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QUARTERLY

The *KRR1* gene encodes a protein required for 18S rRNA synthesis and 40S ribosomal subunit assembly in *Saccharomyces cerevisiae*★✪

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The newly discovered *Saccharomyces cerevisiae* gene *KRR1 (YCL059c)* encodes a protein essential for cell viability. Krr1p contains a motif of clustered basic amino acids highly conserved in the evolutionarly distant species from yeast to human. We demonstrate that Krr1p is localized in the nucleolus. The *KRR1* gene is highly expressed in dividing cells and its expression ceases almost completely when cells enter the stationary phase. *In vivo* depletion of Krr1p leads to drastic reduction of 40S ribosomal subunits due to defective 18S rRNA synthesis. We propose that Krr1p is required for proper processing of pre-rRNA and the assembly of preribosomal 40S subunits.

In the course of functional analysis of unknown open reading frames found during the sequencing of chromosome III of *Saccharomyces cerevisiae* we have identified a novel gene *KRR1 (YCL059c)* required for cell viability. It encodes a protein containing a characteristic nuclear targeting sequence highly conserved in the evolutionarily distant species from yeast to human (Gromadka *et al.*, 1996). The results of large-scale analysis of yeast gene expression, with *lacZ* inserted at random location (Burns *et al.*, 1994), indicated nucleolar localization of the Krr1 protein. Therefore to further characterize the function of Krr1p

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Abbreviations: DAPI, 4′,6-diamino-2-phenylindole dihydrochloride; pre-rRNA, precursor ribosomal RNA; RNP, ribonucleoprotein; rRNA, ribosomal RNA; snoRNA, small nucleolar RNA.

we took into consideration its possible role as a component of the complicated machinery of synthesis, modification and processing of rRNA and /or assembly of pre-ribosomes, the machinery which organizes the subnuclear structure — the nucleolus (Nierras *et al.*, 1997).

Pre-ribosome biogenesis requires coordinated synthesis of ribosomal RNA (rRNA) and ribosomal proteins. The *S. cerevisiae* genom contains 100–220 copies of rDNA located on chromosome XII (Petes, 1979). A single rDNA copy consists of two transcription units: the 35S rDNA precursor transcribed by RNA polymerase I and 5S rDNA transcribed by RNA polymerase III. The newly synthesized 35S rRNA is immediately processed into the final 18S rRNA of the 40S subunit, and 25S and 5.8S of the 60S subunit and packed with ribosomal proteins to generate ribosomes (reviewed in Venema & Tollervey, 1995). There are up to 45 different proteins in the 60S ribosomal subunit and 32 in the 40S subunit. (Warner, 1989; Verschoor *et al.*, 1998, and references therein). The ribosomal precursors contain also a number of non-ribosomal proteins required for the proper maturation and assembly of the highly structured pre-rRNAs with ribosomal proteins, which are then selectively dissociated to yield mature ribosomal subunits. The proteins must be imported from the cytoplasm, where they are synthesized, and again must cross the nuclear envelope when exported to the cytoplasm as ribosomal subunits. This process requires an additional set of proteins classified as importins and exportins (reviewed in Rout *et al.*, 1997; Weis, 1998; Ohno *et al.*, 1998; Izaurralde & Adam, 1998).

Although studies on all aspects of ribosome biogenesis have advanced rapidly the understanding of the mechanisms regulating the synthesis of equimolar quantities of ribosomal proteins and rRNA in order to construct and supply the ribosomes adequately to cellular growth rates is far from complete. Also the role and mechanism of action of the many proteins associated with pre-ribosomes is unknown.

The completion of the sequence of the *S. cerevisiae* genome and its global computional analysis brought to light the number of novel proteins predicted to be involved in ribosome biogenesis, making the picture of this process even more complicated. According to the data available in the MIPS protein data base, 80 ORFs are assigned as encoding large cytoplasmic ribosome subunit and 56 as encoding the small one. To these genes one has to add 39 ORFs encoding proteins involved in rRNA synthesis and 54 ORFs encoding proteins involved in rRNA processing. So far products of 41 ORFs have been localized to the nucleolus. It appears that most of the products of genes involved in rRNA synthesis and processing are essential, as is the case of *KRR1* (Gromadka *et al.*,1996). In this work we present data indicating that depletion of the Krr1 protein has an impact on the 40S ribosomal subunit biogenesis resulting from a decrease in the production of 18S rRNA. The primary defect is apparently due to inefficient processing of 35S rRNA at the A_0, A_1 and A_2 clevage sites.

MATERIALS AND METHODS

Media and growth condition. The *S. cerevisiae* strains and plasmids used in this study are described in Table 1 and Table 2, respectively. *Escherichia coli* DH5α was used for plasmid preparation (Sambrook *et al.*, 1989). Standard complete YEPD, YEPGal, minimal SD and SC-dropout media were used (Adams *et al.*, 1997). For biochemical analysis, cells were grown in liquid media at 30°C with vigorous areation. Growth rate was followed by counting cells in Thomas' camera or measurement of absorbance at 600 nm.

Genetic analysis. Standard media and procedures were used for crossing, sporulation

Table 1. Strains used in this study*

Strains	Relevant genotype
W303-1B	*MATa*
FA29/1**	*MATa KRR1/krr1*Δ*::HIS3*
GR18/1-6	*MATa/a KRR1/krr1*Δ*::HIS3* pRG33(UAS*GAL10*-P*CYC1*-*KRR1, URA3, CEN6*)
GR18/1-6-1A	*MATa krr1*Δ*::HIS3* pRG33
GR18/1-6-1B	*MATa KRR1* pRG33
GR18/1-6-1C	*MATa KRR1* pRG33
GR18/1-6-1D	*MATa krr1*Δ*::HIS3* pRG33
GR19	*MATa/a KRR1/krr1*Δ*::HIS3* pRG38 (UAS*GAL10*-P*CYC1*-*UBI-HA-KRR1, URA3, CEN6*)
GR19/1	*MATa KRR1* pRG38
GR19/2	*MATa krr1*Δ*::HIS3* pRG38)
GR19/3	*MATa krr1*Δ*::HIS3* pRG38)
GR19/4	*MATa KRR1* pRG38)
GR24/2	*MATa/a trp1-1/TRP1::HA-KRR1 KRR1/krr1*Δ*::HIS3*
GR24/2-2A	*MATa TRP1::HA-KRR1 krr1*Δ*::HIS3*)
GR24/2-3A	*MATa TRP1::HA-KRR1 krr1*Δ*::HIS3*

*All strains are derivatives of W303 (Thomas & Rothstein, 1989) and harbour the following additional mutations: *ade2-1, leu2-3,112, trp1-1, his3-11, ura3-1, can1-100, mit*$^{+}$*, rho*$^{+}$. All GR strains were constructed in this study. **Gromadka *et al.*, (1996).

and tetrad analysis (Rose *et al.*, 1990). Diploid strains were obtained by separating zygotes from a mixture of cells by micromanipulation.

DNA manipulations. Routine DNA manipulations: plasmid preparation, subcloning, transformation and transfection of *E. coli,*

Table 2. Plasmids used in this study

Plasmids	Description
pYeDP13	*E. coli/S. cerevisiae,* UAS*GAL10*-P*CYC1,* T*PGK, URA3,* 2μ (Nasr *et al.*, 1994)
pGUR	*E. coli/S. cerevisiae,* UAS*GAL10*-P*CYC1*-*UBI-HA,* T*PGK, URA3,* 2μ (C. Cullin unpubl.)
pRS304	*E. coli/S. cerevisiae, TRP1* (Sikorski & Hieter, 1989)
pRS316	*E. coli/S. cerevisiae, URA3, CEN6* (Sikorski & Hieter, 1989)
pRG14	*E. coli/S. cerevisiae, KRR1, URA3,* 2μ, (Gromadka *et al.*, 1996)
pRG32	*E. coli/S. cerevisiae,* PCR product carrying *KRR1*(primers RG104 and RG105) cloned by gap-repair between UAS*GAL10*-P*CYC1* and T*PGK* into plasmid pYeDP13 (*URA3,* 2μ)
pRG33	*E. coli/S. cerevisiae, Sca*I-*Sca*I fragment carrying *CEN6/ARS* cassette (from pRS316) cloned by gap-repair into pRG32 (replacement 2μ cassette)
pRG38	*E. coli/S. cerevisiae,* PCR product carrying *KRR1* (primers RG109 and RG110) cloned by gap-repair between UAS*GAL10*-P*CYC1*-*UBI-HA* and T*PGK* into plasmid pGUR (*URA3,* 2μ)
pRG44	*E. coli/S. cerevisiae,* PCR product carrying *HA-KRR1* (primers reverse and product of PCR from primers RG115 and RG61) cloned by gap-repair into plasmid pRG14 after digestion with *Bam*HI/*Bal*I (*TRP1,* 2μ)
pRG45	*E. coli/S. cerevisiae, Pst*I-*Kpn*I fragment carrying *HA-KRR1* cloned between same site of pRS304 (*TRP1*)

and agarose gel electrophoresis were carried out as described in Sambrook *et al.* (1989). To release plasmid DNA from yeast cells for the transformation of *E. coli* and to prepare chromosomal DNA for PCR, the procedures described by Hoffman & Winston (1987) were used. Oligonucleotide primers were prepared using a Beckman Oligo 1000 M DNA Synthesizer according to manufacturer's instructions. Sequencing reactions were carried out using an ABI Prism BigDye terminator cycle sequencing ready reaction kit with unlabelled internal primers. Sequencing reactions were analyzed on an ABI310 Genetic Analyser (Perkin-Elmer).

Plasmid construction. Plasmids and primers are listed in Table 2 and Table 3, respectively. Plasmid pRG32 was constructed by gap-repair using a PCR generated cassette of pRS316 (Sikorski & Hieter, 1989), which were co-transformed into yeast cells. Plasmid pRG38 was constructed by gap-repair using a PCR generated cassette. Yeast cells were co-transformed with plasmid pGUR (C. Cullin, unpubl., vector) digested by *Not*I/*Bsu*36I and the PCR generated cassette of *KRR*1 (primers RG109, RG110) which was flanked by at least 50 nucleotides identical to the plasmid sequence and selected for Ura^+.

The plasmid pRG44 was made in two PCR steps. First the *KRR1* gene with the HA-epitope was generated. This product was used as primer in second PCR to generate HA-*KRR1* fusion under control of the *KRR1* promoter. Yeast cells were co-transformed with this product and plasmid pRG14 (Gromadka *et al.*, 1996) digested with *Bam*HI/*Bal*I and selected for Trp^+. Plasmid pRG45 was constructed by

Table 3. Primers used in this study

Primers [1) 2) 3)]	Description
RG61	*GAT TTC TCT TTC CAT TTG C*
RG104	GTA GCA TAA ATT ACT ATA CTT CTA TAG ACA CGC AAA CAC AAA TAC ACA CA*A TGG TGT CTA CAC ATA ACA G*
RG105	TTC CAA TAA TTC CAA AGA AGC ACC ACC ACC AGT AGA GAC ATG GGA GAT CC*C TAA TTT TGG TTT GGC TTG T*
RG109	CTC CCC GCG CGT TGG CCG ATT CCT ACC CAT ACG ACG TTC CAG ACT ACG CT*A TGG TGT CTA CAC ATA A*
RG110	CAA AGA AGC ACC ACC ACC AGT AGA GAC ATG GGA GAT CCC CCG CGA ATT CC*T TAC TAA TTT TGG TTT G*
RG115	*AAT GTA GAC AGC AAA CAA AAG GGT CAC ACA AAT ATC CAA ATT GGC AAA CG***A TGG GCG GCC GCT ACC CAT ACG ACG TTC CAG**
reverse	CAG GAA ACA GCT ATG ACC

[1)] Italicized – sequence of chromosome III, Accession Number X59720. [2)] Underlined – vector sequence. [3)] Bold – sequence encoding HA-epitope (YPYDVPDYA)

(Orr-Weaver *et al.*, 1983). Yeast cells were co-transformed with plasmid pYeDP13 (Nasr *et al.*, 1994) digested with *Bam*HI/*Eco*RI and the PCR generated cassette of *KRR*1 (primers RG104, RG105) flanked by at least 50 nucleotides identical to the plasmid sequence and selected for Ura^+. Plasmid pRG33 was constructed by gap-repair using the 3.2 kb *Pst*I fragment of pRG32 carrying UAS_{GAL10}-P_{CYC1}-*KRR1* hybrid and the 1.7 kb *Sca*I fragment inserting the 1.9 kb *Pst*I/*Kpn*I fragment from plasmid pRG44 into plasmid pRS304.

PCR conditions. PCR was performed in a Perkin Elmer GeneAmp PCR System 9600 with the kit "Expanded High Fidelity PCR System" Boehringer Mannheim according to the manufacture instruction.

RNA extraction. Total RNA was extracted from cells grown in 100 ml of YEPD medium to A_{600} of 0.2–0.3 using the acid-phenol

method as described (Ausubel *et al.*, 1997). An inoculum was pregrown in YEPGal over night.

Northern blotting. RNA was separated by electrophoresis in vertical 1.2% agarose formaldehyde gels as described in Current Protocols (Ausubel *et al.*, 1997). Equal amounts (4 μg) of RNA were loaded in each lane. RNA was transferred to Hybond-N (Amersham) according to the manufacturer's instruction.

Small RNAs were resolved in 6% polyacrylamide (19 : 1) gels containing 7 M urea and 0.6 × Tris-borate/EDTA electrophoresis buffer (TBE) buffer. RNA, in 50% formamide, heated at 95°C for 2 min, then chilled on ice was applied on the gels. The RNA was subsequently transferred electrophoretically to Hybond-N (Amersham) at 300 mA, overnight, in 0.5 × TBE.

Prehybridization was at 65°C. Hybridization with oligonucleotide probes was at 37°C in 6 × SSPE, 0.5% SDS, 5 × Denhardt's reagent and 200 μg/ml denatured herring sperm DNA. The filters were washed three times at 37°C for 5 min in 6 × SSPE, 0.1% SDS, once at 42°C for 15 min, and exposed to X-ray film.

Probes were prepared as follows: ten picomoles of oligonucleotides were 3′-end labeled with 2 μl of (γ-^{32}P)ATP (>7000 Ci/ mmol, >100 mCi/ml, end-labeling grade, ICN Biomedical, Inc.) by using T4 polynucleotide kinase. The sequence of probes are listed in Table 4.

Polysome analysis and total ribosomal subunit quantification. Yeast were grown in 100 ml of YEPD or YEPGal medium at 30°C to an A_{600} of about 0.7. Polysome preparation and gradient analysis were done according to Weaver *et al.* (1997). Ten A_{260} units of polysomes were loaded on a 30 ml sucrose gradient (7–47%) and centrifuged for 5 h at 22 000 r.p.m. at 4°C in SW 28.1 rotor. The sucrose gradients were analyzed at 254 nm.

Western immunoblot analysis. 6 × 10^7 cells grown in YEPD were harvested by centrifugation, resuspended in 80 μl of 1.85 M NaOH and 3.7 μl β-mercaptoethanol and incubated at 0°C for 10 min. Proteins were precipitated with 83 μl 50% trichloroacetic acid (TCA), incubated at 0°C for 10 min and collected by centrifugation. The pellet was rinsed with 0.5 ml of 1 M Tris/base, and resuspend in 85 μl of sample buffer (100 mM Tris/HCl, pH 6.8, 4 mM EDTA, 4% SDS, 20% glycerol, 0.002% bromphenol blue, 4% β-mercaptoethanol) and boiled for 4 min. Proteins from 7 × 10^6 cells were separated on SDS/12% polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting. Mouse monoclonal anti-HA antibody (clone 16B12, BabCo) was used as primary antibody at 1 : 1000 dilution, and goat anti-mouse alkaline

Table 4. Oligonucleotides used for Northern hybridization:

sequence	complementary to sequence*
CCAGATAACTATCTTAAAAG	A_0-A_1 cleavage sites
CATGGCTTAATCTTTGAGAC	18S RNA
GCTCTTTGCTCTTGCC	D-A_2 cleavage sites
TGTTACCTCTGGGCCC	A_2-A_3 cleavage sites
CCAGTTACGAAAATTCTTG	A_3-B_L cleavage sites
GGCCAGCAATTTCAAGTTA	E-C_2 cleavage sites
CTCCGCTTATTGATATGC	25S RNA
GGTTATGGGACTCATCAACC	snoRNA U3
ATGTCTGCAGTATGGTTTTAC	snoRNA R30

* Cleveage sites are shown in Fig. 4G.

phosphatase conjugated antibody was used as the secondary antibody at 1:2500 dilution, and detected with the Amersham CDP-*Star* detection system according to manufacturer's instruction.

Indirect immunofluorescence microscopy. Detection of the cellular HA-Krr1p protein by indirect immunofluorescence microscopy was performed after Pringle *et al.* (1991). As the first antibody anti-HA mouse antibody was used (raw ascites fluid, clone 16B12, BabCo) at 1:750 dilution, and goat anti-mouse Cy3 conjugated antibody (Jackson Immuno-Research Lab., Inc.) was used as the secondary antibody at 1:250 dilution. The samples were stained for DNA with DAPI (4′,6-diamino-2-phenylindole dihydrochloride) at final concentration of 0.04 mg/ml for 2 min. Than cells were washed twice with water. Samples were viewed at 600× magnification on Mikrophot-SA microscope equipped with filters for epifluorescence and photographs were taken through a 60× objective on Kodak Gold 400 film.

RESULTS

Immunolocalization of epitope-tagged Krr1p

Previous data pointed to nucleolus localization of Krr1p (Burns *et al.*, 1994; Gromadka *et al.*, 1996). In order to confirm the localization of the protein we constructed a fusion between Krr1p and influenza virus hemaglutinin epitope HA. The sequence, which codes for a 9-amino-acid epitope, was inserted in frame before the first ATG of the *KRR1* coding sequence. 200 nucleotide sequence of the *KRR1* promoter region preceded the insertion. The heterozygous diploid *krr1*Δ*::HIS3 trp1/KRR1 trp1* was transformed with *KRR1-HA* fusion on the integrative pRG45 vector (Table 1). The hybrid gene under its own promoter was integrated into the *TRP1* locus. After selection for tryptophan prototrophy diploids were sporulated and tetrad analysis was performed. The Trp^+ His^+ spore clones, in which the *KRR1-HA* fusion and *KRR1* null allele co-segregated, were viable and formed normal sized colonies. In liquid media the mutant grew with the same growth rate and efficiency as the wild type. This indicated that the HA tagged protein was functioning in the cell as well as the wild type protein. Therefore we reasoned that the subcellular localization of HA-Krr1p should parallel that of native protein. Consequently indirect immunofluorescence microscopy was used to examine the subcellular localization of HA-Krr1p.

As shown in Fig. 1 the immunofluorescence is restricted to the nucleus. In the experiment presented the inoculum was pregrown in YPD medium untill late stationary phase. There were two hours of lag before cells started the growth. Already in cells entering from the lag into the exponential phase of growth the increased amount of the HA-Krr1 protein spreading throughout the nucleus was visible, whereas in the exponential phase the intensive fluorescence became compactly arranged in a crescent-shaped region around one side of the nucleus and did not superimpose the DAPI staining. Such a picture is characteristic for proteins localized into yeast nucleolus (Weaver *et al.*, 1997; Kressler *et al.*, 1998). However, in cells entering stationary phase the fluorescence declined and appeared much more diffuse in the nucleus. On the basis of microscopic observation, we concluded that Krr1p is localized predominantly in the nucleolus and its synthesis depends on the growth phase.

Expression of *KRR1* changes during the cell growth

The changes in the synthesis of Krr1p in response to the growth rate have been confirmed by quantitative immunoblot analysis. The level of the HA-Krr1 protein was assayed in cells grown in reach glucose medium. The culture was inoculated with cells from late sta-

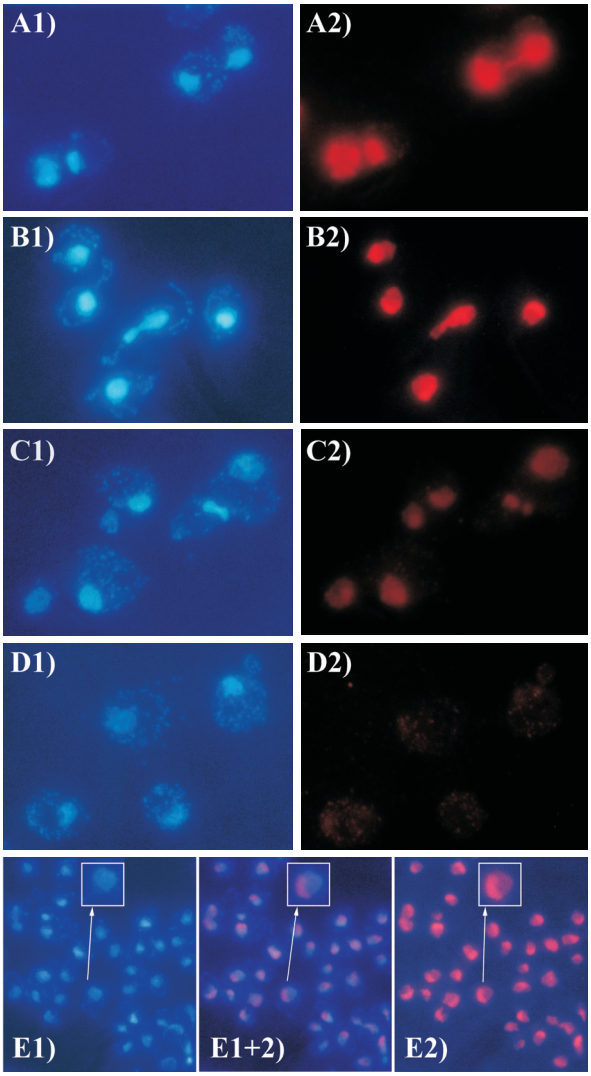

Figure 1. Nucleolar localisation of Krr1p.

The same fields were viewed for DNA by DAPI staining (left) and for HA-Krr1p by anti-HA antibody immunofluorescence (right). Cells of strain GR24/2-2A (*TRP1::HA-KRR1, krr1*Δ*::HIS3*) were incubated with mouse anti-HA antibody followed by Cy3 conjugated goat anti-mouse antibody. The samples were taken at different growth phases: 2 h lag phase (A), early log phase (B), late log phase (C), and stationary phase (D). In the last row (E) – the sample was taken at early log phase, pseudocolours were assigned to digitised micrographs (E1 and E2) and images were merged (E1+2).

tionary culture. As presented in Fig. 2A after two hours of lag cells started to divide with generation time of 70 min, after 12.5 h the growth slowed. Samples for Western blotting were taken at several time points (Fig. 2A). The results presented in Fig. 2B clearly demonstrate the variation of Krr1p synthesis with growth rate. The increased synthesis of Krr1p preceded the entrance of the cells into the logarithmic phase of growth, it was highest in early log phase, decreased in cells entering stationary phase and almost completely ceased at late stationary phase. Such a pattern is characteristic for changes in the synthesis of rRNA and ribosomal proteins during the growth cycle of yeast cells (Ju & Warner, 1994).

Construction of conditional alleles of *KRR1*

To further analyze the essential Krr1p function we used the "promoter shut-off" approach. The *KRR1* coding sequence was put under the control of the conditional UAS_{GAL10}-P_{CYC1} promoter in the centromeric vector pRG33 (Table 2). In this system, expression of the gene is induced by galactose and repressed by glucose (Johnston & Davis, 1984). Since this promoter did not create protein depletion sufficient to give a clear-cut phenotype, to construct an allele of *KRR1* which would be more successfully and rapidly downregulated the galactose system was combined with a ubiquitin-dependent degradation signal. In this system protein produced at a low level in the presence of glucose is rapidly degraded (Park *et al.*, 1992). The fusion was made using the plasmid pGUR (Table 2).

The effect of Krr1p depletion was studied in strains GR18/1-6-1D and GR 19/2 bearing the *KRR1* deletion. In these strains Krr1p was produced from the plasmid borne UAS_{GAL10}-P_{CYC1}-*KRR1* or UAS_{GAL10}-P_{CYC1}-*UBI-HA-KRR1* allele, respectively. The transformants grew normally compared with isogenic *KRR1* strain both on plates and in liquid galactose medium, whereas in glucose medium the growth rate of *krr1*Δ*::HIS3* spore clones bearing the UAS_{GAL10}-P_{CYC1}-*KRR1* construct was significantly reduced and spore clones bearing the UAS_{GAL10}-P_{CYC1}-*UBI-HA-KRR1* construct did not grow (Fig. 3). In liquid YPD media wild-type cells pregrown in YPgalactose medium grew with the doubling time 1.5 h,

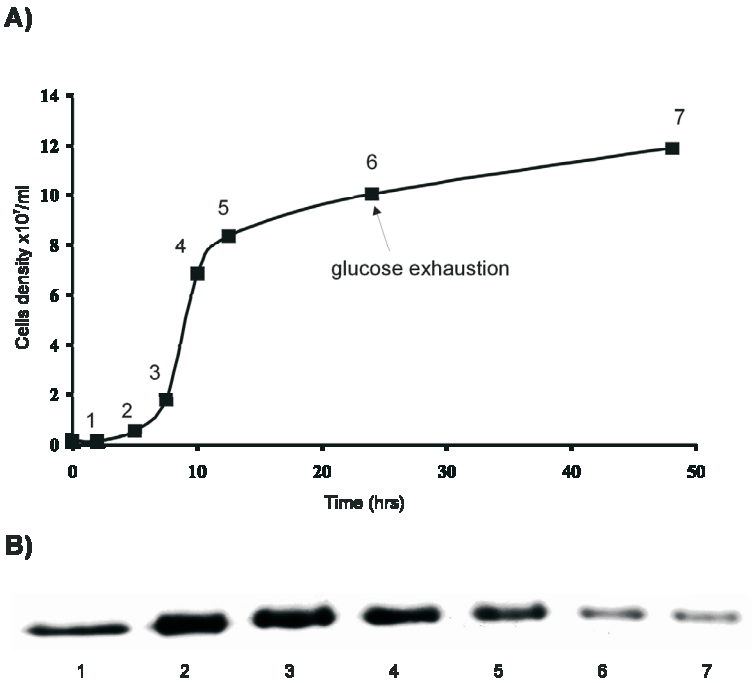

Figure 2. Kinetics of Krr1p synthesis during growth of GR24/2-2A in YEPD medium.

(A) The growth curve. The time of sample withdrawing are numbered. (B) Western blot showing Krr1p level in cells collected at the marked growth intervals.

whereas the GR18/1-6-1D strain grew with the doubling time 2.5 h and the growth rate of GR19/2 was strongly affected with the doubling time of 4–5 h. After 30 h of growth the GR18/1-6-1D strain reached the same cell density as the wild-type, whereas the cell density of GR19/2 culture was one fifth of that of the wild-type. In liquid culture cells did not cease growth completely, probably due to residual Krr1p synthesis. For further studies we chose the strain GR19/2 in which the Krr1p function is more severely affected.

18S rRNA maturation is impaired in Krr1p depleted cells

The nucleolar localisation of Krr1p together with the observed response in its synthesis to transitions in the growth rate pointed to a role of Krr1p in nucleolar processes of rRNA maturation and/or ribosome assembly. This led us to analyze the effect of Krr1p depletion on rRNA processing. Steady state levels of mature 18S and 25S RNAs (Fig. 4A) and of pre-rRNAs (Fig. 4B-F) were determined by

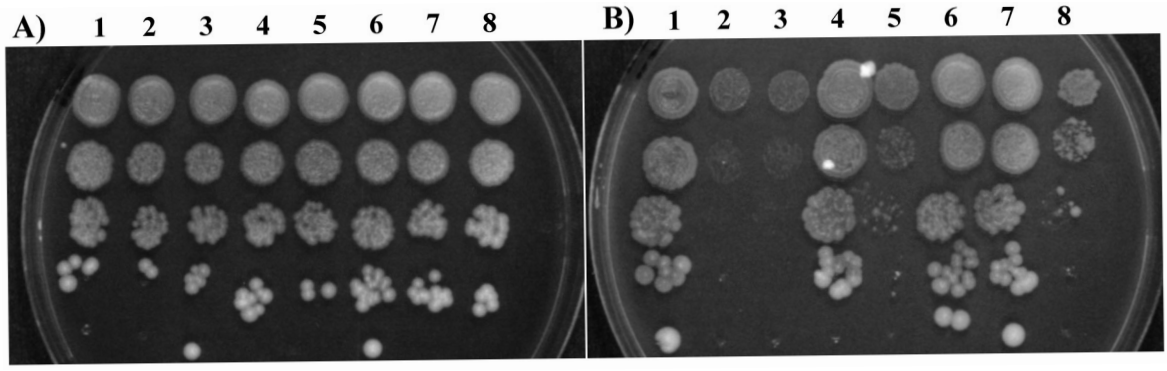

Figure 3. Effect of growth conditions on growth of Krr1p depleted cells.

Growth phenotypes of complete tetrads derivative of GR19 diploid (columns 1–4) and GR18/1-6 diploid (columns 5–8) grown on YEPGal **(A)**, and YEPD **(B)** medium. Clones 1, 4, 6 and 7 carry *KRR1* allele, clones 2, 3, 5 and 8 carry *krr1*Δ*::HIS3* allele complemented by UAS_{GAL10}-P_{CYC1}-*UBI-HA-KRR1* gene fusion (2 and 3) and UAS_{GAL10}-P_{CYC1}-*KRR1* gene fusion (5 and 8). Saturated overnight cultures in YEPGal medium were diluted and 5 μl drops of undiluted culture and serial 33-fold dilutions were spotted onto YEPD and YEPGal media.

Northern hybridization. To isolate RNA the wild-type and mutant GR19/2 cells pregrown in galactose medium were transferred to glucose medium and total RNA was isolated from wild-type cells after five generations (lane 1) and from mutant after two and five generations (lane 2 and 3, respectively). As shown in Fig. 4A, a clearly reduced level of 18S rRNA was observed in the mutant. To determine whether the reduced level of 18S rRNA resulted from defects in rRNA processing different oligonucleotides, hybridizing to defined regions of the 35S pre-rRNA transcript (Fig. 4G, Table 4), were used to monitor the processing intermediates. In Krr1p depleted cells the accumulation of 35S rRNA was observed. The loss of 27S A_2 rRNA (Fig. 4D) and 20S rRNA (Fig. 4C) and accumulation of aberrant 23S RNA (Fig. 4B, C, D) indicate the absence of cleavage at sites A_0, A_1 and A_2, since accumulation of 23S RNA results from direct cleavage of 35S rRNA at the A_3 site. As shown in Fig. 5E and F the processing of 27S A_3 rRNA and the unchanged level of 25S rRNA (Fig. 4A) demonstrate correct 25S rRNA maturation in Krr1p depleted cells. The observed defect in A_0, A_1 and A_2 cleavage in the GR19/2 mutant was not caused by changed levels of U3 and R30 snoRNAs since in all samples tested the steady-state level of these RNAs was the same (data not shown). The prolongation of growth of the mutant strain in glucose medium (5 generations, 24 h) slightly reduced the differences of hybridization patterns between wild-type and mutant (Fig. 4 B–D path 1 and 3), which probably re-

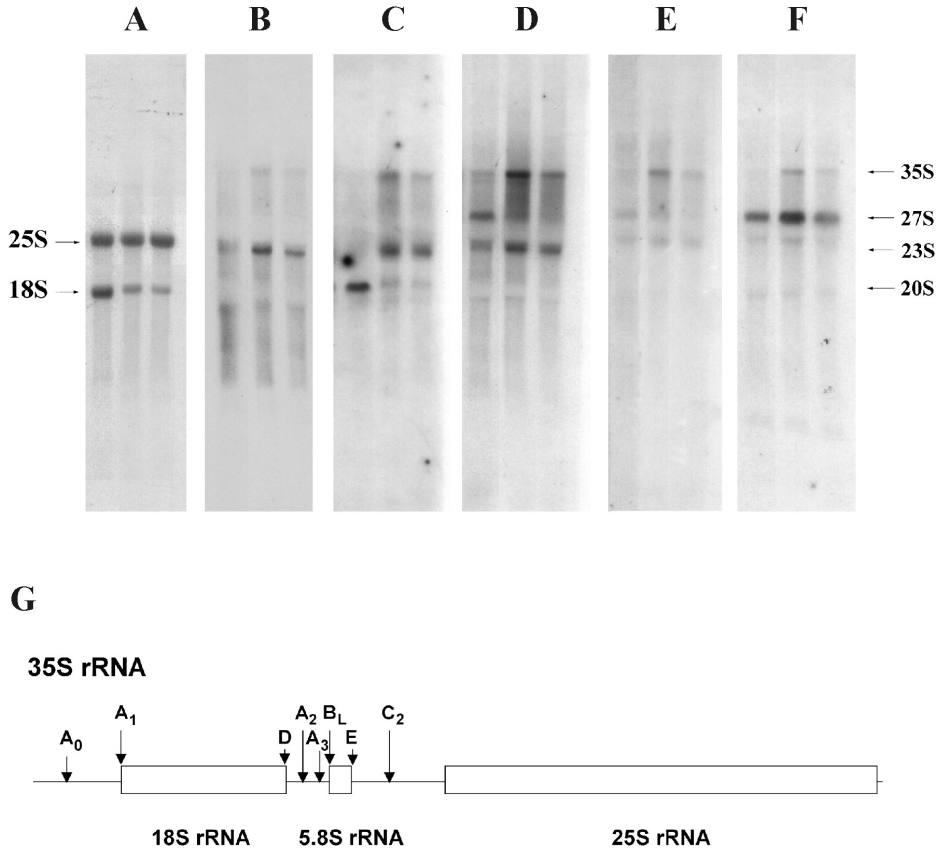

Figure 4. Maturation of rRNA.

Pre-rRNA processing in strain with the *KRR1* gene expressed from plasmid pGR38 (strain GR19/2 lanes 2 and 3) as compared with wild-type (lane 1). RNA was extracted from cells shifted from YEPGal to YEPD for 8 h (lanes 1 and 2), and 24 h (lane 3). Probes against **(A)** mature 18S and 25S rRNA, **(B)** A_0/A_1 site, **(C)** D/A_2, **(D)** A_2/A_3, **(E)** A_3/B_{1L}, **(F)** E/C_2, and **(G)** simplified overview of 35S pre-rRNA with marked processing sites and position of oligonucleotides used to probe the blot (Table 4). Processing intermediates and products are indicated.

flects the fact that the decreased amount of Krr1p only slowed down the 18S rRNA processing; otherwise the complete depletion of protein and block in rRNA processing leads to cell death. In the control experiment no differences in rRNA levels were seen between wild type and GR19/2 strains grown in galactose medium (not shown).

Depletion of Krr1p affects the level of 40S ribosomal subunit

Ribosome assembly begins as soon as the pre-rRNA is synthesized. Packing of newly synthesized rRNA with ribosomal proteins takes place in the nucleolus, therefore to test whether the delay in pre-rRNA processing caused by Krr1p depletion affects also the ribosome formation, polyribosome profiles from the strains *KRR1/* UAS_{GAL10}-P_{CYC1}-*UBI-HA-KRR1* and *krr1*Δ/UAS_{GAL10}-P_{CYC1}-*UBI-HA-KRR1* grown in galactose and glucose medium were analyzed. Results presented in Fig. 6 show that polyribosome profiles of the wild-type and mutant strain grown in galactose medium are identical (Fig. 5A and 5C). Shifting the wild-type cells to glucose medium did not changed the ribosomal profile (Fig. 5B) whereas the lowered level of Krr1p in mutant cells grown in glucose led to severe depletion in 40S subunits, a large excess of free 60S subunits, and reduced amounts of mono- and polyribosomes (Fig. 5D). The results of polyribosome gradient analysis are in good agreement with the observed reduction in 18S rRNA level in Krr1p depleted cells.

DISCUSSION

In this paper we describe functional analysis of the novel gene *KRR1* (uncharacterized open reading frame *YCL059c*) whose product appears to be an essential component of the machinery of rRNA maturation and ribosome biogenesis. Our initial functional analysis showed that Krr1p is essential for cell viability (Gromadka *et al.*, 1996). Subsequent exper-

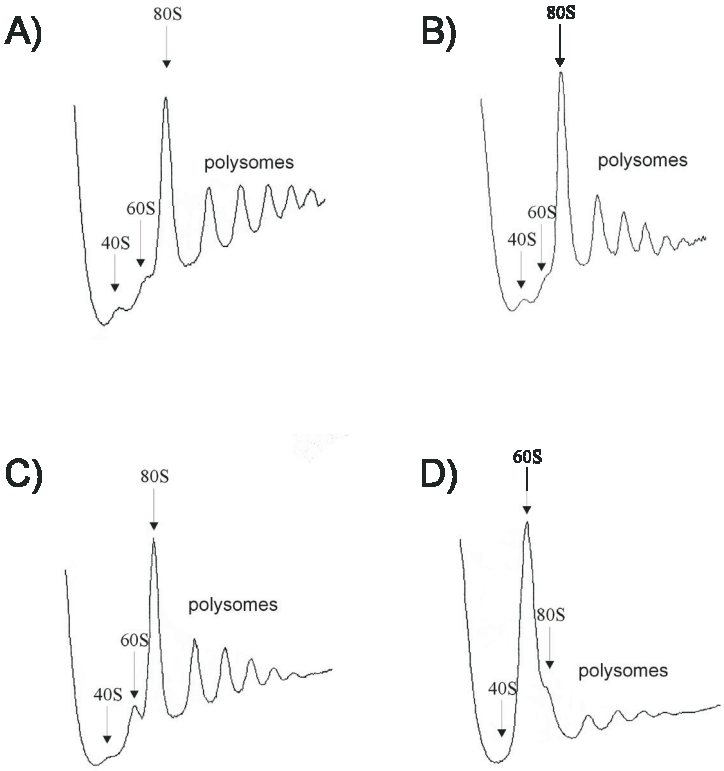

Figure 5. Aberrant polysome formation in Krr1p depleted mutant.

(A) and **(B)** GR19/4 (*KRR1* pRG38) grown in YEPGal and YEPD, respectively; **(C)** and **(D)** GR19/2 (*krr1*Δ*::HIS3,* pRG38) in YEPGal and YEPD, respectively.

iments with the HA-Krr1p fusion showed that the protein is localized exclusivly in the nucleus and in intensively dividing cells, collected in the exponential phase of growth, it is associated with the nucleolus (Fig. 1). Single copy of the fusion gene was integrated into the genome of *krr1*Δ strain and expressed from the wild-type *KRR1* promoter, which means that we have avoided the possible miss-localization of the protein resulting from its over-expression. The changes of Krr1p content depending on the growth rate observed by microscopic inspection were confirmed and quantified by Western analysis (Fig. 2B). The amount of the Krr1 protein in cells from stationary phase of growth constituted about one fifth of that synthesized in exponentially growing cells. A most interesting feature is the increase of the Krr1 protein synthesis prior to the first division as if the *KRR1* gene got the signal "cells are ready to divide". Studies on the effects of growth rate on the levels of rRNA in yeast cells revealed that in nutritional shift-up experiments the synthesis of rRNA and ribosomal proteins increases in parallel with increased growth rate (Waldron & Lacroute, 1975; Waldron, 1977; Kief & Warner, 1981). Ju & Warner (1994) in their experiments followed the rate of ribosome synthesis during the yeast growth cycle. They demonstrated that transcription of rRNA begins to decline at an early stage in the growth cycle reaching 50% of the initial value and it is accompanied by a decrease in the ribosomal protein mRNAs to about 25% of the maximum, however, after a short period of an increased turnover of ribosomes their number remains stable. The observed changes in Krr1p content resemble more closely that described for rRNA synthesis than for the ribosomal proteins. Our results together with the literature data led us to conclude that Krr1p plays a role in processing of pre-rRNA and / or in the formation of intermediates of pre-40S subunit within the nucleolus.

The growth of *KRR1* deleted strain bearing the conditional UAS_{GAL10}-P_{CYC1}-*UBI-HA-KRR1* allele was strongly affected when cells were grown on glucose medium. The reduced level of Krr1p in such conditional mutant grown in glucose medium led to the impairment in 18S rRNA synthesis which seems to be the primary defect leading to the deficiency in 40S ribosomal subunits. The analysis of the steady-state level of pre-rRNA processing intermediates indicates that Krr1p is required for proper clevage of pre-rRNA at sites designates as A_0, A_1 and A_2 (Venema & Tollervey, 1995). The properties of GR19/2 mutant resembled those described for Rrp7p-depleted cells (Baudin-Bailleu *et al.*, 1997). These authors proposed that the deficiency in the 40S subunit level was a consequence of impaired accumulation of 18S rRNA in mutant cells. However, they did not exclude the possibility that the primary defect may be preribosome assembly, rather than the impairment in pre-rRNA processing. We expect that further experiments on isolation and characterization of suppressors alleviating the effect of Krr1p depletion will provide a tool to find other proteins involved in the formation of protein-rRNA complexes.

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