

Expression of the yeast *NAM9* gene coding for mitochondrial ribosomal protein*

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We studied expression of the *NAM9* gene of *Saccharomyces cerevisiae* that was previously reported to code for a mitochondrial ribosomal protein. Increase in *NAM9* gene dosage is accompanied by the increase in both mRNA and protein. The levels of the *NAM9* transcript and protein are both reduced in cells growing on glucose as compared to cells growing on galactose as a carbon source. Nam9p accumulates to the same level in *rho*⁰ and *rho*⁺ cells. These results confirm previous data indicating diverse regulation of different mitochondrial ribosomal protein genes and suggest that expression of Nam9p is not co-ordinated with the expression of other mitochondrial ribosomal components.

Expression of numerous ribosomal genes must be perfectly co-ordinated to achieve balanced accumulation of ribosomal components enabling proper assembly of the subunits. In *Escherichia coli* this co-ordination is achieved at the level of translation through an autogenous feedback mechanism where the accumulated ribosomal protein is the repressor of its own synthesis [1]. In contrast, in the yeast *Saccharomyces cerevisiae* expression of genes coding for proteins of cytoplasmic ribosomes is co-ordinated at the level of transcription by global regulators Abf1p and Rap1p [2]. The rate of synthesis of individual proteins could be additionally modulated at the level of splicing, mRNA stability, translation and degradation of proteins synthesised in excess [3].

In mitoribosomes of *S. cerevisiae* the two ribosomal RNAs and a single protein of the

37S subunit are encoded by mitochondrial DNA. The remaining 70–80 mitoribosomal proteins are nucleus-encoded, synthesised in the cytoplasm and transported to mitochondria [4]. Thus genes in both mitochondria and the nucleus must be expressed in a concerted manner to ensure the production of equimolar amounts of mitoribosomal components. Moreover, the synthesis rates have to be coordinately regulated in response to carbon source. Until now 28 nuclear genes of *S. cerevisiae* coding for mitochondrial ribosomal proteins have been described. Data on the regulation of expression were reported for 12 mitoribosomal genes (for references see [5]). The results of these studies do not yet allow to construct a consistent model of the regulation of mitoribosomal genes expression.

We studied the expression of the *NAM9* gene of *S. cerevisiae*. Nam9p was previously

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reported as a mitoribosomal protein based on phenotypes of mutant as well null alleles of *NAM9*, immunological properties of β -galactosidase-Nam9 fusion protein and, finally, sequence homology to the ribosomal protein S4 of bacteria, chloroplasts and eukaryotes [6, 7]. This paper presents experiments where extra copies of the *NAM9* gene were introduced into yeast cells to determine the extent to which the expression of Nam9p is co-ordinated with the expression of other mitoribosomal proteins. Transcription and translation of *NAM9* was studied in response to carbon source, the presence of mitochondrial genome and mutation within the *NAM9* locus.

MATERIALS AND METHODS

Strains, media and growth conditions.

The *S. cerevisiae* strains used in this study were: KL14-4A *MAT a his1 trp2* (P.P. Slonimski laboratory), MB43-15C *MAT a NAM9-1 ura3-1 leu2-3 his3* [7] and W303-1B *MAT a ura3-1 leu2-3 his3 trp1 ade2 can^R* (Ro Rotstein laboratory). Derivatives of *rho^o* were constructed as described in literature [8]. Rich yeast media YPG and YPGALA, containing respectively 2% glucose or 2% galactose, as well as WO-ura and WO-leu minimal medium, supplemented with 2% glucose or 2% galactose and the appropriate amino acids, were described previously [6].

Plasmids. pH13 and pAD6 are centromeric plasmids, carrying respectively *NAM9* and *NAM9-1* alleles [7]. pFL-NAM9 is a multicopy plasmid encoding *NAM9*; pFL-NAM9-1 and YEp-NAM9-1 are multicopy plasmids encoding the mutant *NAM9-1* allele [9]. Multicopy plasmid are derivatives of Yep351 [10] and pFL44L [11].

To construct an epitope-tagged versions of *NAM9* and *NAM9-1* first the 448 bp *Bam*HI-*Eco*RI fragment of pH13 was transferred to Bluescript vector (Stratagene) and then the sequence encoding epitope from influenza virus hemagglutinin protein (HA-epitope) was introduced using site-directed mutagenesis with the oligonucleotide 5'TGC AAC AAT ACA CGA CGA TCA AGC GTA GTC TGG GAC GTC GTA TGG GTA TTT

ACC ATT TCT CAA GTA3'. The *Aat*II restriction site created with this procedure was used for selection and appropriate insertion of the HA epitope was finally confirmed by DNA sequencing. 3' Terminal *Bam*HI-*Sal*I fragment of *NAM9* (or *NAM9-1*) sequence inserted in pH13, pAD6, pFL-NAM9 and pFL-NAM9-1 were substituted with the *Bam*HI-*Sal*I fragment containing the HA epitope created as described above, resulting in pH13/HA, pAD6/HA centromeric and pFL-NAM9/HA, pFL-NAM9-1/HA multicopy plasmids, respectively.

mRNA isolation and hybridisation.

Cells of KL14-4A or its *rho^o* derivative were grown to the mid-log phase in liquid YPG or YPGALA medium. MB43-15C cells harbouring appropriate plasmid were grown to mid-log phase in WO-ura and WO-leu medium. The harvested cells were broken with glass beads and RNA was isolated by phenol extraction. RNA, 10 μ g, was loaded on 1.5% agarose gel in 1 \times Mops, 6.3% formaldehyde buffer, electrophoresed overnight and capillary transferred in 20 \times SSC to Hybond-C extra membrane (Amersham Manual Instructions). Hybridisation was carried out at 42°C. The *Eco*RI-*Hind*III or *Ava*I-*Hind*III restriction fragments of the *NAM9* gene and the *Xho*I-*Hind*III fragment of the yeast actin gene *ACT1*, used as probes, were isolated from agarose by electroelution and labelled with [α -³²P]ATP using the random prime system (Amersham).

Immunoblot analysis. Cells of W303-1B strain or its *rho^o* derivative harbouring appropriate plasmid encoding HA-tagged Nam9p (or Nam9-1p) were grown to mid-log phase ($A_{600} = 1$) in liquid WO-ura glucose or galactose medium supplemented with appropriate amino acids. The total proteins (35 μ g) extracted from trichloroacetic acid-treated cells were resolved by 10% polyacrylamide/SDS gel electrophoresis and electrophoretically transferred to Hybond-C extra membrane (Amersham). Membrane was blocked in 5% skim milk powder in TBST buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 30 min at room temperature. Mouse 12CA5 monoclonal antibody, specific for HA epitope (purchased from Berkeley Antibody Company) was

added at a 1:2000 dilution, and incubation was carried out overnight at room temperature. Blot was washed in 5% skim milk powder in TBST buffer and incubated for 2 h with goat anti-mouse antibody conjugated to alkaline phosphatase (DAKO) at a 1:2000 dilution. Alkaline phosphatase was detected with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) (Promega).

Quantitation of RNA and protein. The levels of mRNA and protein on Northern and Western blots, respectively, were measured by scanning densitometry using Ultrascan XL Enhanced Laser Densitometer (LKB). *NAM9* mRNA levels were normalised to actin mRNA levels.

RESULTS AND DISCUSSION

Effect of gene dosage on *NAM9* expression

Extra functional copies of *NAM9* were introduced into yeast cells using multicopy plasmids, derivatives of Yep351 [10] and pFL44L [11] (see Materials and Methods).

Total cellular RNA was analysed by Northern blotting using two independent *NAM9* fragments as probes (see Materials and Methods). Both probes revealed the same transcript of 1.8 kb. The size of the transcript is consistent with the size of the *NAM9* ORF and the location of putative initiation and termination signals.

The amount of *NAM9-1* mRNA in cells transformed with Yep351-*NAM9-1* was approximately 32 times higher than in control cells transformed with Yep351 vector alone (Fig. 1A). This result is similar to those obtained for other mitoribosomal proteins: transcription was increased in proportion to the gene dosage [12, 13, 14].

The relative amounts of Nam9p in cells containing different numbers of functional *NAM9* copies is shown in Fig. 1B. For immunodetection of Nam9p we inserted a synthetic DNA sequence encoding an epitope from the influenza virus hemagglutinin protein (HA epitope) at the 3' end of the *NAM9* gene, generating the pAD6/HA single copy and pFL-*NAM9*/HA multicopy plasmids (see

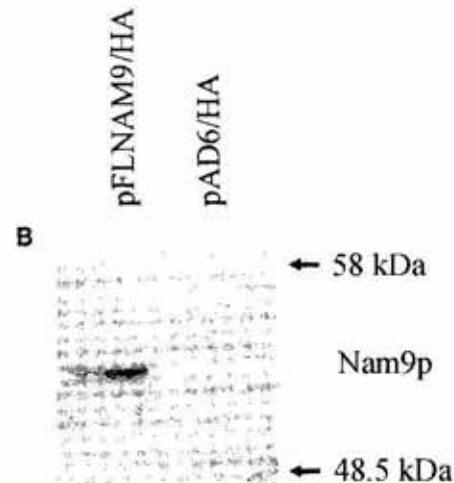
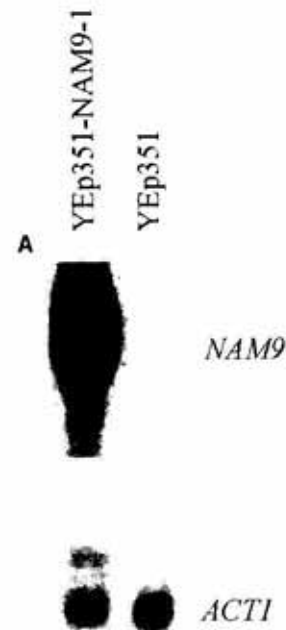


Figure 1. *NAM9* expression in strains with elevated *NAM9* gene dosage.

A. Northern analysis. RNA samples were prepared from MB43-15C cells transformed with either YEp351-*NAM9-1* (Yep351-derivative carrying *NAM9-1* gene) or Yep351. Cells were grown in minimal glucose WO-leu medium. Total RNA (10 μ g per lane) was separated by electrophoresis, transferred to a membrane and hybridised with the 32 P-labelled *AvaI-HindIII* fragment from the *NAM9* coding region. As a control for RNA loading, the same blot was hybridised with the 32 P-labelled *XhoI-HindIII* fragment of the yeast *ACT1* gene encoding actin. B. Western analysis. Protein samples were prepared from W303-1B cells transformed with either the multicopy pFL-*NAM9*/HA or the centromeric pAD6/HA plasmid carrying an HA-epitope tagged version of the *NAM9* gene. Cells were grown in minimal WO-ura galactose medium. Samples of total cell protein (35 μ g per lane) were subjected to immunoblot analysis with the HA-specific 12CA5 antibody, as described in Materials and Methods.

Materials and Methods). The resulting fusion protein Nam9p-HA, expressed from the plasmid, restored growth phenotypes of mutants in the same fashion as did plasmid-expressed Nam9p (data not shown). This indicated that the presence of the HA epitope tag did not impede the function of Nam9p.

Extracts from cells bearing pAD6/HA and pFL-NAM9/HA plasmids were subjected to SDS/PAGE and subsequent immunoblot analysis. A band of about 53 kDa corresponding to the Nam9p-HA fusion protein was detected with an antibody specific for HA. The level of Nam9p-HA expressed from the single copy plasmid, pAD6/HA, was below detection limit, whereas when overexpressed from pFL-NAM9/HA it was readily detected (Fig. 1B). No signal was observed in the control cells transformed with pAD6 plasmid or pFL44L vector (not shown). These data show that the steady-state level of Nam9p-HA was increased in the cells harbouring multiple copies of the *NAM9-HA* gene. This result strongly suggests that expression of Nam9p from the multicopy plasmid is not co-ordinated with the level of expression of other mitochondrial ribosomal proteins. Nam9p seems to be relatively stable in an unassembled form and accumulates in the cell if its rate of synthesis is increased.

Another mitoribosomal protein, Mrp1p, also accumulates in the cell when overexpressed from a multicopy plasmid [15]. Expression of other mitoribosomal genes, however, seems to be co-ordinated probably by degradation of proteins synthesised in excess. In the case of *MRP7* overdose, accumulation of mRNA, but not protein, is observed [13].

Effect of mitochondrial DNA on expression of the *NAM9* gene

The expression of wild type *NAM9* was analysed in isonuclear yeast strains with and without mitochondrial genotypes. The results of Northern and Western blot analyses are shown in Fig. 2. The level of the *NAM9* transcript is two times higher in *rho*⁰ cells. The level of Nam9 protein is comparable in *rho*⁺ and *rho*⁰ cells (we consider the small difference observed, below 30% as not signifi-

cant). Expression of Nam9p in the *rho*⁰ cells indicates that it is not linked to expression of mitochondrially-encoded mt rRNAs.

Regulatory effects connected with the presence/absence of products encoded in the mtDNA on the expression of nuclear-encoded mitoribosomal proteins are diverse. Some genes (*MRPL16*, *MRP7*) are not differentially transcribed in *rho*⁺ and *rho*⁰ cells [13, 16]. Transcription of *MRP20*, *MRP49*, *MRP2*, *MRP13* genes [12, 17, 18] is elevated in *rho*⁰ cells, which is consistent with the retrograde mode of regulation [19], but proteins are often absent in *rho*⁰ cells, even if

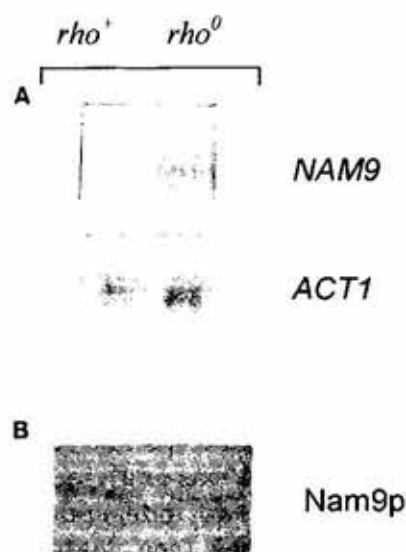


Figure 2. *NAM9* expression in isonuclear strains with different mitochondrial genomes.

A. Northern analysis. RNA samples were prepared from strains KL14-4A *rho*⁺ and KL14-4A *rho*⁰ growing in YPG medium. Hybridisation was carried out as described in legend to Fig. 1. B. Western analysis. Protein samples were prepared from W303-1B *rho*⁺ and W303-1B *rho*⁰ cells transformed with the pFL-NAM9/HA plasmid. Cells were grown in minimal WO-ura galactose medium. Immunoblot analysis was carried out as described in legend to Fig. 1.

their respective transcripts levels are elevated [18]. This is probably due to degradation of proteins when assembly of mitoribosomes is impossible in the absence of mitochondrial rRNAs. Other proteins, Mrp1p and Mrp7p, similarly to Nam9p accumulate in *rho*⁰ cells at a level comparable to that observed in the isogenic *rho*⁺ cells. Therefore Nam9p belongs to the category of mitoribosomal proteins whose expression is not linked to other mitoribosomal components —

whether to nuclear-encoded proteins or mitochondrial-encoded rRNAs.

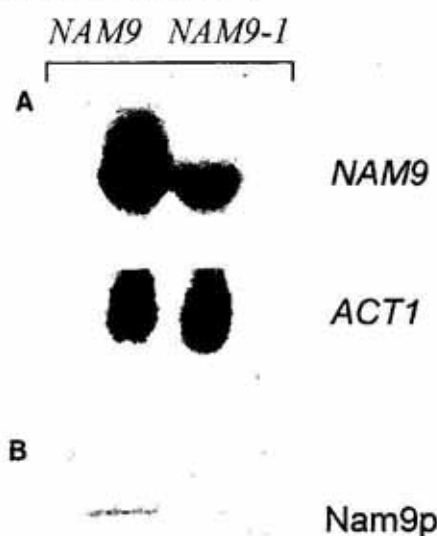


Figure 3. Expression of wild type and mutated Nam9p.

A. Northern analysis. Total RNA was isolated from the strain MB43-15C transformed with the centromeric plasmids pAD6 and pH13 carrying the wild type *NAM9* and the mutated *NAM9-1* allele, respectively. Cells were grown in minimal WO-ura glucose medium. RNA was hybridised to the *NAM9* probe, as was described in legend to Fig. 1. As a control for RNA loading the same blot was hybridised with the actin probe (*ACT1*). B. Western analysis. W303-1B cells harbouring pFL-NAM9/HA or the pFL-NAM9-1/HA plasmid, carrying the epitope-tagged *NAM9* alleles, were grown in WO-ura medium supplemented with 2% galactose. Immunoblot analysis was carried out as described in legend to Fig. 1.

The level of Nam9p is affected by the *NAM9-1* missense mutation

The *NAM9-1* mutation is a single amino acid substitution Ser82 → Leu in the region homologous to the ribosomal protein S4 from *Escherichia coli*. This mutation affects growth on media containing nonfermentable carbon sources and acts as a nuclear suppressor of some mitochondrial *mit⁻* ochre mutants [6, 7]. The amount of the transcript was determined for two transformants of *NAM9-1* mutant: with single copy plasmid carrying the wild type and analogous plasmid carrying the mutated *NAM9* gene (pAD6 and pH 13, respectively) (Fig. 3A). The level of the mRNA was 2.5 times higher for the transformant harbouring the wild type gene. The copy number of pH 13 and pAD6 were the

same as was confirmed by hybridisation to an *URA3*-specific probe (not shown). A slight decrease of the *NAM9* transcript was also observed in the originally isolated *NAM9-1* mutant, as compared with the wild type isogenic strain (Murawski, unpublished). The mechanism leading to the different mRNA levels is unknown. It is possible that the mutation specifically changes the structure of *NAM9* mRNA and affects its stability. Several examples of mRNA-destabilising sequences within ORF have been described [20].

The wild type Nam9p accumulated in the cells is 9.7 times higher (Fig. 3B) as compared to mutated Nam9-1p. Our observations lead to the conclusion that overexpressed wild type protein is more abundant than the overexpressed mutated protein. We can only speculate about the bearing of these results on the physiological level of mutated Nam9-1p in the cell.

Sequence analysis of the 5' upstream region of the *NAM9* gene: looking for possible regulatory elements

The principal function of the mitochondrial translation system in yeast is to synthesise several subunits of the respiratory enzyme complexes, therefore the synthesis of mitoribosomal proteins is expected to be repressed during non-respiratory growth in the presence of glucose. In fact glucose repression is a common feature in the regulation of nuclear genes coding for mitochondrial proteins. No common *cis*-acting sequence controlling the synthesis of mitoribosomal proteins has been found by computer search of the available *MRP* genes [21]. The role of the HAP2/3/4 complex in the co-ordination of *MRP* transcription has been discussed [18]. About half of the *MRP* genes, including *MRP1*, 2, -L6, 7, 13, -L13, 20, 49 and *YMR26* which are known to respond to glucose repression, have 5' sequences that matched to the HAP2/3/4 consensus sequence, although this match could be not perfect [5, 18, 21].

The sequence 5'-TGATTGAT-3' found at position -234 to -226 upstream of the AUG in the *NAM9* gene differs from the HAP2/3/4 consensus TNPuTTGGT by one nucleotide.

Therefore the HAP2/3/4 complex could be involved in the regulation of *NAM9* transcription. Surprisingly a sequence -220(5'-CGTTAAGCACGAT-3')-208, localised close to the putative HAP2/3/4 motif in the *NAM9* gene, matches perfectly the consensus sequence of the ABF1 transcription factor (CGTNNNNNPuPyGAPy). ABF1 has been described as a multifunctional DNA binding protein involved in coupling of the rate of mitochondrial biosynthesis to cellular growth [22] as well as in the co-ordination of cytosolic ribosomal protein synthesis [2]. Close localisation of HAP2/3/4 and ABF1 binding sites was detected previously in the promoter region of the *QCR8* gene encoding a subunit of the mitochondrial ubiquinol-cytochrome oxidase [23, 24]. ABF1 is likely to interact with the HAP2/3/4 complex influencing the basal and induced *NAM9* transcription levels. Identification of ABF1 and HAP2/3/4 consensus sequences as possible regulatory elements is consistent with the mitoribosomal character of the Nam9p.

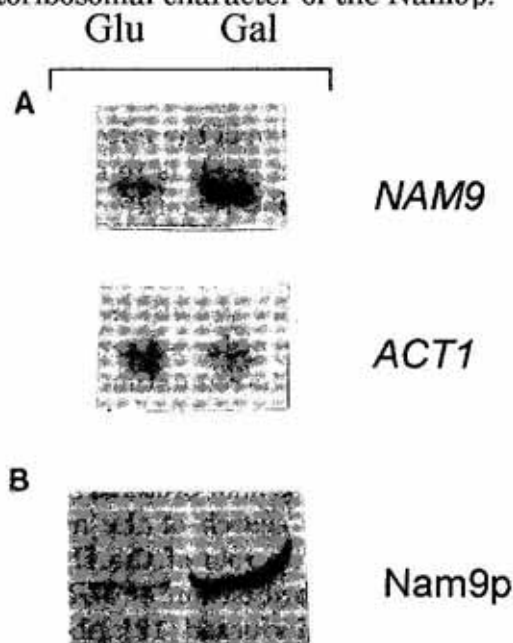


Figure 4. Catabolite repression of *NAM9*.

A. Northern analysis. Yeast strain KL14-4A was grown in YPG 2% glucose (Glu) or YPGALA 2% galactose (Gal) medium. Hybridisation was carried out as described in legend to Fig. 1. B. Western analysis. Yeast strain W303-1B harbouring the pFL-*NAM9*/HA plasmid, carrying the epitope-tagged *NAM9* gene, was grown in WO-ura medium supplemented with appropriate 2% glucose (Glu) or 2% galactose (Gal). Immunoblot analysis was carried out as described in legend to Fig. 1.

NAM9 expression is controlled by carbon source

Steady state levels of the *NAM9* mRNA and protein were determined in the wild type cells growing under catabolite repression in 2% glucose and in derepressed conditions in 2% galactose. The results of Northern and Western blot analyses are shown in Fig. 4. The level of *NAM9* transcript was 2.9 times higher in derepressed cells (Fig. 4A), thus the transcription of this gene appears to be regulated by glucose repression. The response to carbon source was even more pronounced for the Nam9 protein levels (Fig. 4B). The difference was sevenfold implicating a posttranscriptional component in the regulation of the *NAM9* expression.

All twelve mitochondrial ribosomal proteins studied so far in respect of the control of gene expression were regulated to various extent in response to glucose (for references see [5]). Although the primary control seems to occur at the level of transcription, the differences in protein levels often are higher than those observed for the corresponding transcripts. This indicates a posttranscriptional modulation of the response to catabolite repression. The most pronounced is the case of the *MRP7* gene, regulated by carbon source only at the level of translation [13].

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