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GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast

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Among the few proteins of the eukaryotic nucleolus that have been characterized, four proteins, nucleolin, fibrillarin, SSB1 and NSR1, possess a common structural motif, the GAR domain, which is rich in glycine and arginine residues. In order to examine whether the presence of this domain is characteristic of a family of nucleolar proteins, we investigated whether other yeast genes encode proteins containing GAR domains. We report here the sequence and the characterization of a new yeast gene, *GAR1*, which encodes a protein of 205 residues containing two GAR domains. GAR1 is a non-ribosomal protein, localized in the yeast nucleolus, which is essential for cell growth. Immunoprecipitation with anti-GAR1 antibodies shows that GAR1 is associated with a subset of snoRNAs, including snR10 and snR30. Depletion of GAR1 by expression under the control of a regulated *GAL* promoter, impairs processing of the 35S primary transcript of pre-rRNA and prevents synthesis of 18S rRNA. GAR1 is thus the fifth member of a family of nucleolar proteins containing GAR domains, and is involved in rRNA metabolism.

Key words: GAR domain/nucleolus/pre-rRNA processing/*Saccharomyces cerevisiae*/snRNA

Introduction

In eukaryotic cells, most of the steps of ribosome biogenesis take place in the nucleolus, a large structure located within the interphase nucleus (for reviews, see Hadjiolov, 1985 and Warner, 1990). In addition to the pre-rRNA and some of the ribosomal proteins, the nucleolus contains a set of proteins and RNAs that are involved in ribosome biogenesis but that are not part of mature cytoplasmic ribosomes. This set of 'non-ribosomal components' can be roughly divided into (i) factors involved in rDNA transcription (polymerases and transcription factors), (ii) those involved in maturation of pre-rRNA (methylases, snoRNPs and maturases), (iii) those involved in assembly of the pre-ribosomes, (iv) those involved in the shuttling of components back and forth between cytoplasm and nucleus and (v) those involved in all the other steps that lead to a functional nucleolus; i.e. assembly of the nucleolus, interactions between the nucleolus, the nuclear matrix and the nuclear membrane, and the dynamic rearrangement of the whole structure during the cell cycle. Most studies have been devoted to the transcription of the rDNA genes and, more recently, to the maturation of the transcript (reviewed by Reeder, 1990 and by Sollner-Webb and Mougey, 1991), while little is known about the other steps.

Some of the major components of the nucleolus have been characterized and their sequences established by cloning and sequencing of the corresponding genes or cDNAs. It is striking that the most homologous region between four nucleolar proteins: nucleolin (Lapeyre *et al.*, 1987; Caizergues-Ferrer *et al.*, 1989), fibrillarin (Lapeyre *et al.*, 1990; Ochs *et al.*, 1985; Schimmang *et al.*, 1989), SSB1 (Jong *et al.*, 1987) and NSR1 (Lee *et al.*, 1991), is a long domain very rich in glycine, arginine and, to a lesser extent, phenylalanine residues that we will refer to as the GAR domain (for Glycine Arginine Rich). In nucleolin and fibrillarin, most of the arginines of the GAR domain are dimethylated (Lapeyre *et al.*, 1986; Lischwe *et al.*, 1985a,b); this has not been assessed for other proteins containing GAR domains (GAR proteins). Based both on global amino acid composition and internal repeated motifs, a consensus structure for nucleolar GAR domains can be derived (see Discussion).

Nucleolin and NSR1 both contain consensus RNA recognition motifs (also termed RNA binding domains) (Figure 9A; see Dreyfuss *et al.*, 1988; Mattaj, 1989; Kenan *et al.*, 1991, for reviews) and nucleolin has been shown to interact directly with the pre-rRNA (Bugler *et al.*, 1987; F.Amalric, unpublished). In addition, nucleolin shuttles back and forth between nucleus and cytoplasm (Borer *et al.*, 1989) while NSR1 binds to nuclear localization signal peptide conjugates *in vitro* (Lee and Mélèse, 1989). These results would be consistent with roles for these proteins in the transport of ribosomal components.

The two other nucleolar GAR proteins, fibrillarin (designated NOP1 in yeast) and SSB1, also contain sequences resembling consensus RNA recognition motifs and are found associated with small nucleolar RNAs (snoRNAs). In vertebrates, the association of fibrillarin with U3, U8 and U13 has been demonstrated using sera from patients with auto-immune diseases (Lischwe *et al.*, 1985a; Tyc and Steitz, 1989), although no direct binding has yet been shown. In yeast, NOP1 is associated with the 11 yeast snoRNAs characterized to date (Schimmang *et al.*, 1989; Henriquez *et al.*, 1990) and is required for pre-rRNA processing (Tollervey *et al.*, 1991). Fibrillarin is highly conserved in evolution (Lapeyre *et al.*, 1990; Jansen *et al.*, 1991; Aris and Blobel, 1991a), and human or *Xenopus* fibrillarin can functionally replace yeast NOP1 (Jansen *et al.*, 1991). In initial experiments, U14 (previously snR128) and U3 (previously snR17) were precipitated with low efficiency by anti-NOP1 sera, possibly because of epitope masking (Hughes *et al.*, 1987; Schimmang *et al.*, 1989). However, in the buffer conditions used here (see Materials and methods), U3 and U14 are immunoprecipitated with efficiencies similar to that of other snoRNAs (D.Tollervey,

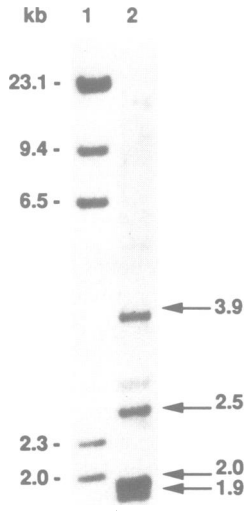
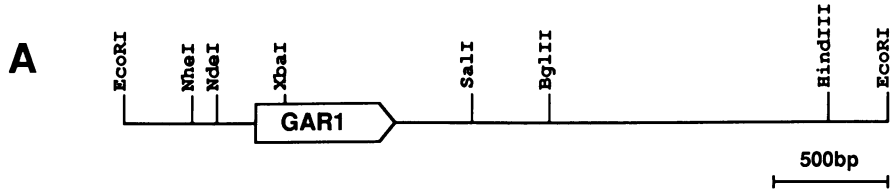


Fig. 1. Southern analysis of yeast genomic DNA. 10 μg of total yeast genomic DNA were digested with *Eco*RI and the fragments were separated by electrophoresis on a 1% TAE−agarose gel prior to transfer under vacuum onto charged nylon membrane. The filter was hybridized at low stringency with a ^{32}P-labelled fragment of cDNA encoding the GAR domain of *Xenopus* fibrillarin as a probe. Lane 1, radioactive molecular weight standards, with the sizes indicated on the left. Lane 2, genomic DNA digestion. Four fragments give strong signals that are indicated by arrows, the sizes being shown on the right.

unpublished). SSB1 has been shown to be associated relatively strongly with snR10 and more weakly with other snoRNAs (Jong *et al.*, 1987; Clark *et al.*, 1990). The snoRNAs are believed to be involved in rRNA maturation, and this has now been demonstrated for U14 (Li *et al.*, 1990; Jarmolowski *et al.*, 1990) and U3 (Kass *et al.*, 1990; Savino *et al.*, 1990; Hughes and Ares, 1991) which are essential in yeast (Hughes *et al.*, 1987; Zagorski *et al.*, 1988). In addition, the deletion of the non-essential snR10 leads to a severe defect in rRNA processing (Tollervey and Guthrie, 1985; Tollervey, 1987). U3, U14 and snR10, as well as several non-essential yeast snoRNAs, have been shown to be associated with pre-rRNA species (Tollervey, 1987; Tyc and Steitz, 1989). An additional snoRNA, snR30, has been shown to be essential for cell viability (Bally *et al.*, 1988; D.Tollervey, unpublished) but has not been further characterized.

In view of the low number of nucleolar proteins so far characterized, we have attempted to isolate additional species. In particular, we investigated whether other nucleolar proteins containing a GAR domain exist in yeast. Using a cDNA probe corresponding to the GAR domain of *Xenopus* fibrillarin, we isolated three different genes which encode proteins containing GAR domains; two of them were found to correspond to the previously characterized genes *NOP1* (fibrillarin) and *SSB1*, while the third appeared to be a new gene that we called *GAR1*.

We report here the sequence and the characterization of the *GAR1* gene which encodes a protein containing two GAR



B

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-355 GCTAGCAAGGACATATACGATTGACATTTTTGAATGTACTTTCAAATACTTGTGAATCAAACTAAGATATGATTCCTGCATATTCAGGTATATACTATATATTTTAATTTCATTAGCAT

-236 ATGTGTCATACGGTTTTTAAAACTTATGAGCAGGAAAAAGGT[GTCACGTG]ATCAGATGGTCTTTTTTTCAAACTTTGACGTCGGAATTTTTTGTAGTGAAAAATTTTTCGAGATGAG

-119 GAAAGGAAGAAAAAATTAATTTGTTAGCCTTATAACGTGAGCACCTTTTCGATAGAGTATCTGATTGCTCTCTTCTACCAAAAGATCACCTGAGGATAATTTTTAACCAAAAGAAAATA

   1 ATG AGT[TTC AGA GGA GGT AAT AGA GGT GGC CGT GGC GGC TTC CGT GGC GGC TTT CGT GGT GGA CGT]ACA GGC AGT GCT AGA TCA TTT CAA
 (1) M  S [F  R  G  G  N  R  G  G  R  G  G  F  R  G  G  F  R  G  G  R ]T  G  S  A  R  S  F  Q

  91 CAA GGA CCA CCA GAC ACT GTT CTA GAA ATG GGA GCT TTT CTA CAT CCA TGT GAG GGT GAT ATT GTT TGC CGT TCT ATC AAC ACT AAG ATT
(31) Q  G  P  P  D  T  V  L  E  M  G  A  F  L  H  P  C  E  G  D  I  V  C  R  S  I  N  T  K  I

 181 CCA TAT TTC AAT GCG CCA ATA TAT TTG GAA AAC AAG ACT CAA GTC GGT AAA GTT GAC GAA ATA TTG GGT CCA TTA AAC GAA GTA TTT TTC
(61) P  Y  F  N  A  P  I  Y  L  E  N  K  T  Q  V  G  K  V  D  E  I  L  G  P  L  N  E  V  F  F

 271 ACT ATC AAG TGT GGT GAC GGT GTC CAA GCA ACC AGT TTT AAA GAA GGT GAT AAA TTC TAC ATT GCC GCT GAC AAA CTA TTG CCT ATT GAA
(91) T  I  K  C  G  D  G  V  Q  A  T  S  F  K  E  G  D  K  F  Y  I  A  A  D  K  L  L  P  I  E

 361 AGA TTT TTA CCT AAG CCT AAG GTG GTA GGC CCA CCA AAA CCA AAA AAC AAG AAG AAG AGA AGT GGT GCC CCA[GGT GGC CGT GGT GGT GCT
(121) R  F  L  P  K  P  K  V  V  G  P  P  K  P  K  N  K  K  K  R  S  G  A  P[ G  G  R  G  G  A

 451 TCA ATG GGT CGC GGT GGT AGC AGA GGA GGT TTC AGA GGA GGC CGT GGT GGT AGT TCT TTC AGA GGA GGC CGT GGT GGC AGT TCT TTC AGA
(151) S  M  G  R  G  G  S  R  G  G  F  R  G  G  R  G  G  S  S  F  R  G  G  R  G  G  S  S  F  R

 541 GGT GGA TCT CGT GGC GGA TCA TTT AGA GGA GGA TCT CGT GGC GGA TCT CGT GGT GGT TTC AGA GGA GGT AGA AGA[ TAA TCTAGCTAGAAAAA
(181) G  G  S  R  G  G  S  F  R  G  G  S  R  G  G  S  R  G  G  F  R  G  G  R  R[ -

 633 AATAAAAAAAAAAACCATCCTTTATCTTTCACAATTCTTCATTTCTTCCAACTTACTATATCTGCCGAAGCACGTCTAACGTCGAAAAAAAAAAAAGAAATTACCGTTTGTTCTGTAT

 752 ACTATATATATATATTGTTGATTGATTACCTGTAATAAATGATTAAATGAAAAAGAGAAATTCAGTAATACCAGAAGCCGCCGTAGTTAAACAAACCACCACGGTTCTTACGGTTG

 871 TCTTAATATTATATCCATACAAAGTTTACGAATGGAACACTATAAATAAAACTTTTCTTTTCTTTGTCCATCTCAGGTTTATGCTCCCATTCAGTTTCTTCCTTAATACCCCTTTGCAA

 990 TCTTTTCAACTTGAGGGTAAATTGTGGACCCAGTTCTTGCAAACCAACCCTTTCATTATCTTTGAAGACATACCGATGCCTTCTGAAGAAAATGTAATCTCTTTGATTATGTAGGGTGA

1109 TAACTTGTCGAC
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Fig. 2. The *GAR1* gene encodes a 205 residue polypeptide. **A:** Physical map of the 3.9 kb *Eco*RI insert containing the *GAR1* gene. The positions of the main restriction sites are indicated, as well as the position of the *GAR1* ORF. **B:** Sequence of the *Nhe*I−*Sal*I fragment of 1475 nt containing the *GAR1* gene. Number 1 refers to the position of the putative initiation codon, which is followed by an ORF of 615 nt. The amino acid translation is given below the nucleotide sequence. The two GAR domains are boxed, the small one starting at nt +7 and the large one at nt +433. These two domains (20 and 61 residues) represent nearly 40% of the protein. Within the promoter region, a CDE1 consensus sequence is boxed at nt −187. This sequence of the *GAR1* gene has been deposited in the EMBL nucleotide sequence database under the accession number X63617.

domains. The GAR1 protein is localized exclusively in the nucleolus and is thus the fifth member of the family of nucleolar proteins containing GAR domains.

Results

Cloning of the genes encoding proteins of the GAR family

In order to identify new genes encoding proteins containing GAR domains in *Saccharomyces cerevisiae*, we used a fragment of cDNA from *Xenopus* fibrillarin corresponding to the GAR domain of this protein (Lapeyre *et al.*, 1990) to probe Southern transfers of total genomic DNA from strain YNN281, digested with either *Eco*RI or *Hin*dIII. Hybridizations were made under various conditions of stringency in order to obtain a small number of hybridizing bands with good signal to noise ratios. In the optimized conditions, four strong hybridization signals are detected, corresponding to *Eco*RI fragments of 3.9, 2.5, 2.0 and 1.9 kb (Figure 1, lane 2), as well as a weak signal from a fragment of 2.7 kb. The signals for the fragments at 2.7 and 2.0 kb are probably due to non-specific hybridization to fragments of the repeated rDNA. Preparative agarose gel electrophoresis was performed with digested genomic DNA. Fragments corresponding to the size of those giving signals in the Southern hybridization were cut out of the gel, purified and used separately to construct partial genomic libraries in λ ZAPII (Stratagene). Recombinant clones were isolated for each fragment; the fragments of 1.9 and 2.5 kb correspond to the genes *SSB1* (Jong *et al.*, 1987) and *NOP1* (Schimmang *et al.*, 1989; Henriquez *et al.*, 1990) respectively, while the 3.9 kb *Eco*RI fragment corresponds to a previously unidentified gene, encoding a protein containing two GAR domains.

The 3.9 kb EcoRI fragment contains the GAR1 gene, which is essential for cell viability

The 3.9 kb *Eco*RI insert was mapped (Figure 2A) and the gene was localized within the fragment by hybridization with the *Xenopus* fibrillarin probe. Sequence analysis reveals the presence of a 615 bp open reading frame that could encode a protein of 205 residues (Figure 2B), with a predicted molecular weight of 21.5 kDa and an extremely basic pI of ~12, due to the presence of 19% basic residues. The protein contains two GAR domains, a short one located at the N-terminus and a long one located at the C-terminus, resulting in a high content of glycine (>24%) and arginine (>12%) residues. A secondary structure analysis (Chou and Fasman, 1979) reveals that the two GAR domains are prone to forming many β-turns, due to the numerous doublets of glycine.

Northern blot analysis using poly(A)$^+$ RNA prepared from *S.cerevisiae* and a *Xba*I−*Bgl*II fragment of the isolated insert as a probe, detects a single transcript of 0.8 kb (data not shown). This size is consistent with the predicted ORF of 615 nucleotides and the position of a putative TATA box at −85 (relative to the ATG), assuming a poly(A) tail of ~100 residues. Curiously, the higher eukaryotic consensus signal for polyadenylation (AATAAA) is found three times at positions 633, 787 and 915, while none of the consensus signals described for *S.cerevisiae* (Irniger *et al.*, 1991) are detected within the 500 nucleotides downstream from the stop codon (Figure 2B). A CDE1 box, which matches perfectly with the consensus, was identified at position −207. This sequence, which is found in centromeres of *S.cerevisiae*, is also present in several yeast promoters and is recognized by the factor CPF1 (Mellor *et al.*, 1990).

To perform the gene disruption experiment, we constructed a mutation *in vitro* which replaces most of the *GAR1* gene promoter and part of the coding sequence with the selective marker *URA3* (see Materials and methods). The partially deleted *gar1::URA3* gene was then inserted at the *GAR1* locus in a diploid strain by homologous recombination. Correct integration at the *GAR1* locus was verified by Southern analysis (Figure 3A). The non-transformed strain yields the intact 3.9 kb *Eco*RI fragment, while three independent transformants yield the 3.9 kb fragment and a 4.6 kb fragment corresponding to the predicted size for a recombinant chromosome containing the *gar1::URA3* gene, instead of the normal *GAR1* copy. Diploid cells were viable, showing the mutation to be recessive, while haploid progeny containing only the disrupted *GAR1* gene

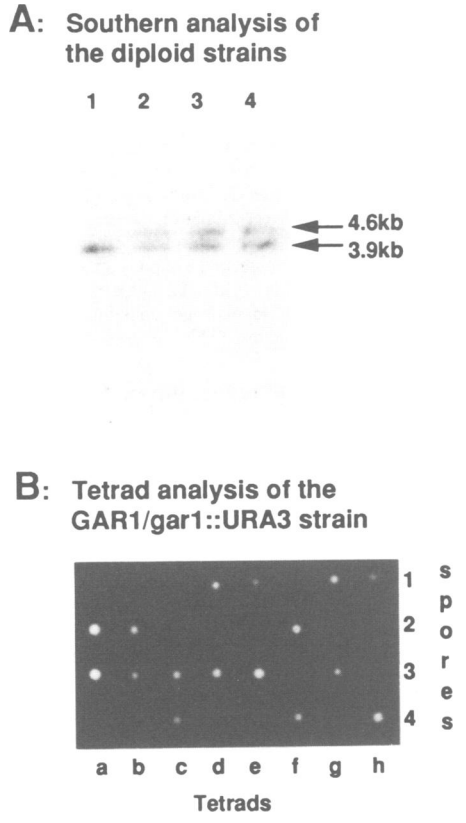

Fig. 3. Disruption of the *GAR1* gene. **A:** Lane 1, DNA extracted from the parent diploid strain; lanes 2−4, DNA extracted from three independent *URA*$^+$ transformants. The *GAR1* gene was destroyed *in vitro* by replacing part of the promoter and of the coding sequence by a fragment of DNA bearing the *URA3* gene. The 3.9 kb *Eco*RI fragment containing the *GAR1* gene is thus converted to a 4.6 kb *Eco*RI fragment bearing *gar1::URA3* gene. This linear fragment was used to transform a *ura3*$^-$ diploid strain. Genomic DNA was prepared from the transformants, digested by *Eco*RI and analysed by Southern blotting with the 3.9 kb *Eco*RI fragment as a probe. As expected, the untransformed strain has a 3.9 kb band, while all the transformants have two fragments of 3.9 and 4.6 kb. **B:** A diploid heterozygous for *GAR1/gar1::URA3* was sporulated and tetrads were dissected with a micromanipulator. The figure shows the dissection of eight tetrads (lanes a−h), each of the four spores being deposited on agar plates (rows 1−4). Spores were grown for two days on YPD medium at 30°C.

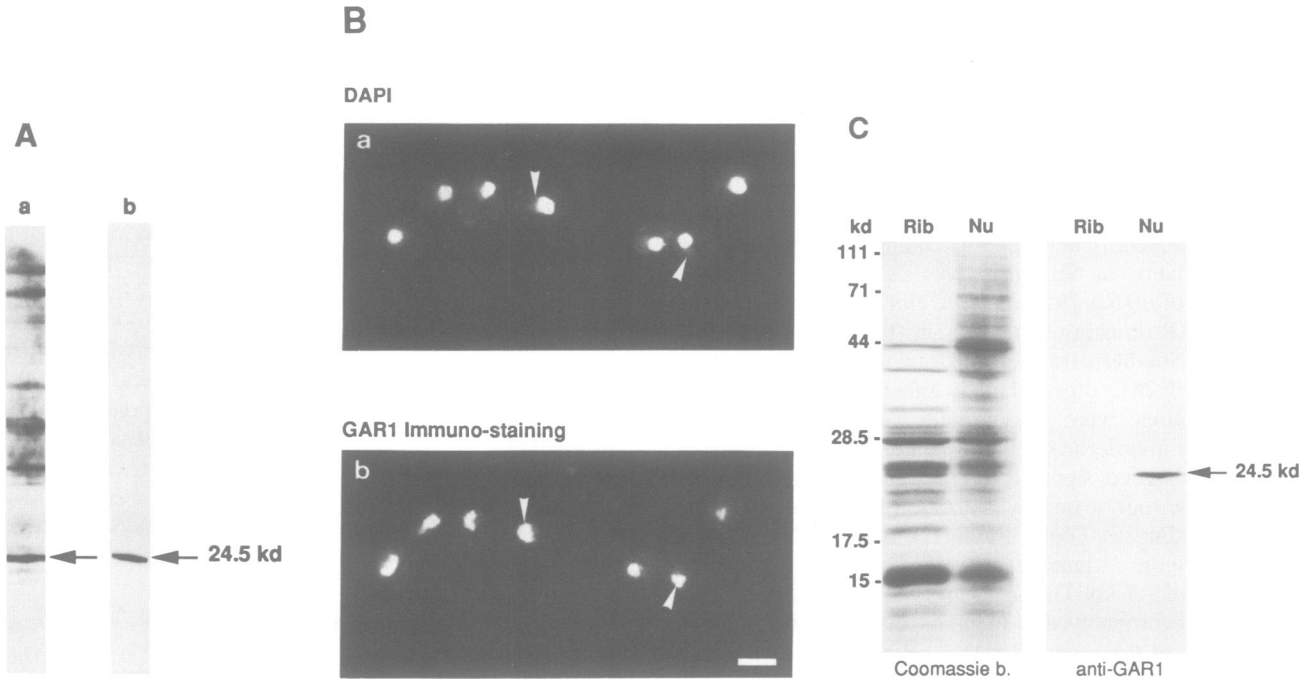

Fig. 4. Characterization of the GAR1 protein. **A:** Preparation of antisera. Anti-GAR1 antibodies were obtained in rabbits by immunization with a recombinant protein produced in *E.coli*. In a Western blot experiment using total yeast nuclear extract, crude anti-GAR1 serum #2 gives a relatively high background (lane a), while the immuno-purified anti-GAR1 antibodies react strongly with a nuclear protein of 24.5 kDa (lane b), with a good signal to noise ratio. **B:** Indirect immunofluorescence. Nuclei were first stained with DAPI (panel a). The white arrow points to the nucleolus, which is slightly stained. Indirect immunofluorescence was then performed with antibodies affinity purified against GAR1 from serum #2 and the complexes were revealed by a second antibody (Texas-Red labelled goat anti-rabbit) (panel b). In this case, the staining (white arrows) corresponds precisely to the nucleolus. Bar, 5 μm. **C:** GAR1 is a non-ribosomal protein. Cytoplasmic ribosomes (Rib.) or total nuclear extracts (Nu.) were separated on a 12.5% polyacrylamide gel containing SDS. The gel was either stained with Coomassie blue (Coomassie b.) or Western blotted using antibodies affinity purified against GAR1 from serum #2 (anti-GAR1). A single peptide of 24.5 kDa is detected only in the nuclear extract.

were non-viable, indicating that the *GAR1* gene is essential for yeast cell growth (Figure 3B).

GAR1 is a non-ribosomal protein localized in the nucleolus of the yeast cell

Recombinant GAR1 protein produced in *Escherichia coli* was purified from inclusion bodies by SDS−PAGE, electro-eluted from the gel and used to raise antibodies in rabbits (see Materials and methods). The antisera were immunopurified using the same recombinant protein as an affinity ligand. In Western blots of yeast nuclear extracts, total antisera or immunopurified IgGs recognize a protein with an apparent molecular weight of 24.5 kDa (Figure 4A). Two sera gave good immune responses (designated sera #2 and #3); antibodies affinity purified against GAR1 from both sera decorate a single protein band of the same apparent molecular weight (shown for serum #2 in Figure 4).

Immunocytochemistry was used to localize the protein within the cell. DAPI staining of fixed yeast cells labels both mitochondrial and nuclear DNA. Examination of the nuclear labelling (Figure 4B) reveals two different structures; the bulk of nuclear DNA is strongly stained and occupies about half of the nuclear volume, while weaker staining is observed in the other half of the nucleus. Numerous studies have shown that this lightly stained region of the nucleus corresponds to the yeast nucleolus, also known as the dense crescent (Sillevis Smitt *et al.*, 1973; Yang *et al.*, 1989). For instance, anti-fibrillarin antisera specifically label this region of the nucleus (Hurt *et al.*, 1988; Aris and Blobel, 1988). Double labelling of the same cells was performed with DAPI and by indirect immunostaining with the immunopurified anti-GAR1 IgGs (Figure 4B). The antibody staining is clearly localized in the nucleolus. Globular substructures within the nucleolus appear to be more heavily decorated than the remainder of the nucleolus. Even with the low background obtained in this experiment, we wanted to rule out any possibility that GAR1, which is a small basic protein, could be a ribosomal protein partially accumulated in the nucleolus. To demonstrate this, ribosomes were prepared from yeast cells. Comparison of ribosomal and nuclear proteins by Western blotting using immunopurified anti-GAR1 IgGs (Figure 4C), shows that GAR1 is present only in nuclei and not on cytoplasmic ribosomes. We conclude that GAR1 is a non-ribosomal protein exclusively localized within the yeast nucleolus.

GAR1 is associated with snoRNAs

The ability of two different anti-GAR1 sera (sera #2 and #3) to immunoprecipitate snoRNAs from lysates of *S.cerevisiae* was tested. Antibodies affinity purified against GAR1 from both sera specifically precipitate snR10 and snR30 but do not detectably precipitate the snoRNAs snR190, U3 or U14 (Figure 5, lanes 3 and 4) nor the nucleoplasmic snRNAs U4 and U6 (data not shown). No specific immunoprecipitation was detected using the pre-immune serum (Figure 5, lane 2). The same pattern of immunoprecipitation was observed using the crude anti-GAR1 sera (data not shown). For the crude sera, the same level of immunoprecipitation was observed using a *NOP1*$^+$ strain and a strain in which the GAR domain is deleted from NOP1 (data not shown; R.Jansen, E.C.Hurt and D.Tollervey, unpublished)

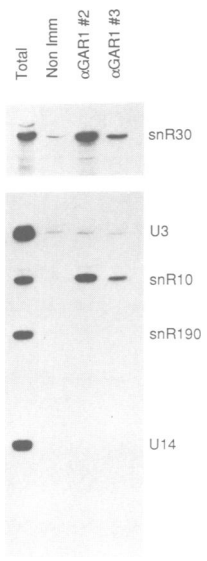

Fig. 5. GAR1 is associated with a subset of snoRNAs. Lane 1, Total RNA from lysate used for immunoprecipitation; lane 2, RNA recovered following immunoprecipitation with non-immune rabbit serum; lanes 3–4, RNA recovered following immunoprecipitation with antibodies affinity purified against GAR1 from serum #2 (lane 3), or from serum #3 (lane 4). RNA was recovered following immunoprecipitation, separated by PAGE, transferred to Hybond-N and hybridized with anti-sense riboprobes for the snoRNAs indicated.

GAR1 is required for pre-rRNA processing

To analyse the function of GAR1, its expression was placed under the control of an inducible *GAL10* promoter (see Materials and methods). Transfer of the *GAL::gar1* strain to glucose medium represses the synthesis of GAR1, and Western blotting shows the level of GAR1 protein to fall progressively (data not shown). Following transfer to glucose minimal medium, the growth rate of the *GAL::gar1* strain (2.5 h/generation) is similar to that of the *GAR1*$^+$ parent strain for 10–12 h. After this period, growth of the *GAL::gar1* strain is progressively impaired. After 48 h of growth on glucose medium, the doubling time of the *GAL::gar1* strain is >24 h, although some growth is still observed. This probably occurs because repression by the *GAL10* promoter is not complete and some residual GAR1 synthesis is therefore to be expected.

To assess the effects of depletion of GAR1 on snoRNA accumulation, the steady state levels of U3, U14, snR10, snR30 and snR190 were examined by Northern hybridization after transfer of the *GAL::gar1* strain to glucose medium. Comparison of RNA extracted from equivalent numbers of cells following growth for 12, 24, 36 and 48 h on glucose medium revealed no differences in the levels of any of the snoRNAs tested (data not shown).

To assess the effects of depletion of GAR1 on rRNA accumulation, the steady state levels of 18S and 25S rRNA were examined following growth of the *GAL::gar1* strain for 12, 24, 36 and 48 h on glucose medium (Figure 6, panel A). GAR1 depletion severely inhibits the accumulation of 18S rRNA, but has much less effect on the accumulation of 25S rRNA. To examine the effects of depletion of GAR1 on pre-rRNA processing, the steady-state levels of pre-rRNAs were measured by Northern hybridization with probes specific for the internal transcribed spacer regions of pre-rRNA (Figure 6, panels B–D). In the strain depleted of GAR1 a number of alterations in the pre-rRNAs are detected; the level of 35S is increased while the major processing intermediates, 32S, 27SA and 20S are strongly reduced. A RNA species smaller than 25S is detected which, from its size and hybridization pattern, is likely to be the pre-rRNA species previously designated 23S. This species is detected in other pre-rRNA processing mutants (Tollervey, 1987; Li *et al.*, 1990; Tollervey *et al.*, 1991; Hughes and Ares, 1991) and probably extends from the 5′ end of the primary transcript to the 5′ end of 5.8S rRNA, and is created by the cleavage of 35S at site B_1, rather than at the normal cleavage sites, A_1 and A_2 (see Figure 8). Primer extension from oligonucleotide 3 in ITS2 (see Figure 8), shows that cleavage at site B_1 is correct at the nucleotide level in strains depleted of GAR1 (data not shown).

To determine whether the absence of 18S rRNA is due to inhibition of its synthesis, pulse–chase analyses of pre-rRNA processing were performed. *GAR1*$^+$ and *GAL::gar1* strains were labelled with [^{3}H-methyl]methionine (Figure 7) or [^{3}H]uracil (data not shown), following growth for 12 or 24 h on glucose medium. Intact cells were pulse labelled for 2 min at 30°C and chased with a large excess of unlabelled methionine or uracil for the times indicated. Under these conditions, processing is very rapid in the *GAR1*$^+$ strain and even at the earliest time point, most 35S and 32S pre-rRNAs have already been processed (Figure 7, *GAR1*$^+$). In the *GAL::gar1* strain, processing of 35S pre-rRNA is slower and the normal, major processing intermediates, 32S and 27SA pre-rRNAs, are absent. Little synthesis of 18S rRNA is detected even 12 h after transfer to glucose medium (Figure 7, 12 h), and this level is further reduced 24 h after transfer (Figure 7, 24 h). The aberrant processing intermediate, 23S, is also detected by pulse labelling.

From the relative intensities of 25S rRNA and its precursor, 27SB pre-rRNA, as compared with 18S rRNA and its precursors, 23S and 20S pre-rRNA, it is clear that massive and very rapid degradation of the 5′ region of the pre-rRNA must be occurring. Full length 35S pre-rRNA is synthesized and accumulates in the GAR1 depleted strain. From the data in Figures 6 and 7, no pre-rRNA intermediate in size between 35S and 27S is detected. It is therefore likely that cleavage of 35S pre-rRNA is at site B1 to yield 27SB which is stable and processed to 25S rRNA. The remainder of the molecule is the species designated 23S, but as judged by Northern hybridization or pulse–chase labelling the relative yield of this pre-rRNA is low. It seems probable that this is due to extremely rapid degradation of the pre-rRNA; how such rapid degradation can be triggered by cleavage at site B_1 remains to be determined.

No clear differences were observed between cells labelled with [^{3}H-methyl]methionine or [^{3}H]uracil (data not shown), indicating that, unlike depletion of NOP1 (Tollervey *et al.*, 1991), depletion of GAR1 does not affect methylation of pre-rRNA.

Discussion

We report here the characterization of GAR1, a new protein of the yeast nucleolus which belongs to a small family of nucleolar proteins, characterized by the presence of related sequences rich in glycine and arginine residues, the GAR domain. We have shown that GAR1 is associated with small

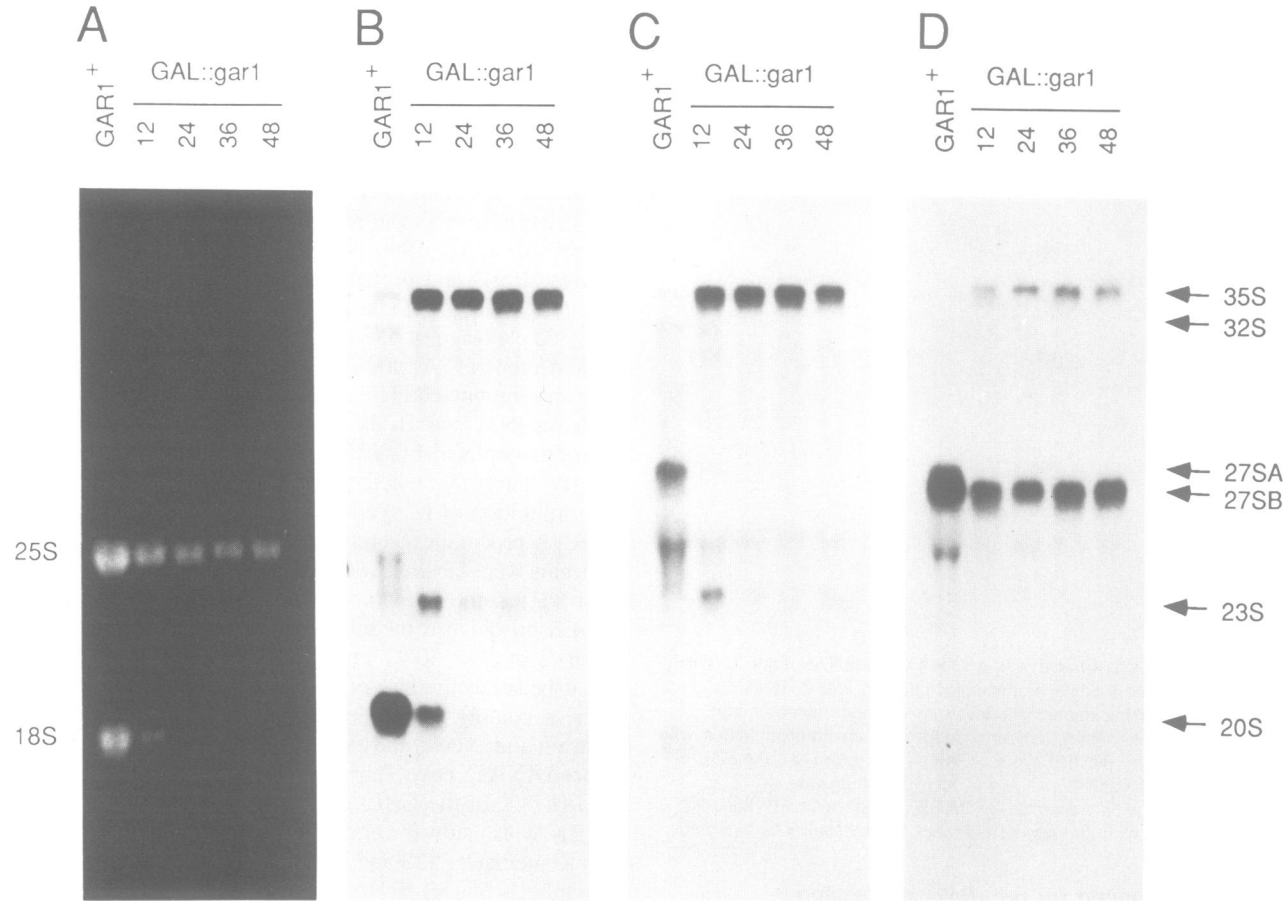

Fig. 6. Steady state levels of rRNA and pre-rRNA in cells depleted of GAR1. **Panel A**, rRNA visualized by staining with ethidium bromide; **panel B**, Northern hybridization with oligonucleotide 1, specific for the 5′ region of internal transcribed spacer 1; **panel C**, Northern hybridization with oligonucleotide 2, specific for the 3′ region of internal transcribed spacer 1; **panel D**, Northern hybridization with oligonucleotide 3, specific for the 5′ region of internal transcribed spacer 2. Lane 1, RNA from the *GAR1*$^+$ strain grown for 12 h in glucose medium; lanes 2–5, RNA from the *GAL::gar1* strain grown in glucose medium for 12, 24, 36 and 48 h, respectively. All three oligonucleotides are expected to hybridize to 35S and 32S pre-rRNA, in addition, oligonucleotide 1 should hybridize to 20S and 23S pre-rRNA, oligonucleotide 2 should hybridize to 27SA and 23S pre-rRNA and oligonucleotide 3 should hybridize to 27SA and 27SB. See Figure 8 for a diagram of the positions of the oligonucleotides and pre-rRNA species. RNA was separated on an agarose–formaldehyde gel and transferred to Hybond membrane. Mature rRNA was visualized on the filter under UV irradiation and duplicate filters were hybridized with the oligonucleotide probes.

nucleolar RNAs and is required for normal rRNA processing.

There are striking similarities in the phenotypes of several mutations affecting pre-rRNA processing. The effects of depletion of the nucleolar proteins GAR1 and NOP1, and the essential snoRNAs U3 and U14, using regulated *GAL* promoters, and of the deletion of the gene encoding snR10, have been reported to date (Tollervey *et al.*, 1991; Li *et al.*, 1990; Hughes and Ares, 1991; Tollervey, 1987). All lead to the accumulation of the 35S pre-rRNA primary transcript, the absence of cleavage of 35S at the normal sites (A_1 and A_2) and its cleavage instead at a site (B_1) normally used in a subsequent cleavage event (see Figure 8). This leads to the synthesis of 27SB and a species designated 23S containing the entire 5′ region of the pre-rRNA up to the 5′ end of 5.8S rRNA, and the absence of the normal processing intermediates 32S and 27SA. In strains depleted of GAR1, NOP1 or U14, the 23S species is apparently degraded very rapidly, since little synthesis of 18S rRNA is detected even by pulse–chase labelling (Tollervey *et al.*, 1991; Li *et al.*, 1990). 23S pre-rRNA is not, however, obligatorily a 'dead end' intermediate, since *snr10*$^-$ strains are apparently able to process it to 18S rRNA (Tollervey, 1987). The simplest interpretation is that the major processing pathway in wild-type strains (35S → 32S → 27SA + 20S) is more sensitive than the alternative pathway (35S → 27SB + 23S) to alterations in many nucleolar components. It should be noted, however, that depletion via regulation by *GAL* promoters is never complete and even at late time points of depletion the phenotype cannot be assumed to be equivalent to that of a null mutation. It may therefore be that the residual processing in the depleted strains is also dependent on these nucleolar components. The question remains of why these mutations so closely resemble each other? This similarity makes it likely that the primary effects of all are at the same step. One explanation is that they are all required for the functioning of a large pre-rRNA processing complex which carries out a number of the pre-rRNA cleavage reactions.

Affinity purified anti-GAR1 antibodies immunoprecipitate the snoRNAs snR10 and snR30, but not other snoRNAs tested (U3, U14 and snR190) nor the nucleoplasmic snRNAs U4 and U6. GAR1 is apparently not required for the synthesis or stability of these snoRNAs since snR10 and snR30 both accumulate to normal levels during depletion of GAR1. Association with GAR1 may, however, be required for the function of the snoRNPs. The phenotype

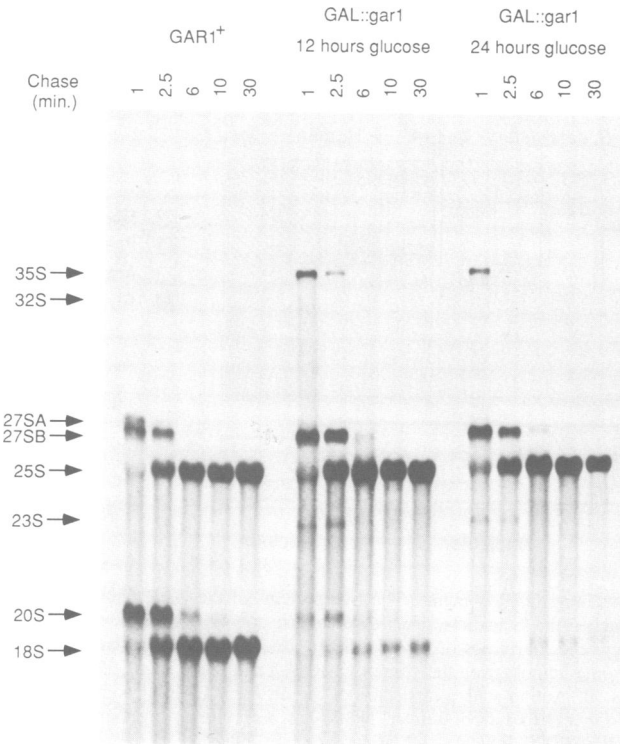

Fig. 7. Pulse−chase labelling of pre-rRNA in wild-type cells and in cells depleted of GAR1. Pre-rRNA was pulse-labelled for 2 min with [^{3}H-methyl]methionine and chased with a large excess of unlabelled methionine for the times indicated (1, 2.5, 6, 10 or 30 min). RNA was extracted, separated on an agarose−formaldehyde gel and transferred to Genescreen+ membrane. Labelled bands were visualized by fluorography. **Left panel**, labelling of *GAR1*$^+$ strain grown for 12 h in glucose medium; **central panel**, labelling of *GAL::gar1* strain following growth for 12 h on glucose medium; **right panel**, labelling of *GAL::gar1* strain following growth for 24 h on glucose medium.

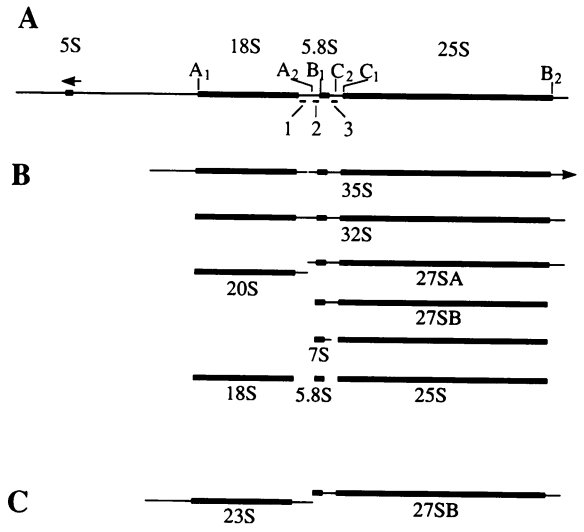

Fig. 8. Structure of the yeast rDNA repeat and pre-rRNA processing pathways. **A**: Structure of the rDNA repeat from *S.cerevisiae*, showing the major cleavage sites (A−C) and the location of the hybridization probes (1−3) used for the Northern hybridizations shown in Figure 6. **B**: The major pre-rRNA processing pathway in wild-type cells. The species 32S is clearly a major processing intermediate, but it is not at present clear whether it is an obligatory intermediate between 35S and 20S/27SA. **C**: Probable structure of the 23S/27SB cleavage products of 35S in GAR1 depleted strains. The endpoints of these species have not yet been identified at the nucleotide level, but hybridization analyses (Figure 6) show that the 27SB species does not include the internal transcribed spacer 1 (ITS1) region, while 23S extends through ITS1 beyond site A_2, and also includes sequences in the external transcribed spacer (ETS) region (data not shown). Moreover, pre-rRNA cleavage at site B_1 is correct at the nucleotide level in strains depleted of GAR1. From these data and the sizes of these RNAs, we predict that the locations of 23S and 27SB are as shown. The figure is reprinted from Tollervey *et al.* (1991) by kind permission from Oxford University Press.

of *snr10*$^-$ mutants resembles that of strains depleted of GAR1, and snR30 is essential for viability, although its function has yet to be determined. We were concerned that antibodies against different GAR proteins might cross-react with the GAR domains. On Western blots, the affinity purified anti-GAR1, anti-NOP1 (Schimmang *et al.*, 1989) and anti-SSB1 (Clark *et al.*, 1990) antibodies decorate single bands, showing that the antibodies do not cross-react with the denatured proteins. Moreover, the snoRNAs precipitated by the three antibodies differ; anti-NOP1 immunoprecipitates all snoRNAs tested (Schimmang *et al.*, 1989), whereas anti-SSB1 immunoprecipitates mainly snR10 and snR11 but does not immunoprecipitate an RNA of the size of snR30 (Clark *et al.*, 1990). The immunoprecipitation by anti-GAR1 antibodies at least, is not due to cross-reaction with the GAR domain of NOP1, since precipitation is still observed in a strain carrying a deletion of the NOP1 GAR domain. Thus, while cross-reaction of the GAR proteins under native conditions cannot be fully excluded, it seems most probable that the snoRNAs are associated with at least three GAR proteins. It is not certain whether GAR1, NOP1 and SSB1 are components of the snoRNPs or components of larger nucleolar structures, of which the snoRNPs are also components. The differences in immunoprecipitation might argue for their presence in distinct snoRNPs. However, disruption of the nucleolar structures required to release the snoRNPs for immunoprecipitation might leave components of the same complexes differentially associated.

It is striking that many constituents of the nucleolar apparatus seem to be modular proteins built from a small number of units: basic repeats, acidic/serine blocks, RNA recognition motifs and GAR domains (Figure 9A). The other units are also found in nucleoplasmic proteins but the GAR domain appears to be restricted to nucleolar proteins. Other proteins with glycine rich regions have been reported, most notably hnRNP A1 and A2 (Cobianchi *et al.*, 1986; Burd *et al.*, 1989). However, the amino acid composition of the nucleolar GAR domains and the glycine-rich domains of A1 and A2 are very different. In particular there is a large under-representation of arginines in A1 and A2; the average percentage of arginine in nucleolar domains is ~25%, while it drops to 6−8% in A1 and A2. Conversely, the percentage of non-GRF residues, which is very low in nucleolar proteins, reaches ~40% in A1 and A2 (Figure 9B). The nucleolar domains are very compact without being interrupted by long stretches of non-RGF residues and are formed by the internal repetition of short stretches of 5−12 residues containing the sequence RGGXGGR or RGGXRGG, where X is generally a phenylalanine or, less frequently, serine, tyrosine or alanine. We conclude that the GAR domain is a distinct conserved motif restricted to nucleolar proteins. The GAR domains are located in different regions of the molecule; centrally for SSB1, C-terminally for nucleolin and NSR1 and N-terminally for fibrillarin. GAR1, which contains two GAR domains, has one at each end of the protein. In all the available sequences, proline

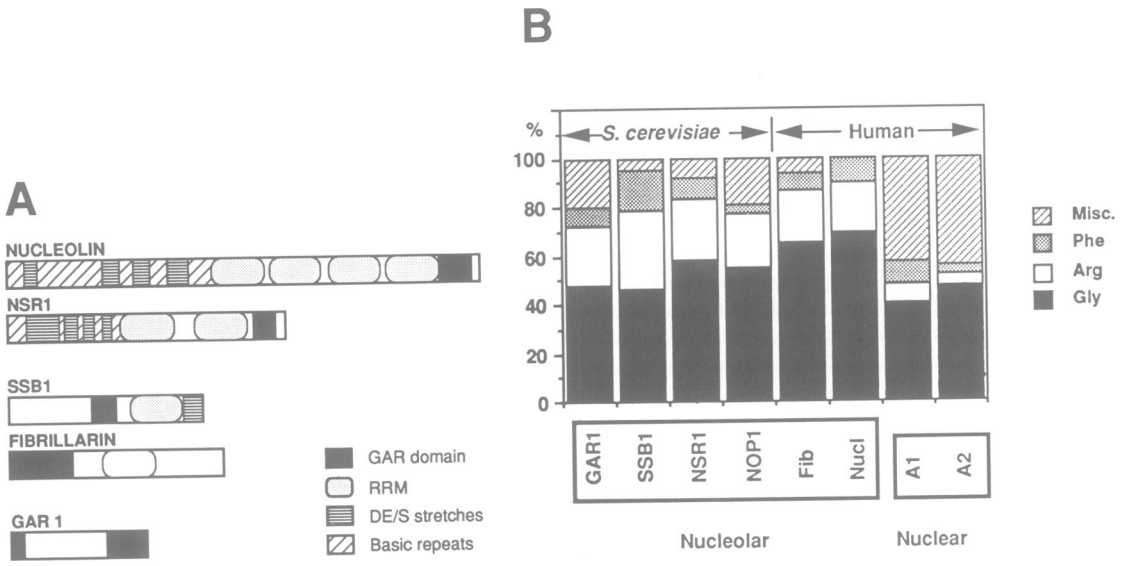

Fig. 9. Analysis of the GAR domain. **A:** Structure of the nucleolar proteins containing GAR domains. The five proteins of the GAR family have been schematically drawn. Diagonal hatched lines, basic domains; horizontal hatched lines, acidic/serine rich domains; dotted rounded boxes, RNA recognition motifs; black boxes, GAR domains. Nucleolin and NSR1 are made of the four types of module; SSB1 lacks a basic region but has the other three motifs; fibrillarin has one GAR domain and a sequence which has some homology to the consensus RNA recognition motif, although it is significantly divergent (open rounded box), while GAR1 has two GAR domains. **B:** Amino acid composition of the different GAR domains. Six nucleolar and two nuclear proteins have been analysed. Four are from *S.cerevisiae* and four from human. NOP1 has been chosen together with human fibrillarin to illustrate the differences between yeast and human. The codes used to indicate each amino acid are indicated on the right, with the non RGF residues pooled in 'Miscellaneous'. The percentage of each amino acid is indicated on the left.

residues are found between the GAR domain and the remainder of the protein. Structurally, the doublets of glycine in the GAR domain are predicted to form β-turns and would be expected to give a highly flexible structure. This flexibility, and the separation of the GAR domain from the body of the protein by proline residues, suggests that this region is organized as a structurally independent domain, accessible for interactions with other components. The GAR domain is a positively charged, repeated structure, obviously suggesting the possibility that it interacts with nucleic acids. There is evidence of a direct role for the GAR domain of nucleolin in RNA structure modification without sequence specificity (Ghisolfi *et al.*, 1992) and an arginine mediated protein-RNA interaction has recently been described (Calnan *et al.*, 1991). It is thus possible that GAR domains are involved in RNA metabolism by modifying the conformation of the RNA. For instance, the GAR domains might open the structure of the pre-rRNA so as to render it accessible to other components such as ribosomal proteins and snoRNPs. The cloning of GAR proteins from yeast should allow us to test the function of the GAR domain in ribosome synthesis *in vivo*.

Materials and methods

Strains, media and microbiological methods

The haploid strain YNN281 (*MAT***a**, ade2-101, his3-Δ200, lys2-801, trp1-Δ1, ura3-52, CAN$^+$), the diploid strains +D4 (*MAT***a**/α, ade1/ade1, ade2/ADE2, gal1/gal1, HIS5/his5, his7/HIS7, LEU2/leu2, lys2/LYS2, LYS11/lys11, tyr1/TYR1, ura1/ura1; provided by L.H.Hartwell) and JR26-19B×JU4-2 (*MAT***a**/α, ade2-1/ade2-1, ade8/ADE8, can1-100/can1-100, his4/HIS4, his3/HIS3, leu2-3/leu2-3, lys1-1/lys1-1, ura3-52/ura3-52; provided by O.Fasano) and the diploid transformant heterozygous for the *GAR1* alleles (*MAT***a**/α, ade2-1/ade2-1, ade8/ADE8, can1-100/can1-100, his4/HIS4, his3/HIS3, leu2-3/leu2-3, lys1-1/lys1-1, ura3-52/ura3-52, GAR1/gar1::URA3) were used. Strains were grown in YPD liquid medium/plates containing 1% yeast extract, 2% bacto-peptone, 2% glucose or selective medium/plates complemented with the appropriate nutrients [SD medium (2% glucose, 0.7% yeast nitrogen base) or SG medium (2% galactose, 0.7% yeast nitrogen base, plus nutrients); Sherman *et al.*, 1986]. Yeast diploids were sporulated by growing the cells on YPA plates (1% yeast extract, 2% bacto-peptone, 1% potassium acetate, 2% agar) for four days at 30°C. Tetrad analysis was performed by incubating sporulated diploids for 10 min with glusulase and dissecting the ascus into the four tetrad spores on YPD plates. Yeast transformation was performed by the PEG method (Klebe *et al.*, 1983).

Recombinant DNA work

Plasmid or phage DNA preparation, use of λ ZAPII (Stratagene), enzyme digestion and gel analysis were performed as described (Sambrook *et al.*, 1989), or according to the supplier's instructions. DNA sequencing was carried out using a Sequenase kit (USB Corp.). Oligonucleotides were synthesized using an Applied Biosystems synthesizer and used without further purification.

Southern blot analysis under low stringency conditions

Total yeast genomic DNA was extracted according to Johnston (1988), then digested and subjected to electrophoresis as described. DNA was stained and transferred to charged nylon membrane (Zeta probe) under vacuum (Vacu-blot, Hoefer Scientific). After air drying, the filters were pre-incubated at 37°C for 5 h in denatured prehybridization buffer containing 25% formamide, 6× SSC, 5× Denhardt's, 10% dextran sulphate, 0.1% SDS, 0.1% PPi, 100 μg/ml of sheared *E.coli* DNA, 100 μg/ml of poly(A) and 100 μg/ml of tRNA. The denatured multiprime labelled (Amersham) probe, corresponding to the *Eco*RI−*Bst*EII cDNA fragment encoding the GAR domain of *Xenopus* fibrillarin (Lapeyre *et al.*, 1990), was added to the same medium, at a final concentration of $2-4 \times 10^5$ d.p.m./ml. Hybridization was performed for 15 h at 37°C. Filters were then washed four times at room temperature and four times at 42°C in 2× SSC, 0.1% SDS before autoradiography at −70°C for 24 h with intensifying screen.

Isolation of the genomic GAR1 gene

To construct the partial genomic DNA library, 50 μg of *S.cerevisiae* genomic DNA were digested with *Eco*RI and electrophoresed on a 18×25 cm 1% agarose−TAE gel. After ethidium bromide staining, migration was measured using molecular weight standards (λ DNA cut by *Hin*dIII) and the repeated rDNA fragments, which provide good internal markers. 0.5 cm width bands were cut and DNA (about 0.5 μg) was purified using the 'Geneclean' kit (Bio 101). The purified *Eco*RI DNA fragments were then ligated with the *Eco*RI arms of the λZAPII vector and encapsidated *in vitro* (Stratagene). The screening of the partial genomic library, thus obtained, was performed in one step using the probe and the low stringency hybridization conditions

described above. Phages libraries were constructed rather than plasmid libraries because screening was performed under low stringency conditions and because, in this case, phage λ gives a lower background. With the λ ZAPII vector, the plasmid containing the insert can then be recovered easily by super-infection with a helper phage (Stratagene).

Gene disruption

Gene disruption was performed by the method of Rothstein (1983). The 0.4 kb *Nde*I−*Xba*I fragment of the *GAR1* gene, which contains most of the promoter and part of the coding sequence, was removed and replaced by a 1.1 kb *Bgl*II−*Bgl*II fragment containing the *URA3* gene, all the extremities being filled in by the Klenow fragment of *E.coli* DNA polymerase I. The recombinant molecule was then digested with *Eco*RI and *Sal*I and 10 μg of the linear 2.5 kb fragment, containing the *URA3* gene flanked by 5′-non-coding and 3′ coding sequences of the *GAR1* gene, were used to transform the diploid *ura3*$^-$ strain JR26-19B×JU4-2. *URA*$^+$ transformants were obtained which carried the *gar1::URA3* gene disruption at the homologous locus, as demonstrated by Southern analysis performed under high stringency conditions, according to Sambrook *et al.* (1989). One of the transformants, heterozygous for *GAR1*, was used for tetrad dissection.

Overproduction of the GAR1 protein in E.coli; generation and affinity-purification of anti-GAR1 antibodies

Recombinant GAR1 protein was produced in *E.coli* using the T7 system [kindly provided by F.W.Studier (Studier *et al.*, 1990)]. To fuse the 5′ side of the *GAR1* ORF with the T7 *gene10* leader, a PCR reaction was performed with two oligonucleotides, one complementary to the 5′ side of the ORF and the other complementary to the region +394 to +375. The primer complementary to the 5′ side of the ORF carried an additional *Bam*HI site, 6 nt upstream of the ATG. The 400 nt PCR fragment thus obtained was cut by *Bam*HI and *Xba*I and used to replace the natural 5′ side of the gene. A 1.5 kb *Bam*HI−*Bgl*II fragment was recovered and inserted, in frame with the T7 *gene10* leader, at the unique *Bam*HI cloning site of the plasmid pET3-a. Plasmid pET3-GAR1 was used to transform the *E.coli* strain BL21 (DE3) which contains the pLysS plasmid. Production of T7 RNA polymerase, which is cloned under a *pLAC* promoter, is achieved by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the liquid medium. The T7 RNA polymerase actively transcribes the *gene10−GAR1* fusion gene and leads to the overproduction of the GAR1 recombinant protein. At the end of IPTG induction, cells were harvested and inclusion bodies were prepared using a standard procedure (Harlow and Lane, 1988). Recombinant GAR1 was purified from inclusion bodies by SDS−PAGE, electro-eluted from the gel and used to immunize rabbits. Three rabbits were intravenously injected four times with 10 μg of protein. Antibodies against GAR1 were affinity purified from the immune serum using the recombinant protein as an affinity ligand. To prepare the affinity column, 200 μg of GAR1 were coupled with CNBr-activated Sepharose (Pharmacia). IgGs were further purified on a protein A−Sepharose column (Pharmacia).

Immunoblotting and indirect immunofluorescence

The affinity purified antibodies were used for immunoblotting and indirect immunofluorescence in 1:100 and 1:10 dilutions, respectively. Western blotting was performed using standard methods (Harlow and Lane, 1988) and detection was achieved with an ECL kit (Amersham).

Indirect immunofluorescence microscopy on formaldehyde-fixed yeast cells was performed after Hall *et al.* (1990), with the following modifications; the diploid strain +D4 was grown in YPD medium to an OD_{600} of 0.5. Cells were fixed, converted into spheroplasts and resuspended in 0.5 ml of SP (1.2 M sorbitol, 0.1 M potassium phosphate buffer, pH 6.5) per 30 ml of starting culture. Fixed, permeabilized cells were attached to poly-L-lysine-coated coverslips and processed for immunofluorescence microscopy by incubation for 45 min with the affinity purified antibodies against the GAR1 protein (1:10 dilution). The second antibody (Texas Red-labelled goat anti-rabbit IgGs from Amersham) was used at a 1:50 dilution. Samples were mounted in Moviol containing 0.2 μg/ml DAPI and viewed on a Leitz microscope.

Purification of nuclei and ribosomes

Nuclei from strain YNN281 were prepared according to Aris and Blobel (1991b). Isolation of ribosomes and ribosomal proteins was performed as described by Warner and Gorenstein (1978).

Construction of the GAL::gar1 strain

In order to delete part of the promoter of the *GAR1* gene, a 1.7 kb *Bcl*I−*Bgl*II fragment, starting at position −188, was replaced with a 1.5 kb *Bam*HI−*Bgl*II fragment starting at position −6 (described above). The *Hin*dIII−*Bam*HI fragment of pLGSD5, which contains the *URA3* gene and the UAS_G from the *GAL1−GAL10* intergenic region (Guarente *et al.*, 1982), was isolated and cloned, by blunt-end ligation, in the *Nde*I site of the *GAR1* gene deleted of part of its promoter. A linear *Eco*RI−*Sal*I fragment containing the entire construct, *GAR1* 5′ flanking region−*URA3*−*GAL10*−*GAR1* coding region, was used to transform yeast. Transformation of YNN281 with selection for *URA3*, results in the precise replacement of the *GAR1* gene with the construct in which the promoter region is replaced by the *GAL10* promoter, which is subjected to galactose induction and glucose repression. Southern analysis confirmed that the construct was correctly integrated at the *GAR1* locus in the *GAL::gar1* strain.

Immunoprecipitation and RNA analysis

This was done as previously described (Schimmang *et al.*, 1989), except that the cell lysis buffer for immunoprecipitation contained 150 mM KAc, 20 mM Tris−Ac pH 7.5, 5 mM MgAc, 5 mM VRC (BRL), 0.2% Triton X-100 and 2 mM PMSF. The same buffer, without VRC and PMSF, was used for antibody binding and washing. Immunoprecipitation was for 2 h at 4°C using lysate prepared from the equivalent of 5 OD_{600} units of cells (~10^8 cells). Under these conditions the lysate is present in excess.

Extraction of RNA, hybridization of pre-rRNA and pulse−chase labelling

RNA extraction, gel electrophoresis and hybridization were performed as described (Tollervey, 1987). Pulse−chase labelling of pre-rRNA was performed as described (Tollervey *et al.*, 1991). The oligonucleotides used for hybridization of pre-rRNA and the probes used for hybridization of snRNAs and snoRNAs were those previously described (Tollervey *et al.*, 1991).

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