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NUCLEIC ACID BINDING CHARACTERIZATION OF THE GAR DOMAIN OF NUCLEOLIN

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List of Abbreviations

cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
DFC	dense fibrillar component
DMA	dimethylarginine
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ETS	external transcribed spacer
FC	fibrillar center
GAR	glycine-arginine rich region
GC	granular component
hnRNP	heterogeneous nuclear ribonucleoprotein
kDa	kilodalton
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
snoRNA	small nucleolar ribonucleic acid
rDNA	genes encoding ribosomal RNA
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
Tris	Tris[hydroxymethyl]aminomethane

Abstract

Nucleolin is a non-ribosomal, nucleolar-specific phosphoprotein that is ribosome synthesis. The carboxy terminal domain of nucleolin is rich in glycine and arginine and as such is called the GAR domain. The GAR domain was tagged with histidine residues and expressed in *Escherichia coli* cells. The polypeptide was purified to homogeneity. RNA corresponding to a specific recognition site in pre-rRNA was synthesized. Nitrocellulose binding assays indicate that the GAR domain of nucleolin binds nucleic acid albeit in a non-specific manner.

Introduction:

The nucleolus is the most visible structure within the nondividing eukaryotic nucleus and functions in the synthesis of ribosomes. The number of nucleoli in a nucleus differs depending on the species and the stage in the cell's reproductive cycle. In an electron microscope, the nucleolus, which is roughly spherical in shape, appears as a mass of densely stained granules and fibers. It is made up of nucleolar organizers (specialized regions of some chromosomes that contain multiple copies of genes for the synthesis of ribosomes) and pre-ribosomal RNA in various stages of production. Assembly of ribosomal subunits (large and small) occurs within nucleoli where pre-rRNA is processed (cut and modified) and where ribosomal proteins associate with newly synthesized rRNA (Dorit *et. al.*, 1991). A cell that is actively growing can produce approximately 10,000 ribosomes per minute (Campbell, 1993).

More specifically, the nucleolus performs the function of biosynthesizing the large and small ribosomal subunits. Ribosomal RNAs, [18S, 5.8S, 28S] and [5S], and about 85 structural proteins are needed for assembly of the ribosomal subunits. The structural proteins are synthesized in the cytoplasm and are translocated into the nucleus and then on to the nucleolus for assembly. RNA polymerase III transcribes the 5S rRNA from multiple copy genes that are found outside the nucleolus. RNA polymerase I transcribes 18S, 5.8S, and 28S rRNAs inside the nucleolus. All three are transcribed in the form of a large pre-ribosomal transcript (47S for mammalian cells). The pre-ribosomal transcript also contains spacer sequences - in front of the 18S region, between the 18S and 5.8S regions, and between the 5.8S and the 28S regions. The 47S pre-RNA transcript is cleaved by exo- and endonucleolytic reactions. These reactions give rise to intermediates before the formation of mature

18S, 5.8S, and 28S rRNAs. Nucleolar-specific proteins and small nucleolar RNAs (snoRNAs) are needed for these reactions. Complexes formed between the snoRNAs and nucleolar-specific proteins are involved in pre-rRNA cleavage and base modifications of the transcript. Posttranscriptionally, specific uridine residues are converted to pseudo-uridine and ribose rings at conserved sites are methylated at the 2' position. The cleavage and modification reactions take place concurrently with the assembly of ribosomal subunits with in-coming ribosomal proteins (reviewed by DiMario, in press). The 18S rRNAs and small subunit specific ribosomal proteins are assembled to form the small ribosomal subunits. The 5.8S, 28S rRNAs, imported 5S rRNA, and large subunit specific ribosomal proteins are assembled to form the large ribosomal subunits. These large and small subunits translocate separately out of the nucleoli, through the nuclear envelope and into the cytoplasm for assembly into a whole ribosome. At this point, the ribosome is ready to translate mRNA sequences into proteins (Hadjiolov, 1985).

A cycle of assembly and disassembly of the nucleolus is closely linked to the cell cycle. The nucleolus disassembles during prophase of mitosis - as the nucleolus begins disassembly, transcription of the pre-ribosomal RNA genes declines. The tight compaction of rDNA into the nucleolar organizer regions of the mitotic chromosomes probably accounts for this decline. During telophase and early interphase of the cell cycle, the nucleolar organizers of the mitotic chromosomes and reutilized nucleolar components give rise to the nucleoli in a process called nucleogenesis (Hadjiolov, 1985).

During interphase, three ultrastructural subdivisions are evident in the nucleolus - the fibrillar center (FC), the surrounding dense fibrillar regions (DFRs), and the peripheral granular regions (GRs) (reviewed by Busch and Smetana, 1970).

The FCs contain ribosomal DNA, but the exact function of the FCs remains arguable. Perhaps their function in interphase is analogous to the function of the nucleolar organizers in mitosis (reviewed by DiMario, in press). Detection of both rDNA and the rRNA within the FCs leads some to argue that transcription occurs on the borders between the FCs and the DFRs (reviewed by DiMario, in press).

Early pre-ribosomal RNA processing and intial subunit assembly occurs in the dense fibrillar regions - which usually surround the FCs. Peripheral to the DFRs are the granular regions. GRs are composed of immature ribosomal subunits. It is in the granular regions that the large and small ribosomal subunits continue to mature (Hadjiolov, 1985).

Several nucleolar specific proteins are involved in the processing of pre-ribosomal RNA. Nucleolin is one of these proteins. Nucleolin is found in abundance in cells where ribosome synthesis is high - rapidly dividing somatic cells (Orick *et. al.*, 1973) and amphibian oocytes (Caizergues-Ferrer *et. al.*, 1989; DiMario *et al.*, 1990). It is believed that nucleolin associates with pre-ribosomal RNA in the DFRs of the nucleolus (reviewed by Olson, 1990; Escande *et. al.*, 1985; Herrera *et al.*, 1986).

Nucleolin was named "protein C23" when it was first recognized as a major nucleolar protein (Orick *et. al.*, 1973). Since that time the name "nucleolin" has gained acceptance. Nucleolin is a non-ribosomal, nucleolar-specific phosphoprotein that appears to be present in all higher eukaryotes. The size of vertebrate nucleolin ranges from 90-110kDa depending on the species. The protein can be divided into three distinct sections. The first segment is a highly charged amino terminal section (residues 1-283). This region is characterized by four highly acidic regions which are separated by basic regions rich in proline. The central region has four RNA-binding

internally repeated sequences of about 90 residues each (residues 284-649). The last segment, the carboxyl terminal region, consists almost exclusively of glycine, dimethylarginine (DMA) and phenylalanine (residues 650-713). It is often referred to as the GAR domain because of its glycine and arginine richness (reviewed by Olson, 1990).

Despite the fact that nucleolin is the most highly characterized nucleolar protein, its exact function in the nucleolus remains uncertain. Several models have been proposed and have been summarized by Olson (1990). Nucleolin's being located primarily in the dense fibrillar region of the nucleolus and its association with newly synthesized pre-rRNA, together suggest that nucleolin is one of the first proteins involved with pre-ribosomal RNA during or after its synthesis. Nucleolin is capable of packaging single-stranded nucleic acids. The presence of large quantities of nucleolin suggests that it functions in protecting newly synthesized RNA from being degraded by nucleases before the RNA can be assembled into ribosomes. In addition to binding single-stranded nucleic acids, nucleolin can catalyze the reassociation of complementary strands of denatured DNA. So, it would seem that nucleolin could also have the ability to rearrange the secondary structure of newly synthesized RNA into its most stable conformation (reviewed by Olson, 1990).

Some other possible functions of nucleolin can be inferred from the character of the N-terminal third of the protein - its highly acidic regions separated by basic regions. Perhaps these acidic regions undergo ionic interactions with ribosomal proteins (which are typically basic). Thus, it would appear that nucleolin might facilitate the temporary gathering or storage of ribosomal proteins as part of the preparation for the assembly of the pre-ribosomal RNA. A second function of the acidic regions could be to interact ionically with histones of nucleolar chromatin.

This interaction could serve to hold in place the nascent RNP complex until it moves to the next phase of pre-RNA processing and ribosomal subunit assembly. Or it could serve to destabalize the rDNA chromatin to aid in transcription (Olson, 1990). Other studies have suggested that nucleolin is necessary to sustain a maximal rate of transcription (Egyhazi *et. al.*, 1988). In this case, it would seem that the assembly system for the ribosome is coordinately regulated. In other words, the assembly system may work by feedback inhibition to prevent a build up of nascent RNA when other components needed for assembly are in short supply. That is, the RNA bound form of nucleolin may serve to inhibit transcription until the entire complex is ready to continue the assembly process (reviewed by Olson, 1990).

The most recent finding is that nucleolin associates with the U3 snoRNP particle that is known to make the first cleavage in the ETS region of pre-rRNA. Thus, the study demonstrated that nucleolin plays a role in the early cleavage of pre-rRNA. The study also suggests that nucleolin association with the pre-rRNA substrate is the limiting step in the primary processing reaction. Associations of nucleolin with other snoRNP particles involved with other cleavage and processing events will likely be discovered in the near future (Ginisty *et al.*, 1998).

In order to characterize the GAR domain of nucleolin in more detail, we (Dr. DiMario's lab) have subcloned the segment of the hamster nucleolin cDNA that encodes this domain. The GAR peptide can be expressed in bacteria with six histidine amino acid residues fused to the N-terminus of the GAR protein. This histidine tag permits rapid affinity purification from bacterial cell lysates. Once purified, we will attempt to cystallize the protein in order to ascertain the structure of nucleolin. In addition, the purified protein will be tested for its RNA binding capacity. The full length protein binds nucleic acid, but nucleolin lacking the GAR

domain (GAR truncation) fails to bind nucleic acid *in vitro* and fails to associate with nucleoli in vivo (Heine et al. 1993). Previous studies conducted in the DiMario lab using Western blotting techniques however, have found that the GAR domain of nucleolin by itself does not bind nucleic acids in vitro. Conversely, Ghisolfi *et al.*, (1992) using solution binding assays found that the GAR domain of nucleolin is "capable of interacting with RNA, and indeed all nucleic acids tested, in an efficient but nonspecific manner." It has been the task of this research project to resolve this apparent contradiction.

Materials and Methods:

Plasmid Construction:

To generate RNA for binding assays, the rDNA encoding the 5' ETS of pre-RNA was cloned into the Bluescript plasmid. This plasmid, designated ETS + BS was used to generate the RNA for the binding assays by T3 run-off transcription. The plasmid was propagated in the *E. coli* strain Xl-1 Blue. A second plasmid was constructed for use in the production of the protein corresponding to the GAR domain of CHO nucleolin. In this case, the DNA fragment was ligated in frame within pET30a plasmid sequences encoding six histidine residues to be used as tags for purification. The recombinant plasmid (pET30a-GAR) was propagated in *E. coli* strain BL21(DE3)LysS.

Peptide production and purification:

The following procedures were based on the protocols described in Novagen Inc.'s manual on the pET system. *E. coli* strain BL21(DE3)LysS with plasmid pET30a-GAR was grown in Laurea broth with 50 ug/ml kanamycin at 37° C. When the culture reached an $O.D._{600}$ of approximately 0.6, isopropyl-thio-β-D-galactoside (IPTG) was added to a final concentration of 1 mM was added. The culture was allowed to shake at 37° C for an additional 3 hours before harvesting the cells. The cells were centrifuged for 10 minutes at 6000 rpm (Sorvall HB4 rotor) and resuspended in Tris/EDTA buffer (50 mM Tris-HCl pH 8.0; 2 mM EDTA). The cells were recentrifuged for 10 minutes at 6000 rpm (Sorvall HB4 rotor). The cells were resuspended in 1 X Binding Buffer at a volume of 1/25 of the cell culture volume. The 1 X Binding Buffer originally contained 5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9. To this buffer, 1 mM PMSF, 0.1% Triton and 7 ug/ml

leupeptin was added. The cells were then lysed via sonification. The solution was ultracentrifuged for 20 minutes at 17,500 rpm (SW41 rotor). The supernatant was filtered through a 0.2 um syringe unit filter.

Next, a 2.5 ml nickel affinity purification column was prepared. The His•Bind resin was obtained from Novagen, Inc. To charge and equilibrate the column, the following steps were taken. The column was washed with 3 volumes of deionized water, 5 volumes of 1 X Charge Buffer (50 mM $NiSO_4$), and 3 volumes of 1 X Binding Buffer. The crude *E. coli* extract containing the GAR domain was loaded onto the column. The column was then washed with 10 volumes of 1 X Binding Buffer followed by 6 volumes of 1 X Wash Buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The bound protein was then eluted from the column with 6 volumes of 1 X Elute Buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). After elution, the column was stripped with 3 volumes of 1 X Strip Buffer (100 mM EDTA, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and recharged as described above for reuse.

Fractions eluted from the column were dialyzed twice against 500 mM NaCl (important for keeping the GAR peptide in solution), 10 mM Tris, and 1 mM EDTA in dialysis tubing with a molecular weight cut-off of 6-8 K Da. The protein solution still in its dialysis tubing was then placed in a weigh tray with dry polyvinyl pyrrolidole to concentrate the protein solution down to a volume of 0.5 – 1.0 ml. Lastly, a modified Lowry procedure was conducted according to Markwell *et al.*,(1978) to determine the concentration of the protein solutions.

***In–vitro* transcription:**

E. coli strain Xl-1 Blue harboring plasmid ETS + BS was grown in Laurea broth containing 50 ug/ml of ampicillin. The culture was incubated on a shaker overnight at 37° C. The following procedures are based on the manual for the E.Z.N.A. Plasmid Miniprep Kit produced by Omega Biotek. One and one half ml of the culture was transferred to a 1.5 ml Eppendorf tube and centrifuged at 10,000 x g for 1 minute at room temperature. The supernatant was poured off and this procedure was repeated until 5 ml of the culture had been pelleted. Two-hundred-fifty uL of Solution I/Rnase A (was added to the pellet. The cells were resuspended thoroughly by vortexing. Two-hundred-fifty uL of Solution II was added and the tube was gently mixed until a clear lysate was obtained. Three-hundred-fifty uL of Solution III was added and the tube was gently mixed until a flocculent white precipitate had formed. The solution was centrifuged at 10,000 x g for 10 minutes at room temperature. The supernatant was transferred to a clean HiBind minicolumn attached to a 2 ml collection tube. The assembly was centrifuged at 10,000 x g for 1 minute at room temperature to pass the lysate through the column. The liquid in the collection tube was discarded and the column was washed with 750 uL of Wash Buffer diluted with ethanol. The assembly was centrifuged as described above to pass the buffer through the column. The liquid in the collection tube was discarded and the empty column was again centrifuged as above to dry it. The column was detached from the colllection tube and placed in a clean 1.5 ml Eppendorf tube. Fifty uL of deionized water was added to the column. This assembly was centrifuged as above to elute the DNA.

The plasmid was linearized with the restriction enzyme *Bst* X1. This site is downstream of the rDNA insert, and linearizing the plasmid here allows run-off T3 *in*

vitro transcription. An aliquot of the DNA was run on a 1.0% agarose mini-gel to check for complete digestion. The remaining DNA was extracted in an 1.5 ml Eppendorf tube with equal volumes of phenol, phenol-chloroform, and chloroform-isoamyl alcohol. Next, 1/10 volumes of 3 M Na^+ Acetate, pH 7 along with 2 volumes of 100% EtOH were used to precipitate the linearized DNA. This solution was placed on dry ice for 5 minutes. The sample was centrifuged at 15,000 x g for 15 minutes at 4° C. The supernatant was carefully removed and the pellet was gently washed with 70% EtOH. The tube was dried out in a 37° C water bath. The DNA pellet was dissolved in 20 uL DEPC treated water and stored at –20° C. An aliquot of the DNA sample was as read at 260 and 280nm in a spectrophotometer to determine purity and concentration.

RNA was synthesized using an Ambion T3 mMessage Machine Kit. The following procedures are based on the Ambion Manual. The following were placed into a 1.5 ml Eppendorf tube: nuclease free deionized water needed to bring final volume to 20 uL, 2 uL 10 X Rxn Buffer (salts, buffer, dithiothreitol), 10 uL 2X Ribonucleotide Mix (10mM ATP, CTP, UTP; 2mM GTP and 8mM Cap Analog), 1 ug template DNA, and 2 uL 10X Enzyme Mix (50% glycerol solution with T3 RNA polymerase, placental RNase inhibitor) . This solution was mixed by pipetting up and down and incubated in a 37° C water bath for 3 hours. Next, 1 uL of RNase free DNase I was added to the solution to digest the DNA template. The solution was vortexed, centrifuged and then incubated in a 37° C water bath for 15 minutes. To purify the RNA, 115 uL of DEPC treated water and 15 uL of Transcription Stop solution (5 M ammonium acetate and 0.1 M EDTA) was added. The solution was vortexed, centrifuged, and extracted with equal volumes of phenol-chloroform and chloroform-isoamyl alcohol. To precipitate the RNA, 1 volume of isopropanol was

added and the solution was chilled at –20° C for 15 minutes. The tube was centrifuged for 15 minutes at 4° C. The supernatant was carefully removed and the pellet was resuspended in 20 uL of DEPC treated water.

An aliquot of the RNA was run on an agarose/formaldehyde gel to check the purity and abundance of the transcript. The 5 ul aliquot of the RNA solution mixed with 5 uL water, 5 uL 4X Sample Buffer (10X MOPS and 37% HCHO at a 4:6 ratio), and 10 uL formamide. The tube was sealed and heated at 65° C for 5 minutes. Five uL of "gel juice" (20% Fricol,0.2% bromophenol blue, 0.2% xylene cyanol FF, 100 mM EDTA, pH 8.0) was added to the solution. To prepare the gel, 1.5 g of agarose was melted in 75 ml water, 10 ml 10 X MOPS (200 mM MOPS, 50 mM NaOAc, 10 mM EDTA) and 16 ml 37% HCHO. The solution was allowed to cool to ~70° C in the hood and then poured. The gel was run at 100 V for ~ 2 hours. To stain the gel, the gel was first washed 2 twice in 250 mls of 0.5 M ammonium acetate for 20 minutes each. The gel was stained with 13 uL of 10 mg/ml ethidium bromide in 250 ml of 0.5 M ammonium acetate for 40 minutes. The gel was finally destained in 0.5 M ammonium acetate for 20 minutes.

To synthesize the radiolabelled RNA, the 2X Ribonucleotide Mix was replaced with 7.5 mM each of ATP, GTP, UTP, and [α-^{32}P]-CTP (25 Ci/mmole). The same protocol mentioned previously for the synthesis reaction and termination of synthesis was followed. Labelled RNA was precipitated as described above. The RNA transcript was resuspended in DEPC treated water. The RNA was quantified after being counted via scintillation counting.

The following is an example calculation:

RNA solution had counts of 4,095,000 cpm/uL

Specific Activity of [α-^{32}P]-CTP = 25 Ci/mmole

There are 2 X 10^{12} cpm/Ci, therefore 25 Ci/mmole = 5 X 10^{13} cpm/mmole

(cpm/uL of RNA solution)(uL RNA solution) ÷ (cpm/mmole of [α-^{32}P]-CTP)

= mmole of [α-^{32}P]-CTP incorporated

(4.095 X 10^{6} cpm/uL)(20uL) ÷ 5 X 10^{13} cpm/mmole

= 1.638 X 10^{-6} mmole of [α-^{32}P]-CTP incorporated

1.638 X 10^{-6} mmole of [α-^{32}P]-CTPs ÷ 484 CTPs within the strand of synthesized ETS RNA

= 3.38 X 10^{-9} mmol strands RNA

3.38 X 10^{-9} mmol strands RNA ÷ 20 uL RNA solution =

16.9 uM strands RNA

Filter-binding assays

RNA transcripts (0.1nM) were incubated with 0-5 uM of the purified GAR protein in a buffer for thirty minutes at room temperatuure. This buffer was composed of 20 mM Tris/HCL, pH 7.4, 4 mM $MgCl_2$, 0.2 M KCl, with 20% glycerol, 1 mM dithiothreitol, 0.5 mg/ml tRNA, 4 ug/ml bovine serum albumin). After the thirty minutes had elapsed, the RNA/peptide solutions were filtered through wet nitrocellulse membranes (pore size = 0.45 uM) under gentle suction. The filters were washed with the buffer and dried in a 65° C oven. Scintillation counting was used to determine the amount of ^{32}P retained on the filters.

Results and Discussion:

The amino acid sequence of the C-terminus of nucleolin is shown in Figure 1. This portion of the protein is designated the GAR domain for its arginine and glycine richness.

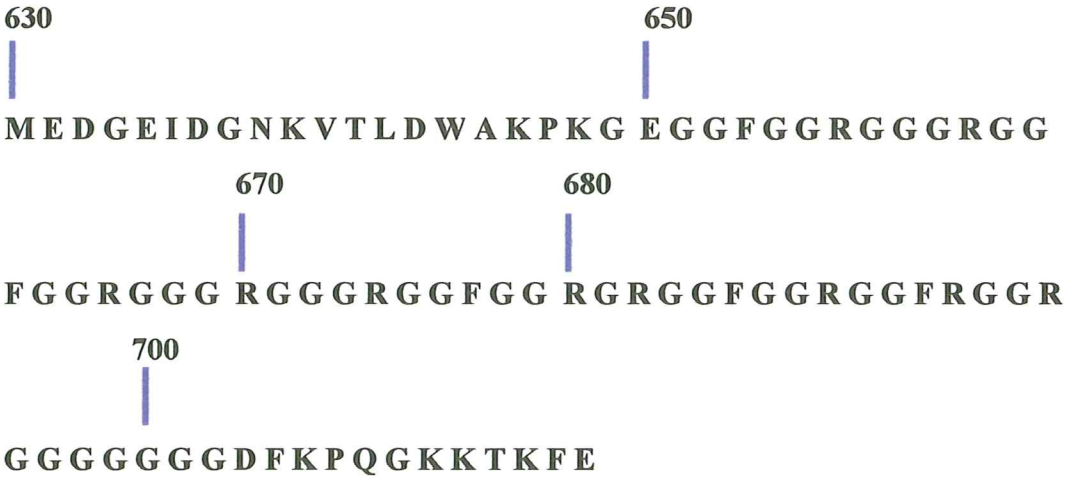

Figure 1. Wild Type CHO GAR Domain Begins at Residue 651 and Ends at 702.

In order to study the RNA-binding capacity of the GAR domain of nucleolin, it was first necessary to synthesize the polypetide. The protein was made using an *E. coli* expression vector and purified (Figure 2).

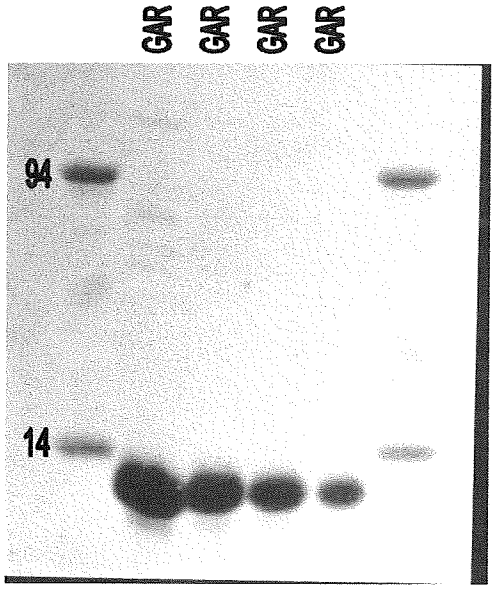

Figure 2. Analysis of the GAR polypeptide by 13% SDS/PAGE, stained with Coomassie Brilliant Blue. Molecular weight markers in kDa are indicated on the right.

To synthesize the RNA for the binding studies, the rDNA encoding the 5' ETS of pre-RNA was cloned in Bluescript plasmid. To verify that digestion of the plasmid with *Bst*X1 was complete, the DNA was run on a 1% agarose gel (Figure 3). The transcript of this insert is shown in Figure 4.

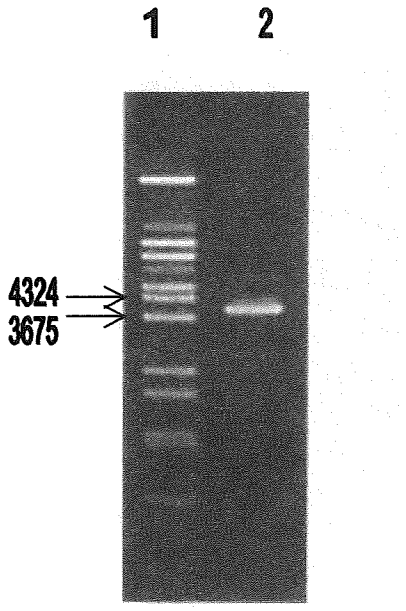

Figure 3. Ethidium stained agarose gel showing linearized ETS+BS. Lane 1 contains markers of lambda DNA digested with *Bst* –EII. The number of base pairs is indicated on the right. Lane 2 contains the digest of the rDNA encoding the 5' ETS of pre-RNA cloned in Bluescript plasmid.

10	20	30	40	50	60
1 agggaacaaa	agcuggguac	cgggcccccc	cucgaggucg	acgguaucga	uaagcuugau
61 aucgaauucC	UGCAGCCCCA	CCGGGAGUUC	CAGGAGCUCG	GGCAGGGGGA	GCCGGCUCGU
121 CCCCCGGCAC	CGGAGGUCCC	CGGGCCCUUU	GGCGCCCGUU	UUUUCGCAAA	GUGCGGCGCC
181 CGCGGGGACU	UGCUCGGCCG	GGCCGGGCCC	CGGCGGCCCC	GGGGCCCCGG	GGCCCUCCCG
241 CGGAGGCCCC	GAUGAGGACG	GAUUCGCCCG	GCCCGCCCCG	GCCGGAGUUC	CGGGAGCCCG
301 GGGAGAGGAG	CCGGCGGCCC	GGCCUCUCGG	GCCCCCCGCA	CGACGCCUCC	AUGCUACGCU
361 UUUUUGGCAU	GUGCGGGCAG	GAAGGUAGGG	GAAGACCGGC	CCUCGGCGCG	ACGGGCGCCC
421 GAAAAAAGGA	CCGGGGCGUU	UCCCGCCUCG	GUCCCCGGUC	UGGGAAGGCU	CCGCGGUCGA
481 GUCUCGCUCC	CCGGCCCGAU	CGAUCUGGCA	ACCCGCGCCC	GGGCGGGGAG	GGCCCUCUGC
541 CCGGCCGACC	CCCCGGCGGG	GCGGCCGCUA	CACGGGCAGG	GAGGCUCCCU	CCCGCCUCGC
601 CCCGGGCGCG	ACCCCCGGAC	CCCCCCCCGG	UCCGGUCCGC	CGCCGGGCCC	ACCCCCCCGG
661 CCGCCCCGGC	GGCCGGGGGG	CGCCCGCGCC	CCCCCCGUGA	GUUCCCCCCG	CACCGUCCGA
721 CCCCGGCAGG	CGCCCGAAGA	AGGCCCGGAC	AGGCGGGGAG	CCCGCCCCGG	GGGACCCCGC
781 UUCCCCCGGC	CGCGGACUCC	UCUCCCCUGG	CCCGGGCCGA	GCACCCCGUU	UCGCCCGACA
841 CCCGCAGAGC	GAGAGAGAAA	GACGGAAAGA	AAGGAGAGUA	GGCCGCGGGC	CCCGUCCCGG
901 CCGCCGCCUC	CCCCCCCCCU	CCCCGGGGGG	GGGGAGCGGC	AGGCCGGGCG	GGGCCCCCGG
961 CCCGGACGGG	AGGGCCCGGG	CGCCGGGAGC	GCCGCCGAGG	GGACGGGCCC	GGGUGACGCC
1021UCAGGGCGCC	GACCCGCCGC	CCCCCCCCCC	CGGCCGCCCC	CGCGCCCGCC	CGCCCGCGCC
1081GGGCCCGGGA	AAGGUGGCUA	CCUGGUUGAU	CCUGCCAGUA	GCAUAcuaga	gcggccgcca
1141 ccgcg					

Figure 4. Conceptual transcription of RNA used in the RNA-GAR binding assay. Actual ETS are in blue font. Non-transcribed promotor sequences are in red font. 18S rRNA fragment is in green font. Black font is BS+(KS) polylinker from T3 start to the *Bst*X1 site. The total number of bases are as follows : A (134); U (106); C(484); G(421).

RNA transcript was made using the T3 Ambion Message Machine Kit and run on a formaldehyde gel to assess purity and abundance (Figure 5)

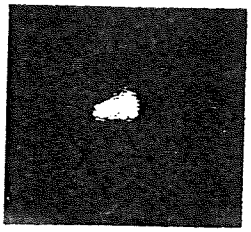

Figure 5. Formaldehyde gel of the RNA transcript

The cpm of the radiolabelled RNA transcript was determined using scintillation counting. The results are shown in Figures 6 and 7.

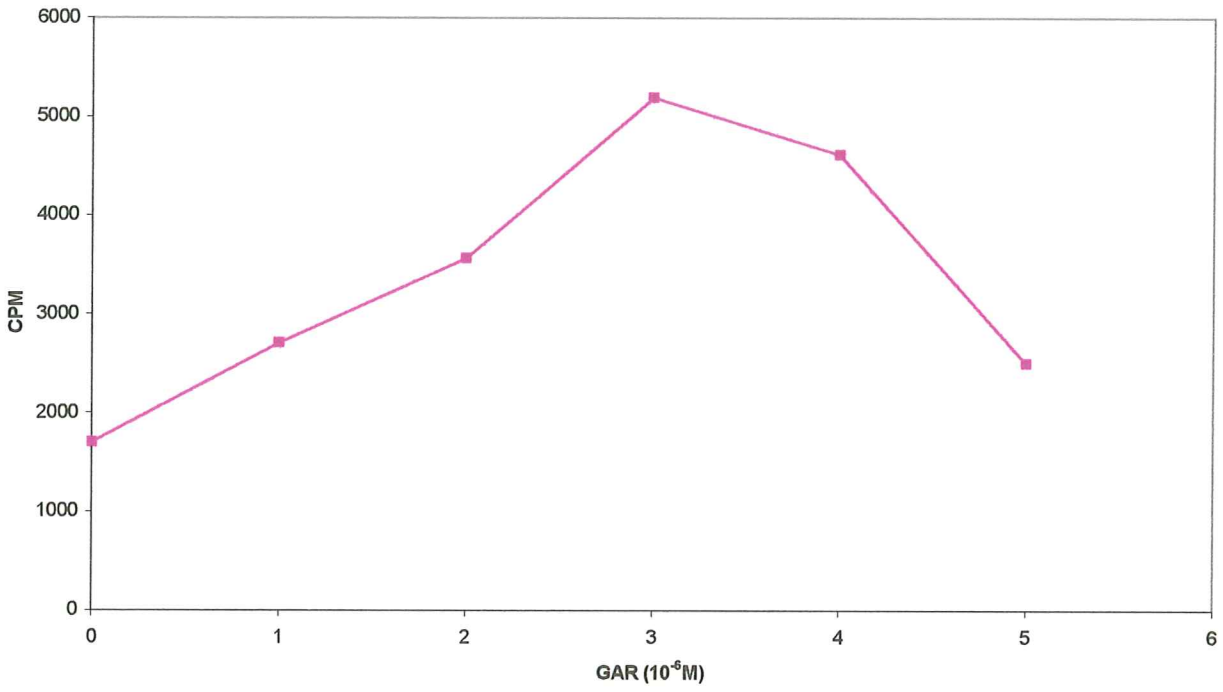

Figure 6. Trial 1

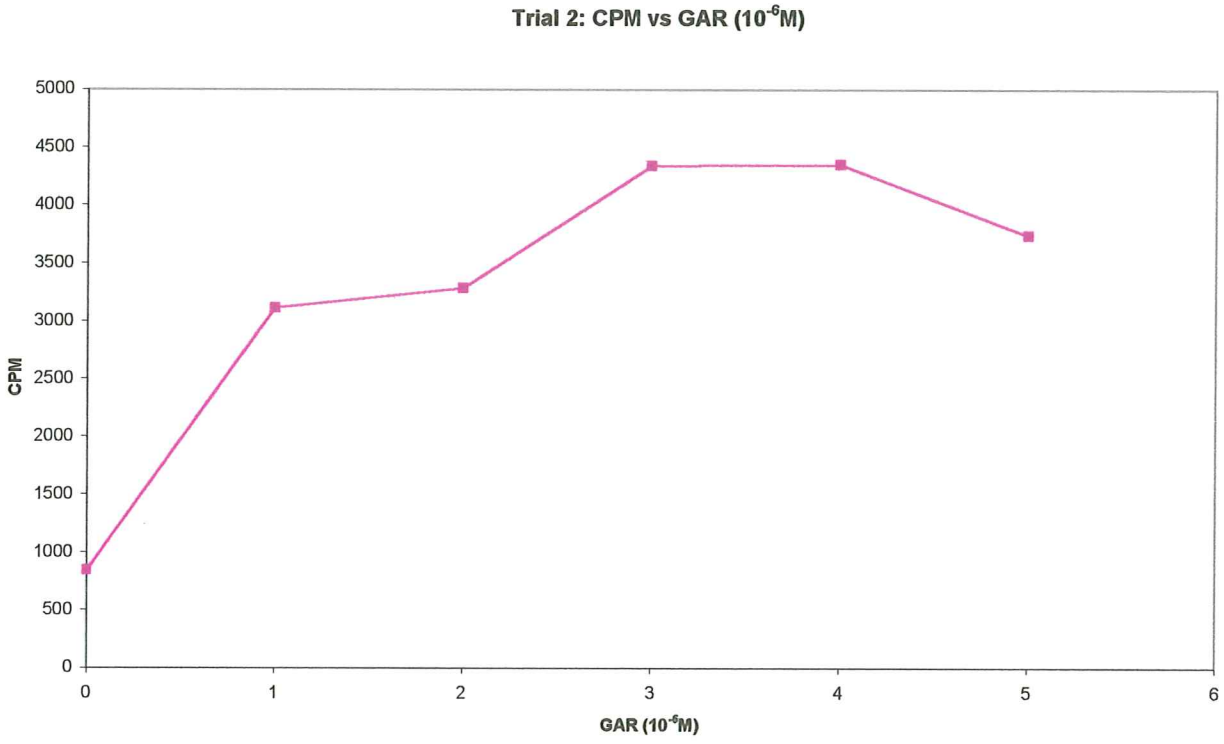

Figure 7. Trial 2.

Both graphs indicate that the GAR domain of nucleolin seems to bind RNA - the cpm increase with increasing concentrations of GAR in the incubation mixture. The graphs also indicate that RNA has maximal binding with the GAR domain of nucleolin at 3 uM. The apparent association constant was calculated to be $1.5 \times 10^6 M^{-1}$. This constant was calculated by estimating the concentration of free protein when the RNA was half saturated with GAR (Gregory *et al*, 1988). There is a plausible explanation for the apparent decrease in binding activity at concentrations greater than 3 uM. Studies have shown that the carboxy terminal domain of the protein, A1, is solely responsible for the aggregation of A1 in solution via A1:A1 protein:protein interactions. In addition, this domain is responsible for associations between A1 molecules bound to nucleic acid (Cobianci *et al.*, 1988; Kumar *et al.*, 1990; Nadler *et al.*, 1991; Casas-Finet *et al.*, 1993). The carboxyl terminus of A1 is glycine-rich and is interspersed with tyrosine and phenylalanine residues (Biamonti *et al.*, 1994). This domain also contains a 36 residue sequence that is analogous to a "RGG box," which is considered a minimal RNA-binding domain (Dreyfuss *et al.*, 1993). This carboxyl terminal domain is believed to exist as a random coil. It is the random coil that provides the binding energy for protein:protein interactions (Pontius *et al.*, 1990). The glycine-rich domain of A1, is similar to the GAR domain of nucleolin (Portman *et al.*, 1994). The fact that A1 is able to form homo-complexes via its glycine-rich domain (Cartegni *et al.*, 1996) introduces the possibility of similar behavior in the GAR domain of nucleolin. Perhaps at concentrations above 3 uM, the GAR polypeptides interact preferentially with themselves.

Thus, it would appear that the results of this project agree somewhat with the results of Ghisolfi *et al.* (1992). However, in that study, the RNA-GAR associations did not decrease at concentrations greater than 3 uM. The results of this project

would seem to contradict the results of previous studies in the DiMario lab on the binding capacity of the GAR domain. It would be interesting to try the Western Blot technique using a series of GAR concentrations – if the concentration used was too high, this could account for the lab's findings that GAR does not bind RNA. Also, in this project, a single point control (5 uM) of BSA was run. The RNA retained on the filters far exceeded the RNA retained retained using 5 uM of GAR (22,316 cpm vs. 2,496 cpm). Thus, while GAR does bind RNA, it appears to be non-specific. It would be interesting to conduct additional control assays using BSA at concentrations of 1-5 uM. These results would lend credit to the claim that GAR's binding is non-specific.

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