stranded DNA (DiMario and Gall, 1990). We have used this filter binding assay in the studies reported here not to define in vivo functions but rather to monitor nucleolin's production in E. coli and to test the potential of wild-type nucleolin and various mutations of nucleolin to bind DNA under a set of defined in vitro conditions. Figure 2A shows a Western blot that was probed with radiolabeled single-stranded DNA to Figure 1. Bar diagrams showing various fusion proteins of Xenopus nucleolin. (A) The fusion protein expressed in E. coli after IPTG induction from either pmyc-XIC23 or pmyc-XIC23-NO38. Starting from the left, the fusion consists of a portion of lacZ (1/2 width checkered box), five myc tags (3/4 width white boxes), and a linker region (3/4 width checkered box). Nucleolin is shown as a series of full-width boxes: the amino terminal third of Xenopus nucleolin consists of alternating basic domains (I) and acidic domains (I). The carboxy terminal two-thirds of nucleolin consists of four RNA-binding domains each containing an RNP consensus sequence (small white inserts). The fourth RNA-binding domain is followed by a GAR domain (I) and then a tail of seven amino acids (I). (B) The full-length fusion protein expressed in Xenopus oocytes from injected mRNA that was in vitro transcribed from pmyc-XIC23-NO38. Translation initiation should have occurred at the initial methionine codon of the first myc tag. (C) The GAR truncation expressed in E. coli after IPTG induction of the β-galactosidase promoter of pmyc-XIC236GAR. The GAR domain and the short carboxy tail were removed by creating a stop codon shortly before the GAR-encoding cDNA by mutagenizing pmyc-XIC23-NO38 at the XbaI site. (D) The GAR truncation expressed from pmyc-XIC236GAR in Xenopus oocytes from injected mRNA that was in vitro transcribed from pmyc-XIC236GAR.
detect _E. coli_-produced nucleolin fusion proteins. Lanes 1 and 2 contain proteins from the protease deficient BL21 strain of _E. coli_ (Studier et al., 1990) that was transformed with pmyc-XIC23. A novel DNA-binding protein of 120 kDa was observed in extracts of IPTG-induced cells (Figure 2A, lane 1), but no DNA-binding protein of this weight was found in extracts of the same cells that were not induced (Figure 2A, lane 2).

A nonprotease deficient _E. coli_ strain, XL1-Blue, was also transformed with pmyc-XIC23. The 120-kDa DNA-binding protein was again observed in this strain only after IPTG induction (Figure 2A, lanes 3 and 4). As a negative control, _E. coli_ cells were transformed with a Bluescript-based plasmid containing a new cDNA insert that when induced with IPTG, produces a fusion protein that is detectable with mAb 3A10, an anti-histone H1 antibody (DiMario and Gall, 1990). This plasmid, referred to simply as p3A10-positive, failed to produce a novel DNA-binding protein either with or without IPTG induction (Figure 2A, lanes 5 and 6, respectively). A prominent single-stranded DNA-binding protein of 61 kDa was observed in all _E. coli_ lysates whether the lysates were prepared from induced or noninduced cells, and whether or not they contained plasmids encoding nucleolin (Figure 2A, lanes 1–6). Because of its presence in all _E. coli_ lysates, we were confident that the 61 kDa was not a proteolytic fragment of the 120 kDa DNA-binding protein (see below).

The apparent molecular weight of the 120 kDa DNA-binding protein and its presence only in lysates of induced cells strongly indicated that it was the nucleolin fusion protein. To test this we used mAb 9E10 that reacts well with the myc epitope (Evan et al., 1985). Figure 2B shows the same blot that was used in Figure 2A, but after it was reprobed with mAb 9E10. The primary 9E10 antibody was detected by immunochromatographic staining. The prominent antigen of Figure 2B, lane 1' co-migrated with the 120 kDa DNA-binding protein observed in Figure 2A, lane 1. Because the 120-kDa antigen was found only in cells harboring pmyc-XIC23 and after IPTG induction, we concluded that this antigen is the *myc*-tagged version of intact Xenopus nucleolin.

mAb 9E10 also labeled a protein of 64 kDa that was found only in *pmyc*-XIC23–transformed cells after IPTG induction. Because it was detected by virtue of the epitope-tags, the 64-kDa protein must have been the amino terminal fragment of nucleolin. Although we could not rule out premature translation termination as cause for its presence, the 64-kDa protein was probably a proteolytic fragment of the 120-kDa antigen. Although similar in size, this 64-kDa antigen migrated slightly behind the 61-kDa _E. coli-specific DNA-binding protein shown in Figure 2A. Because we were able to resolve the 61-kDa DNA-binding from the 64-kDa antigen on the gradient polyacrylamide gels in Figure 2, we were confident that the 64-kDa antigen was not related to the smaller _E. coli-specific nucleic acid-binding protein.
In addition to the intact 120-kDa and truncated 64-kDa antigens, several other proteolytic fragments of \( \sim 108 \) kDa were observed in lysates prepared from induced XL1-Blue cells that contained \( pmyc-XIC23 \) (Figure 2B, lane 3). The \( myc \) tags were still present at their amino termini. Therefore, proteolysis had to occur at the carboxy end of the proteins. Simply on the basis of apparent molecular weights, the difference in size between the intact 120-kDa fusion protein and these 108-kDa fragments suggested that \( \sim 9\% \) of the intact fusion protein was missing. Although the GAR domain and the very carboxy tail actually constitute 9\% of the overall linear length of the \( E.\ coli \)-expressed fusion protein (68 out of 751 total amino acid residues), these crude estimates merely suggested that the GAR domain was missing from the 108-kDa antigens.

To test if the GAR domain is required for in vitro DNA-binding activity, we truncated the fusion protein by engineering a stop codon in the \( pmyc-XIC23-NO38 \) expression plasmid at an unique \( Xba \) I site (compare Figure 1, A and C). The resulting plasmid, \( pmyc-XIC23\Delta GAR \), was expressed in \( E.\ coli \). Labeling with \( mAb \) 9E10 showed that the tagged GAR truncation was indeed present within the cell lysate (Figure 2D, lane 2). However, it failed to bind radiolabeled DNA (Figure 2C, lane 2). The truncation's apparent molecular weight of 107 kDa was in good agreement with the predicted loss of \( \sim 9\% \) of the intact fusion protein of 120 kDa (Figure 2, C and D, lanes 1 and 1'). Failure to bind the probe under these conditions suggested that the GAR domain must either bind nucleic acids directly as suggested by Ghisolfi et al. (1992) or regulate the conformational state of other nucleic acid binding domains within nucleolin by intramolecular peptide-peptide interactions.

**Xenopus Oocyte Expression**

The in vitro binding results with fusion proteins produced in \( E.\ coli \) only suggested potential nucleic acid interactions that may be important for nucleolin association within the nucleoli for preribosomal RNA processing or perhaps ribosome assembly and transport. To approach possible in vivo associations and functions, we produced epitope-tagged versions of nucleolin that were either full length (Figure 1B) or deleted for the carboxy GAR domain (Figure 1D) in *Xenopus* oocytes. These tagged proteins were produced by injecting mRNAs that were in vitro synthesized from \( pmyc-XIC23\Delta NO38 \) or \( pmyc-XIC23\Delta GAR \), respectively. Both mRNAs were synthesized using the T3 promoter just upstream of the \( myc \)-tags. Therefore, unlike translation initiation in \( E.\ coli \), translation initiation in *Xenopus* oocytes should have occurred at the initial AUG codon of the first \( myc \) tag. As a result, the lacZ portion of the fusion should be absent, and the *Xenopus*-produced fusion proteins should be slightly smaller than their counterparts expressed in \( E.\ coli \) (Figure 1, B and D).

Oocyte-expressed nucleolin fusion proteins were tested first for their ability to bind single-stranded DNA. Hand isolated nuclei from noninjected oocytes contain two prominent DNA-binding proteins of 90 and 95 kDa (Figure 3A, lane 1). These are the endogenous versions of *Xenopus* nucleolin that we previously characterized by this filter binding assay (DiMario and Gali, 1990). In addition to the two endogenous nucleolin proteins, hand-isolated nuclei from oocytes that were injected with mRNA encoding \( myc \)-tagged, full-length nucleolin (Figure 1B), contained a novel DNA-binding protein of 102 kDa. The size of the novel protein suggested that the predicted 8.7 kDa \( myc \) tag was fused to a protein of 93.8 kDa, which is in close agreement with the observed apparent weights of either the 90 or the 95 kDa endogenous nucleolin protein. All other detected binding proteins from the nuclei of injected oocytes were common to nuclei of noninjected oocytes (compare Figure 3A, lanes 1 and 2).

The western blot used in Figure 3A was probed with \( mAb \) 9E10 (Figure 3B). Nuclei from noninjected oocytes contained no detectable antigen (Figure 3B, lane 1'), whereas an antigen of 102 kDa was found in the

![Figure 3. Western blots displayed oocyte-expressed myc-tagged nucleolin fusion proteins. (A) The blot was probed with single-stranded radiolabeled DNA. Nuclei from noninjected oocytes contained endogenous nucleolin proteins of 90 and 95 kDa (N), as well as minor binding proteins (lane 1). An equal number of nuclei from injected oocytes contained the endogenous nucleolin proteins and the same minor nucleic acid binding proteins found in noninjected oocytes. They also contained a novel binding protein at 102 kDa (lane 2). (B) The blot used in A was reprobed with \( mAb \) 9E10. No antigen was detected in nuclei from noninjected oocytes (lane 1'). However, the novel 102-kDa nucleic acid-binding protein and a 62-kDa antigen were labeled by \( mAb \) 9E10 (lane 2'). The 62-kDa antigen clearly contained the \( myc \)-tags and at least the amino terminal one-third of nucleolin. (C) A Southwestern blot characterized the GAR truncation. Nuclei from oocytes injected with mRNA transcribed from \( pmyc-XIC23\Delta GAR-NO38 \) contained DNA-binding proteins at 90 and 95 kDa (lane 1). Nuclei from oocytes injected with mRNA transcribed from \( pmyc-XIC23-NO38 \) were used as a positive control (lane 2). (D) The blot used in C was reprobed with \( mAb \) 9E10. The \( myc \)-tagged GAR-truncation comigrated with the 95-kDa endogenous nucleolin.
nuclear extracts from oocytes that had been injected 
with the mRNA (Figure 3B, lane 2). We concluded 
that the 102-kDa antigen was the epitope-tagged 
nucleolin fusion protein because it was found only in 
nuclei that were isolated from injected oocytes, and because it had 
an identical apparent molecular weight when compared 
to the novel DNA-binding protein in the Southwestern 
assay.

As in the E. coli extracts, a myc-tagged proteolytic 
fragment was found in the nuclear extracts from mRNA-
 injected oocytes (Figure 3B, lane 2). Its apparent 
molecular weight of 62 kDa indicated that it contained, 
in addition to the myc-tags, at least the amino terminal 
one-third and perhaps even the first two RNA-binding 
domains within the carboxy terminal two-thirds of nu-
cleolin. The similarity in size between the E. coli-expressed, myc-tagged, 64-kDa proteolytic fragment and the 
myc-tagged 62-kDa Xenopus fragment intrigued us. 
If we assume that these fragments were in fact generated 
from intact nucleolin by proteolysis, and if we allow for 
the presence of the short lacZ peptide on the 64-kDa E. coli 
fragment versus the 62-kDa oocyte fragment, 
then nucleolin expressed in either E. coli or Xenopus 
oocytes may have been cleaved at sites that lie in close 
proximity. Reports have suggested that proteolysis is a 
programmed event in the maturation of the 110-kDa 
CHO nucleolin (Bugler et al., 1982; Bourdon et al., 
1983a) with the size of CHO proteolytic fragments 
ranging from 45–95 kDa (Bourdon et al., 1983b). Chen 
et al. (1991) also suggested that human nucleolin of 105 
kDa actually cleaved itself to generate several fragments 
ranging from 45 to 97 kDa.

In addition to the myc-tagged full-length protein, we 
also tested the oocyte-expressed GAR truncation for its 
ability to bind radiolabeled DNA by Southwestern as-
say. However, a novel DNA-binding protein was not 
evident (Figure 3C, lane 1). Staining the blot with mAb 
9E10 showed that the tagged GAR truncation was in 
fact present within the nuclear extract but that it comi-
grated with the 95-kDa endogenous nucleolin protein 
(Figure 3D, lane 1). In characterizing the oocyte-ex-
pressed GAR truncation, we concomitantly expressed 
full-length myc-tagged nucleolin in separate oocytes as 
a positive control for DNA binding, as a control for 
antibody (mAb 9E10) staining, and as a demonstration of 
a shift in molecular weight between the GAR truncation 
and the intact nucleolin fusion protein (Figure 3, 
C and D, lanes 2, 2). Unfortunately, the comigration 
of the GAR truncation with the 95-kDa endogenous pro-
ten negated our attempts to test the in vitro binding 
capabilities of the GAR truncation by this one-dimen-
sional Southwestern assay.

Because dimethylation does not change the positive 
charge of the arginine side group (Ghisolfi et al., 1992), 
we reasoned that the GAR truncation should be more 
acidic than the endogenous proteins because of the lack 
of the arginine-rich domain. Therefore, a 2-D South-
western blot was employed to separate the endogenous 
forms of nucleolin from the myc-tagged GAR truncation. 
The 2-D protein blot was probed with labeled DNA 
(Figure 4A) and then with mAb 9E10 (Figure 4B). Sev-
eral isoelectric variants of the 90- and 95-kDa endog-
enous nucleolin proteins were evident by DNA-binding. 
These variants exist probably because of various degrees 
of nucleolin phosphorylation. The most acidic DNA-
binding protein of 95 kDa (Figure 4A) colocalized with 
an antigen that stained well with mAb 9E10 (Figure 
4B). This colocalization indicated that the oocyte-ex-
pressed myc-tagged GAR truncation maintained some 
nuclear acid binding capabilities.

Evidence from E. coli expression experiments showed 
that the smallest myc-tagged (i.e., mAb 9E10-positive) 
proteolytic fragment of ~10 kDa could be labeled by 
mAb G1C7. This indicated that the G1C7 epitope re-
sided within the amino terminal region of nucleolin. 
With this knowledge, we used mAb G1C7 to roughly 
estimate the amount of the GAR truncation present 
within the oocyte nuclear extract relative to endogenous 
nucleolin proteins by comparing a combination of mAb 
G1C7 staining intensities and autoradiographic spot 
sizes on the 2-D blots. For this comparison we used 
another aliquot of the same protein sample used in 
Figure 4, A and B. On the basis of G1C7 staining (Figure 
4C), we conservatively estimated that the amount of 
GAR truncation within the lysate was comparable to 
one of the minor isoelectric variants (see arrows in Figure 
4C). But when the DNA-binding signals of the GAR 
truncation and the same isoelectric variant were com-
pared (arrows in Figure 4A), it appeared that the GAR 
truncation failed to bind DNA as efficiently as did the 
minor isoelectric variant.

Finally, mAb G1C7 labels higher molecular weight 
proteins that have less acidic isoelectric points (arrow-
head in Figure 4C). These proteins have not been iden-
tified, and this cross-reactivity precludes us from using 
mAb G1C7 in fluorescence localization experiments de-
scribed below.

Characterization of Anti-Nucleolin Serum R2D2

Before we could pursue the localization of myc-tagged 
nucleolin in Xenopus oocyte nuclei, it was first necessary 
to establish the localization of endogenous nucleolin. 
Because mAb G1C7 labeled proteins other than nu-
cleolin (Figure 4C), a highly specific polyclonal serum 
directed against Xenopus nucleolin was prepared. The 
two versions of nucleolin were purified from S100 cell 
extracts (Figure 5A, lane 1) of cultured Xenopus kidney 
cells by DEAE chromatography and then by poly[G] 
"affinity" chromatography. Nucleolin prepared by this 
two-step procedure was considered highly enriched by 
silver stain analysis (Figure 5A, lane 2), and those frac-
tions containing only nucleolin were used for antibody 
production. The resulting anti-nucleolin serum, R2D2,
labeled only the two nucleolin proteins versus a large complex mixture of cellular proteins (Figure 5B, lanes 1–3).

**Endogenous Nucleolin Localizations**

Previous reports showed that nucleolin primarily associates with the dense fibrillar regions of nucleoli (Noaillac-Depeyre et al. 1989; Biggiogera et al. 1991). Fibillarin is a nucleolar-specific protein that is known to reside within the dense fibrillar regions (Ochs et al., 1985). Therefore, to identify the dense fibrillar regions of the multiple nucleoli, we prepared the contents of nuclei from noninjected oocytes according to the procedures of Gall et al. (1991), and then probed these preparations with mouse anti-fibillarin antibody, mAb 72B9, followed by fluorescein-coupled goat anti-mouse. Figure 6A is a phase contrast micrograph showing several *Xenopus* oocyte nucleoli that were prepared from the same nucleus but ranged in size from 5 to 10 μm. mAb 72B9 staining was restricted to internal regions (dense fibrillar regions) of the nucleoli, whereas the peripheral regions (the granular regions) were not stained. No other nuclear structure was stained by mAb 72B9 (Figure 6B). We next counterstained these same nucleoli with rabbit serum R2D2 and rhodamine-coupled mouse anti-rabbit (Figure 6C). R2D2 intensely stained the same regions that mAb 72B9 stained. The colocalization of mAb 72B9 and our rabbit anti-nucleolin clearly established that fib billarin and nucleolin colocalized to the dense fibrillar regions of the multiple nucleoli. Besides the dense fibrillar regions, however, R2D2 also lightly stained the surrounding granular regions of the nucleoli; the presence of nucleolin within nucleolar granular regions has been previously described (Noaillac-Depeyre et al. 1989; Biggiogera et al. 1991). The staining patterns in Figure 6, B and C indicated that the granular regions of these *Xenopus* multiple nucleoli were very narrow bands surrounding the dense fibrillar regions and that the granular regions constituted a small percentage of the overall nucleolar mass.

We often observed understained spots within the very center of the nucleoli with either mAb 72B9 (Figure 6B) or R2D2 (Figure 6C). We interpreted these regions as the fibrillar centers that were noted to be deficient in fibrillarin (Ochs et al., 1985) and nucleolin (Noaillac-Depeyre et al., 1989; Biggiogera et al., 1991) by antibody staining.

Anti-nucleolin serum R2D2 also lightly stained the RNP material surrounding the nucleoli (Figure 6C). This staining was not simply background fluorescence, because nuclear preparations probed only with the rhodamine-coupled secondary antibody showed no staining of this RNP material. Recall that the R2D2 serum detected only nucleolin on the Western blots (Figure 5D), and this specificity of R2D2 suggested that nucleolin may be more widely distributed throughout the

![Figure 4](image-url). 2-D protein gels were used to separate the oocyte-expressed GAR truncation from endogenous nucleolin proteins. (A) The subsequent Southwestern blot showed several isoelectric variants of nucleolin at the 90 and 95 kDa range. Arrows point to the most acidic DNA-binding protein and an endogenous variant with a stronger binding signal. (B) The blot used in A was reprobed with mAb 9E10. The myc-tagged GAR truncation focused and comigrated with the minor nucleic acid binding protein in A. No other antigen was detected. (C) A separate blot containing proteins from the same nuclear extract as used in A and B was probed with mAb G1C7 to estimate the relative amounts of endogenous versions of nucleolin versus the GAR truncation. By staining intensity and spot size, the endogenous variant noted in (A) appeared to be underrepresented when compared to the GAR truncation.
nucleolar localization of myc-tagged nucleolin, rather than localized solely to the nuclei (Rankin and DiMario, unpublished data).

**Nucleolar Localizations of myc-Tagged Nucleolin Fusion Proteins**

Ultimately, we hope to use tagged nucleolin to characterize nucleolar structure and function in vivo, and nucleolar localization should be the first prerequisite in determining whether or not myc-tagged wild-type nucleolin can function properly in vivo. The results of Figure 3 indicated that tagged wild-type nucleolin translocated into the nuclei. To determine its location within the nuclei, we again injected stages 5 and 6 *Xenopus* oocytes with mRNA synthesized in vitro from pmyc-XIC23-NO38. The oocytes were cultured for 18–24 h at 18°C to allow myc-tagged nucleolin synthesis and translocation into the nuclei. The nuclear contents were then prepared for immunofluorescence microscopy. This oocyte product is depicted in Figure 1B.

Localization of myc-tagged full-length nucleolin to the nuclei by staining with mAb 9E10 and fluorescein-coupled goat anti-mouse is shown in Figure 7B. In addition to the nuclei, surrounding RNP particles were stained, which indicated that the myc-tagged nucleolin associated with these extranucleolar RNP particles, as Figure 6C indicated. We assume that part of this extranucleolar staining was because of an overexpression of the fusion protein and that the majority of tagged nucleolin was actually not associated with the multiple nucleoli. The immunoblots of Figure 3 showed that the majority of myc-tagged nucleolin was intact. This suggested that the immunofluorescence staining in Figure 7B also localized mostly intact nucleolin fusion protein.

Besides labeling endogenous nucleolin, mouse mAb G1C7 (Figure 4C) and rabbit serum R2D2 label myc-tagged versions of nucleolin on Western blots. Therefore, we cannot localize only endogenous nucleolin versus myc-tagged nucleolin by using either antibody. However, if myc-tagged nucleolin colocalized with endogenous versions of nucleolin, the staining patterns with mouse mAb 9E10 and rabbit serum R2D2 should be similar. To test this we counterstained the nucleoli of Figure 7A with R2D2 and rhodamine-coupled goat anti-rabbit IgG. Figure 7C shows similar staining patterns as in Figure 7B. The colocalization of myc-tagged full-length nucleolin with endogenous nucleolin indicated that the myc tags did not interfere with nuclear translocation nor with nucleolar association.

In most cases, staining with the anti-nucleolin serum was very bright, and substructure was not readily discernable. However, some of the larger nucleoli that were separated away from the RNP material showed detailed substructure when stained with 9E10 or R2D2. The top left insets in Figure 7. A–C show such nucleoli from a similarly injected oocyte. Both mAb 9E10 and R2D2 stained the phase-dense regions of these nucleoli. Based upon the localizations of Figure 7, A–C, we concluded that the myc-tagged, full-length nucleolin colocalized with endogenous nucleolin primarily within the dense fibrillar regions of the nucleoli that were defined by the double-labeling experiment of Figure 6.

In the above double-labeling experiments, we first probed the nuclear preparations with mAb 9E10 and fluorescein-coupled goat anti-mouse antibody. We recorded the fluorescein results on film before reprobing with R2D2 and rhodamine-coupled mouse anti-rabbit. The reason for this laborious approach was to eliminate the possibility that fluorescein-coupled goat anti-mouse antibody might cross-react with R2D2 or the rhodamine-coupled mouse anti-rabbit. To control for the possibility that the rhodamine-coupled mouse anti-rabbit IgG cross-reacted with mAb 9E10 or the fluorescein-coupled goat anti-mouse used to detect mAb 9E10, we probed the nuclear contents from a similarly injected oocyte (Figure 7A, top right inset) with mAb 9E10 and fluorescein-coupled goat anti-mouse. We left out rabbit serum R2D2 but reprobed with rhodamine-coupled mouse anti-rabbit. A strong signal was again detected in the fluorescein channel that localized myc-tagged nucleolin to the nucleoli (Figure 7B, top right inset), but no signal was detected in the rhodamine channel (Figure 7C, top right inset). This control was necessary to show that cross-reactivity by rhodamine-coupled mouse anti-rabbit with mAb 9E10 or its fluorescein-coupled secondary antibody did not account for the signals in Figure 7C.
The GAR Truncation Failed to Efficiently Associate with the Multiple Nucleoli

The GAR-truncated protein expressed in E. coli completely failed to bind nucleic acid as compared to the full-length E. coli fusion protein. In addition, whereas the full length fusion expressed in Xenopus oocytes readily bound nucleic acids, the GAR truncation expressed in oocytes appeared to have reduced binding activities when compared to an endogenous isoelectric variant of comparable abundance within oocyte nuclei (Figure 4). Its reduced in vitro nucleic acid binding capabilities next prompted us to determine if the GAR truncation could properly associate with nucleolin in vivo.

We knew from the results of Figure 3D and Figure 4 that the myc-tagged GAR truncation successfully translocated from the site of synthesis in the cytoplasm into the nuclei. To determine if the GAR-truncation associated with the multiple nucleoli, oocytes were again injected with mRNA that was in vitro transcribed from pmyc-XIC23GAR. The oocyte translation product is depicted in Figure 1D. When probed with mAb 9E10, the multiple nucleoli were reproducibly understained (Figure 7E) as compared to nucleoli from oocytes that were injected with mRNA encoding myc-tagged full-length nucleolin (e.g., see Figure 7, B and E).

As with the localization of myc-tagged full-length nucleolin, the tagged GAR truncation associated with extranucleolar RNP material. On the basis of similar RNP staining intensities in Figure 7, B and E, the amount of tagged GAR truncation within the extranucleolar RNP material appeared comparable to the amount of tagged full-length nucleolin within this material. In the absence of any significant nucleolar staining in Figure 7E, this RNP staining served as an internal positive control for the presence of the GAR truncation within the nuclei, as Figure 3D indicated.

Anti-nucleolin rabbit serum R2D2 was again used to localize both the endogenous nucleolin and the GAR-truncation. R2D2 stained the nucleoli that demonstrated the presence of only endogenous nucleolin (Figure 7F) and, as expected, R2D2 stained the extranucleolar RNP material. The differential nucleolar staining with mAb 9E10 versus R2D2 (e.g., compare Figures 7, E and F) clearly demonstrated that the myc-tagged GAR truncation failed to efficiently localize to the multiple nucleoli.

Finally, to control for the possibility that the fluorescein-coupled goat anti-mouse cross-reacted with the RNP material and for autofluorescence in the fluorescein channel, a similar preparation from an injected oocyte (Figure 7D inset) was probed with only this secondary antibody and not mAb 9E10. No signal was detected (Figure 7E inset).

DISCUSSION

We have chosen the Xenopus oocyte to gain more information about the in vivo associations of nucleolin. Ribosomal DNA amplifies in the pachytene stage of oogenesis by rolling circle replication (Brown and Dawid, 1968; Gall, 1968). As much as 30 pg of rDNA per oocyte is then separated into multiple nucleoli; the nucleus of a typical diplotene Xenopus oocyte contains ~1000 extra-chromosomal nucleoli. Transcription of this rDNA produces large quantities of nascent 45S ribosomal RNA that is quickly processed to yield mature 18S, 5.8S, and 28S ribosomal RNA (reviewed by Gerbi et al., 1990). The production of ribosomes in the diplotene oocyte is tremendous; some 300 000 ribosomes are assembled per second within the oocyte (Scheer, 1973), whereas 10–100 ribosomes are assembled per second in a typical nondividing somatic cell (Hadjiolov, 1985). Besides the high rates of ribosome production, other advantages in using Xenopus oocytes to study nucleolin are first, nucleolin is abundant in Xenopus oocytes (DiMario and Gall, 1990), and second, the amplified nucleoli can be prepared for light microscopy such that their structural integrity is well preserved (Callan et al., 1987). These amenities should allow us to study the localization of epitope-tagged wild-type and mutagenized forms of nucleolin by injecting their respective in vitro synthesized mRNAs into Xenopus oocytes. Localization of mutagenized forms of nucleolin should identify what domains are important for nucleolar associations.

The ability of nucleolin to bind both RNA and DNA in vitro has been well documented ( Olson et al., 1983; Bugler et al., 1987; Sapp et al., 1989; DiMario and Gall, 1990; Barrijal et al., 1992). We have shown that epitope-tagged full-length nucleolin expressed in E. coli or in Xenopus oocytes maintains the ability to nonspecifically bind radiolabeled nucleic acids (e.g., DNA) in vitro. However, large proteolytic nucleolin fragments found in E. coli extracts that lacked short regions of their carboxy termini failed to bind radiolabeled DNA. These proteolytic truncations suggested that the GAR domain may be important for this observed in vitro nucleic acid localization.
binding activity. To specifically address this possibility, we truncated nucleolin by introducing a stop codon shortly before the GAR domain. E. coli-expressed fusion proteins truncated in this manner completely failed to bind radiolabeled probes as compared to the full-length E. coli fusion protein. This indicates that the GAR domain is important for in vitro nucleic acid binding.

The GAR-truncation expressed in Xenopus oocytes maintains some detectable binding activity. Specific posttranslational modifications such as phosphorylation within the amino terminal third of nucleolin (Ballal et al., 1975; Geahlen and Harrison, 1984; Belenguer et al., 1990) may explain why the Xenopus-produced GAR truncation maintains some binding activity versus the E. coli-produced GAR truncation. A direct comparison of the Xenopus GAR truncation and the E. coli GAR truncation may not be appropriate because of uncertainties in posttranslational modifications of the two proteins. However, we note that the in vitro binding efficiency of the oocyte-expressed GAR truncation appears to be less than that of an endogenous full-length isolectric variant of similar (probably less) abundance (Figure 4, A and C). This observation suggests that the GAR domain may be required for at least efficient nucleic acid binding by intact nucleolin.

Ghisolfi et al. (1992) showed that on its own, the GAR domain can bind nucleic acids in vitro with a dissociation constant of $0.5 \times 10^6$ M$^{-1}$. In support of our findings, they also showed that the association of the GAR domain with nucleic acid was not as tight as that observed for intact nucleolin that had a dissociation constant of $2 \times 10^7$ M$^{-1}$. The difference in dissociation constants between the GAR domain on its own versus intact nucleolin suggests that intramolecular interactions occur between the GAR domain and the rest of the protein to enhance nucleic acid binding.

Such intramolecular interactions have been previously suggested for proper nucleolin function. For example, Sapp et al. (1989) described a sixfold higher renaturation rate for heat-denatured DNA restriction fragments in the presence of intact nucleolin as compared to the renaturation rate observed for just the 48-kDa carboxy two-thirds of nucleolin. Their results suggested that an interaction between the amino terminal third and the carboxy terminal two-thirds facilitates single-strand DNA renaturation. Second, Olson et al. (1990) showed a substantial increase in chymotrypsin sensitivity within the amino terminal third when intact nucleolin was incubated in the presence of poly[G] versus in the absence of poly[G]. Because the four RNA-binding domains were chiefly responsible for binding poly[G], these latter results indicated that a conformational change takes place within the amino terminal third of nucleolin when the protein’s carboxy two-thirds binds nucleic acid.

We have used radiolabeled single-stranded DNA in our Southwestern assays mostly to monitor the presence and nucleic acid binding capabilities of nucleolin under defined in vitro conditions. Although specific in vitro nucleolin-DNA interactions have been noted (Barrijal et al., 1992), we stress that our Southwestern assays should not be interpreted to reflect in vivo function. The demonstration that the GAR domain is important for nucleic acid interaction in our filter binding assays, however, suggests that it may also play an important role in the proper in vivo association with nucleic acids in the nucleoli. Therefore, in an attempt to study nucleolin’s in vivo associations, we expressed epitope-tagged full-length and GAR-truncated nucleolin in Xenopus oocytes.

Immunofluorescence results showed that full-length myc-tagged nucleolin properly translocates to the nuclei and then associates with nucleoli despite the tags on the amino terminal end and the presence of endogenous nucleolin within the nucleoli. This proper localization suggests that the full-length fusion protein has the potential to function normally in vivo. Previous nucleolar localization studies by Peculis and Gall (1992) showed that Xenopus nucleolar protein, NO38, properly localizes to the nucleolus when a single myc epitope was used as the tag.

The full length myc-tagged nucleolin also localized to extranucleolar RNP particles. This simply may be a result of an overexpression of fusion protein within the oocytes. However, our rabbit anti-nucleolin serum, R2D2, also labeled extranucleolar RNP particles within the nuclei of noninjected oocytes (Figure 6C). This extranucleolar nucleolin may be involved either in shuttling (Borer et al., 1989) or perhaps in the processing of RNAs other than ribosomal RNA. The myc-tagged GAR

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**Figure 7.** Double-labeling experiments localized myc-tagged full length and GAR-truncated nucleolin versus endogenous nucleolin. A and D are phase contrast micrographs of nuclear preparations made from oocytes that had been injected with mRNA encoding myc-tagged full-length nucleolin (A) or the myc-tagged GAR truncation (D). (B) The preparation in A was stained with mAb 9E10 and fluorescein-coupled goat anti-mouse IgG to detect the myc-tagged full-length nucleolin fusion protein. The multiple nucleoli and the surrounding RNP material stained. (C) The preparation in A was reprobed with rabbit anti-nucleolin serum, R2D2, and rhodamine-coupled mouse anti-rabbit IgG to localize endogenous and tagged nucleolin. Similar staining patterns in B and C indicated that the myc-tagged fusion properly localized to the nucleoli. As a control for cross-reactivity, a similar preparation (A, top right inset) was stained with mAb 9E10 and fluorescein-coupled goat anti-mouse (B, top right inset) and then with only the rhodamine-coupled secondary antibody, not R2D2 (C, top right inset). No staining was observed in the rhodamine channel. (E) The preparation in D was stained with mAb 9E10 and the fluorescein-coupled anti-mouse IgG. The multiple nucleoli were not stained above the surrounding RNP material. (F) The preparation in G was reprobed with anti-nucleolin serum, R2D2; the nucleoli were brightly stained above the surrounding RNP material. Bar, 20 µm.
truncation also localized to the extranucleolar RNP material (Figure 7H), perhaps by interactions with its four intact RNA-binding domains. Further investigations into the extranucleolar localization of nucleolin within amphibian oocyte nuclei are underway (Rankin and DiMario, unpublished data).

Our myc-tagged nucleolin GAR truncation failed to efficiently localize to the multiple nucleoli despite its translocation into the nuclei and its accumulation within the extranucleolar RNP material (Figure 7H). Failure of the GAR truncation to specifically associate with nucleoli may be because of an absence of direct interactions that normally occur between the GAR domain and nucleolar macromolecules such as rRNA, or perhaps other nucleolar proteins. Alternatively, various other nucleolin domains such as the four RNA-binding domains or the alternating basic and acidic regions may fail to properly interact with nucleolar macromolecules because of improper tertiary configurations that normally occur when the GAR domain is present.

Other proteins that contain GAR-like domains include fibrillarin (referred to as NOP1 in yeast) that associates with nucleolar U3 snRNA and plays a positive role in pre-rRNA processing (Kass et al., 1990), the yeast nucleolar protein GAR 1 that is essential for pre-rRNA processing (Girard et al., 1992), the yeast nucleolar protein SSB-1 (Jong et al., 1987) that associates with small nuclear RNAs snR10 and snR11 (Clark et al., 1990), NSR1 that is the yeast analogue of vertebrate nucleolin (Kondo et al., 1992a,b), and the vertebrate hnRNP proteins A1 and U (Dreyfuss et al., 1993). In addition, the protein product of the fragile X gene, FMR1, has recently been shown to contain a GAR domain (referred to as the RGG box by Siomi et al., 1993). The FMR1 protein binds RNA in vitro, and deletion of its GAR domain removes the capacity of this protein to bind RNA in vitro (Siomi et al., 1993). We have shown that the GAR domain of nucleolin is also necessary for in vitro nucleic acid binding and specific localization of nucleolin within nucleoli. We are currently testing whether nucleolin’s GAR domain can confer RNA-binding and nucleolar localization characteristics on non-RNA-binding, non-nucleolar proteins.

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Note added in proof. After resubmitting our revised manuscript, Schmidt-Zachmann et al. (1993, Cell 74, 493–504) showed that chicken nucleolin that had been truncated for its GAR domain failed to localize to nucleoli in human-mouse heterokaryons. Schmidt-Zachmann and Nigg (1993, J. Cell Sci. 105, 799–806) further showed that when nucleolin’s GAR domain was attached to cytoplasmic pyruvate kinase, the fusion did not localize to nucleoli. This latter result suggests that by itself, the GAR domain does not act as a nucleolar localization signal.

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