Dual Function of eIF3j/Hcr1p in Processing 20 S Pre-rRNA and Translation Initiation*

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Once the genetic information is transcribed into mRNA, its final translation into a protein requires charged tRNAs and the translation machinery, consisting of the ribosome and soluble translation factors. The assembly of 40 S and 60 S ribosomal subunits occurs primarily in a specialized subnuclear compartment termed the nucleolus, but the final steps occur in the cytoplasm. A large number of proteins and small nuclear RNAs participate in ribosome biogenesis (for review see Ref. 1). In the process of translation initiation, several translation initiation factors (eIFs)\(^\text{1}\) orchestrate the assembly of an 80 S initiation complex to produce the 48 S complex (2).

Hcr1p, a yeast ortholog of the \(j/p35\) subunit of the human translation initiation factor 3 (eIF3) (3), was identified as a high copy suppressor of the temperature-sensitive (Ts\(^-\)) phenotype of the \(rpg1-1\) allele of TIF32/RPG1, encoding the largest subunit of yeast eIF3 (eIF3a) (4, 7). The eIF3 complex stimulates several steps in the translation initiation pathway, including dissociation of 80 S ribosomes into 40 S and 60 S subunits, binding of a ternary complex (TC) consisting of Met-tRNA\(^{\text{Met}}\), eIF2, and GTP to the small subunit (forming the 43 S preinitiation complex) and recruitment of mRNA to the 43 S complex to produce the 48 S complex (2).

Once the genetic information is transcribed into mRNA, its final translation into a protein requires charged tRNAs and the translation machinery, consisting of the ribosome and soluble translation factors. The assembly of 40 S and 60 S ribosomal subunits occurs primarily in a specialized subnuclear compartment termed the nucleolus, but the final steps occur in the cytoplasm. A large number of proteins and small nuclear RNAs participate in ribosome biogenesis (for review see Ref. 1). In the process of translation initiation, several translation initiation factors (eIFs)\(^\text{1}\) orchestrate the assembly of an 80 S initiation complex to produce the 48 S complex (2).


eIF3j/Hcr1p, a protein associated with eIF3, was shown to bind to, and stabilize, the multifactor complex containing eIFs 1, 2, 3, and 5 and Met-tRNA\(^{\text{Met}}\), whose formation is required for an optimal rate of translation initiation. Here we present evidence that eIF3j/Hcr1p is an RNA binding protein that enhances a late step in 40 S ribosome maturation involving cleavage of the 20 S precursor of 18 S rRNA in the cytoplasm. Immunofluorescence staining shows that eIF3j/Hcr1p is localized predominantly in the cytoplasm. The her1\(\Delta\) mutant exhibits a decreased amount of 40 S subunits, hypersensitivity to paromomycin, and increased levels of 20 S pre-rRNA. Combining the her1\(\Delta\) mutation with drs2\(\Delta\) or rps20\(\Delta\), deletions of two other genes involved in the same step of 40 S subunit biogenesis, produced a synthetic growth defect. p35, the human ortholog of eIF3j/Hcr1p, partially complemented the slow growth phenotype conferred by her1\(\Delta\) when overexpressed in yeast. heIF3j/p35 was found physically associated with yeast eIF3 and 43 S initiation complexes in vitro and in vivo. Because it did not complement the 40 S biogenesis defect of her1\(\Delta\), it appears that heIF3j can substitute for eIF3j/Hcr1p only in translation initiation. We conclude that eIF3j/Hcr1p is required for rapid processing of 20 S to 18 S rRNA besides its role in translation initiation, providing an intriguing link between ribosome biogenesis and translation.

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\(\text{1}\) The abbreviations used are: eIF, eukaryotic translation initiation factor; Met-tRNA\(^{\text{Met}}\), methionyl initiator tRNA; RRM, RNA recognition motif; MFC, multifactor complex; kb, kilobase pair(s); ORF, open reading frame; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; WCE, whole cell extract; PIPES, 1,4-piperazinediethanesulfonic acid; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; hc, high copy plasmid; sc, single copy plasmid; P/M, polysome/monosome.
We demonstrated recently that a network of interactions physically links eIF3j/Hcr1p to two of the largest subunits of eIF3, eIF3a/Tif32p, and eIF3b/Prt1p (8). eIF3j binds to an RNA recognition motif (RRM) in eIF3b and makes multiple contacts with eIF3a. Interestingly, eIF3a contains an internal domain related to eIF3j, which is part of its binding domain for the eIF3b RRM. Moreover, eIF3j and eIF3a can bind simultaneously to the eIF3b RRM. These findings, plus the fact that overexpressing eIF3j suppresses the Ts phenotype of mutations in eIF3a and eIF3b, suggest that eIF3j enhances one or more critical functions of the eIF3 complex in translation initiation (8).

We also showed recently that yeast eIF3, together with eIF1, eIF5, and the TC, comprise a multifactor complex (MFC) that occurs free of the ribosomes and is an important intermediate in translation initiation (9). A portion of eIF3j associated with eIF3 also resides in the MFC. The elimination of eIF3j did not prevent binding of MFC components to 40 S ribosomes but affected the stability of the MFC and impeded a step later in the pathway prior to 60 S subunit joining. Consistently, eIF3j was found stably associated with 43–48 S preinitiation complexes in vivo (8). Presumably, it binds to the 40 S ribosome in association with the MFC and remains bound to the initiation complex until 60 S subunit joining occurs.

There is also evidence that eIF3j can interact with 40 S ribosomes independently of eIF3 and other components of the MFC. A temperature-sensitive mutation in the eIF3b subunit (pT7-2) results in the formation of preinitiation complexes until 60 S subunit joining occurs. Deletion of eIF3j suppresses the Ts phenotype (9). 

To place the hTIF32 cDNA (encoding human eIF3a) behind the T7 promoter, the 4.6-kb SacI-BamHI fragment of YEpLVHCR1 (8) was inserted between the SacI and BamHI sites of pT7-7 (14), generating pT7-TIF32. Because BamHI cleaves twice in the middle of hTIF32, SacI-cut YEpLV718 was digested partially with BamHI to isolate the hTIF32 open reading frame (ORF) intact. To insert the TIF32 ORF behind the T7 promoter, the 2.9-kb NdeI-PstI fragment of pGAD-TIF32 (13) was inserted between the NdeI and PstI sites of pT7-7 (14) generating pT7-TIF32. Because NdeI cuts in the middle of TIF32, intact ORF DNA was obtained by partial NdeI digestion of pGAD-TIF32.

An integrating plasmid expressing p35 (human eIF3j) from the constitutive GPD promoter was constructed as follows. A 0.8-kb BamHI-HindIII fragment bearing cDNA encoding human p35 was isolated from YEpMETp35-T (see above) and inserted between the BamHI and HindIII sites of pGAD346 (American Type Culture Collection). The resulting pG26-GPDp35 vector was digested with SacI-HindIII, and the 1.6-kb fragment carrying human p35 cDNA behind the GPD promoter was inserted between the SacI and HindIII sites of YEpLVHCR1 (11) to produce YEpGPDp35. As a last step, the latter integrative plasmid was EcoRI-digested and introduced into the YVLH3 strain.

To create pLV7T-18 S rRNA, we first generated a fragment encoding 18 S rRNA by polymerase chain reaction using S. cerevisiae genomic DNA as a template and the following primers, which introduced NdeI and HindIII restriction sites at the 5'- and 3'-ends, respectively: LV18 S1 (5’-GGAGGCATATGGATCTGTTGAGCTTCGACGACTG-3’) and LV18 S2 (5’-CCAGAAGCTTTAGAATGCTTCGGAAGTTC-3’). This fragment was then digested with NdeI and HindIII and ligated with NdeI- and HindIII-digested pT7-7 (14).

**Experimental Procedures**

**Plasmids**—Table I contains brief descriptions of all plasmids employed in this study. To construct YEpLVHCR1-U, the SalI-KpnI fragment from YEpLVHCR1 (7) was inserted into SalI-Kpn1 digested YCplac33 (11).

| Plasmid                      | Description                                      | Source                  |
|------------------------------|--------------------------------------------------|-------------------------|
| YCplac33                     | Single copy cloning vector, URA3                 | (11)                    |
| YCpLVHCR1-U                  | Single copy HCR1, URA3 plasmid from YCplac33     | This study              |
| YCpLVMETHCR1-T               | Single copy MET-HCR1, TRP1 plasmid from YCplac22 | This study              |
| YEpplac12                    | High copy cloning vector, TRP1                   | (11)                    |
| YEpMETp35-T                  | High copy MET-p35, TRP1 plasmid from YEpplac12   | This study              |
| YpGPDp35                     | Integrative plasmid carrying GPD-p35, URA3      | This study              |
| pGEX-p35                     | Cloning vector for GST fusion                   | (34)                    |
| pGEX-p35                     | GST-p35 fusion plasmid from pGEX-TIF34          | This study              |
| pT7-TIF32                    | hTIF32 ORF cloned under T7 promoter             | This study              |
| pT7-TIF32                    | hTIF3 ORF cloned under T7 promoter              | (35)                    |
| pT7-TIF34                    | Full-length 18 S rRNA cloned under T7 promoter  | (13)                    |

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—Strains YVLH3 (MATa her1Δ::LEU2 ade2-1 trp1-1 can1-100-leu2-3,112 his3-11,15 ura3-1) and YLV314L (MATa leu2Δ::LEU2::rpl1-1 trp1-1::TRP1::rpl1-1Δ ade2-1 can1-100,112 his3-11,15 ade2-1 can1-100-leu2-3,112 his3-11,15 ura3-1) were described previously. Strain W303 (MATa ade2-1 trp1-1 can1-100-leu2-3,112 his3-11,15,15 ade2-1 can1-100-leu2-3,112 his3-11,15 ura3) was obtained from A. Hopper. We purchased the following haploid deletion strains made by the Saccharomyces Deletion Project (Research Genetics): BY4741 (MATa his3Δ1 leu2Δ2 mat1Δ50 ura3Δ0), 16704 (MATa her1Δ::KanMX his3Δ1 leu2Δ2 mat1Δ50 ura3Δ0), 4844 (MATa trp1Δ::KanMX his3Δ1 leu2Δ2 mat1Δ50 ura3Δ0) and 382 (MATa trp1Δ::KanMX his3Δ1 leu2Δ2 mat1Δ50 ura3Δ0), and crossed them to obtain HCR1::her1Δ DR2/
eIF3j/Hcr1p Functions in rRNA Processing and Translation

drs2Δ and HCR1/hcr1Δ RP50A::rp50Δ diploids strains. After sporulation, the tetrads were dissected on YPD media, and the spores were scored for kanamycin resistance (deleted alleles carry kanR cassette), further analyzed by polymerase chain reaction and tested for growth at 30 °C on YPD media. The resulting strains were as follows: YLVh-r (MATa hcr1::KanMX rpg1-1::KanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and YLVh-d (MATa hcr1::KanMX drs2Δ::KanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). The strain YLH13-G35 (MATa hcr1Δ::LEU2 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3::URA3::GPDp35) was produced by transforming strain YLVH13 with YipGDPp35 DNA digested with EcoRV.

Indirect Immunofluorescence Microscopy—Indirect immunofluorescence microscopy was performed as described previously (15), except that rabbit polyclonal anti-Hcr1p antibody (8) was applied at a 1:1000 dilution in 1% (w/v) bovine serum albumin/PEMI (0.1 M PIPE, 5 mM EGTA, 1 mM MgCl2, pH 6.9) at 25 °C for 60 min. Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibodies (Sigma Chemical Co.) diluted 1:200 were used as secondary antibodies. Secondary antibodies were pre-adsorbed overnight at 4 °C on fixed spheroplasts permeabilized with acetone. To diminish background fluorescence in yeast cells due to unbound antibodies, all incubation and washing steps were done in suspension in a test tube. Before observation the cells were resuspended in TBS (Tris-buffered saline: 0.05 M Tris, 150 mM NaCl2, pH 8.0), 100% methanol. The membrane was washed several times in a rehydration buffer (FTTC: excitation 490–495 nm, emission 520–552 nm; Cy3: excitation 540–552 nm, emission 570 nm) using a 100×/1.4 N.A. (numeric aperture) oil immersion objective. Images were recorded at 1280 × 1024 pixel resolution using a Fluoview-cooled charge-coupled device camera using analySIS software.

Northwestern Blot Analysis—Purified recombinant GST fusion proteins (approximately 2 μg) were separated by SDS-PAGE and blotted to a polyvinylidene difluoride membrane (Novex), which was prewetted in 100% methanol. The membrane was washed several times in a rehydration buffer (50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM β-mercaptoethanol) and incubated in a large volume of the latter buffer overnight at 4 °C. The filter was then blocked in a binding buffer (10 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.2% I-Block (Tropix)) and incubated for 4 h in a small volume of the same buffer containing 50 pmol of biotinylated 18 S rRNA, synthesized in vitro from pLVT–17 S rRNA using the T7/SP6 MAXIScript kit (Ambion). The filter was then incubated on a filter blotter for 1 h at 100°C, and the filter was washed with 2× SSC, 0.1% sodium dodecyl sulfate, 100 mM NaCl and stained with 0.1% Coomassie blue R-250 in 50% methanol–5% acetic acid (20). The autoradiogram was then photographed on Polaroid film. The intensity of the 18 S band was quantified using an image analysis software (NIH Image). The data in each lane was normalized to a value of 1.00 for the wild-type strain. As a quality control the 23 S band was used in the analysis. The average densitometric value for 18 S band was 1.76 ± 0.15 (n = 3) in the wild-type strain and 0.85 ± 0.18 (n = 3) in the hcr1Δ mutant. A value of 0.85 was an indication of a complete inhibition of the 18 S subunit. This was used to estimate the kinetic parameters of eIF3j/Hcr1p function in vivo.

Other Biochemical Techniques—Polysome profile analysis and subsequent Western blot analysis of gradient fractions, preparation of whole cell extract, immunoprecipitation, and GST pull-down experiments were conducted as described previously (8). Pulse-chase labeling of pre-rRNA and Northern blot analysis were conducted according to (16).

RESULTS

Deletion of eIF3j/HCR1 Leads to 40 S Ribosomal Deficiency and Hypersensitivity to Paromomycin—We showed previously that deletion of HCR1 leads to a reduction in the level of 40 S ribosomal subunits (8). To analyze this defect in greater detail, we examined ribosomal profiles of isogenic hcr1Δ and wild-type strains. Both strains were grown in YPD medium (17), and cycloheximide was added to one-half of each culture at 5 min prior to harvesting to arrest translating ribosomes and preserve the polyosomes. The cells from these portions of the culture were lysed in a buffer containing cycloheximide and Mg2+. The polyribosomes were resolved by velocity sedimentation in sucrose gradients. As observed previously, deletion of HCR1 led to a reduction in the abundance of free 40 S subunits and a marked increase in the pool of free 40 S subunits (Fig. 1A). A size distribution of inactive ribosomal subunits normally occurs as 80 S couples (18); hence, the presence of a large excess of free 60 S subunits most likely indicates a deficiency in 40 S subunits. To test this prediction, the untreated portions of the two cultures were lysed in the absence of Mg2+ to permit dissociation of 80 S ribosomes into free 40 S and 60 S subunits (Fig. 1B). Quantification of the A254 level in the 40 S and 60 S peaks in this and two other replicate experiments revealed a 60 S/40 S ratio of 2.24 with a standard error of 0.09 for the mutant compared with a value of 1.92 with a standard error of 0.07 for the wild-type. These figures correspond to a reduction of ~14% in the amount of 40 S subunits in the hcr1Δ mutant compared with wild-type. We calculated that the increase in free 60 S subunits observed for the hcr1Δ mutant in Fig. 1A represents ~10% of the total 60 S subunits present in the gradient. Thus, the accumulation of free 60 S subunits seen in the hcr1Δ mutant in Fig. 1A can be accounted for by the ~14% reduction in total 40 S subunits observed from Fig. 1B. Integration of the HCR1 gene into the genome of the hcr1Δ strain restored the 60 S/40 S ratio to the wild-type value (data not shown). These data confirm that eIF3j is required for a normal level of 40 S subunits.

To obtain additional evidence that 40 S ribosome biogenesis is defective in the hcr1Δ mutant, we tested it for sensitivity to the aminoglycoside antibiotic paromomycin, which is known to inhibit strongly the growth of mutants with defects in biogenesis of 40 S ribosomes. (16). The hcr1Δ strain grows more slowly than the wild-type on minimal SD medium (17), but this difference in growth rate was greatly accentuated on SD medium containing 0.5 μg/ml paromomycin (Fig. 1C). The rpg1-1 (eIF3a) and hcr1Δ mutations impair the growth rate on SD medium by similar amounts at 30 °C; however, the difference in growth rate between rpg1-1 mutant and wild-type colonies was nearly the same in the presence or absence of paromomycin (Fig. 1C). Thus, inactivation of eIF3j substantially increased sensitivity to paromomycin, whereas the defect in eIF3a conferred by rpg1-1, known to reduce translation initiation in vivo (6), produced only a subtle increase in drug sensitivity. These findings are consistent with the idea that hcr1Δ impairs 40 S ribosome biogenesis in addition to reducing eIF3 function.

eIF3j/HCR1 Localizes to the Cytoplasm—To explore whether eIF3j might be involved in early steps of 40 S maturation, which take place in the nucleus, or in the final processing events occurring in the cytoplasm, its subcellular localization was analyzed by indirect immunofluorescence. eIF3j antibodies stained intensely the cytoplasm of wild-type cells (Fig. 2A), but stained weakly both the nuclei, visualized by DAPI staining (Fig. 2B). The direct immunofluorescence microscopy of the eIF3j/Hcr1p-FUS3-GFP fusion showed that eIF3j/Hcr1p is localized predominantly in the cytoplasm, consistent with a role in the final steps of 40 S biogenesis occurring in this compartment.

eIF3j/Hcr1p Is Required for Rapid Processing of the 20 S rRNA Precursor and Is an RNA Binding Protein—The direct precurso of 18 S rRNA is 20 S RNA, which is processed at cleavage site D after being transported from nucleolus to cytoplasm. To address whether eIF3j is involved in this event, we examined the kinetics of pre-rRNA processing in hcr1Δ cells by a pulse-chase experiment. Cells were grown in SC medium (17) lacking methionine, and the pre-rRNA was pulse-labeled for 1 min with [methy]-H)methionine and chased with cold methionine for 2, 5, and 15 min. The processing of pre-rRNA to mature 18 S and 25 S RNAs occurs very rapidly and is nearly complete after a 2-min chase in the wild-type strain (Fig. 3A, lanes 1–3). After a 2-min chase in the hcr1Δ mutant, by contrast, a considerable amount of 20 S pre-rRNA remained, and there was a commensurate reduction in the accumulated amount of 18 S rRNA. The ratio of 18 S to 20 S signals after a 2-min chase in the mutant was ~1.5, compared with the corresponding ratio for the wild-type of ~10 (Fig. 3A, compare
lanes 2 and 6). By 5 min of chase, 20 S to 18 S processing was complete in both mutant and wild-type. This experiment was repeated three times, in some cases adding time points of 1.5 and 3.5 min, with results very similar to those shown in Fig. 3A. Taken together, these experiments showed that processing of 20 S to 18 S rRNA was nearly complete by 1.5 min in wild-type cells but not until 3.5 min in the hcr1Δ/H9004 mutant. Thus, we conclude that processing of 20 S to 18 S rRNA is delayed in the hcr1Δ/H9004 mutant. This is the final processing step for the 40 S subunit, which occurs in nascent subunits exported to the cytoplasm. We did not detect an accumulation of 35 S or 32 S precursors, or the 23 S aberrant product, in the hcr1Δ/H9004 mutant, which would indicate defects in nucleolar steps of 18 S rRNA biogenesis. Generally, the 35 S and 32 S precursors are detectable only in mutants where an early step of pre-rRNA processing is delayed. Surprisingly, we did observe a delay in 27 S pre-RNA to 25 S rRNA processing, which is thought to occur in the nucleolus. A similar delay can be observed in the published results on mutations in other genes that impair 20 S to 18 S processing (19, 20), but we are unaware of an explanation for these results.

We also performed Northern blot analysis to verify the aberrant accumulation of 20 S pre-rRNA in the hcr1Δ strain (Fig. 3B). After growing wild-type and hcr1Δ cells in rich medium, total RNA was subjected to Northern analysis using 32P-labeled oligonucleotides as probes to detect 18 S rRNA (lanes 1 and 2), the 3′-end of 20 S pre-rRNA (lanes 3 and 4), or 25 S rRNA (lanes 5 and 6). We quantified the hybridization signals in Fig. 3B and one replicate experiment, and after normalizing for the amounts of mature 25 S rRNA, we calculated that the hcr1Δ/H9004 mutant contained 1.54-fold more 20 S pre-rRNA (lanes 4 versus 3), and 20% less mature 18 S rRNA (lanes 2 versus 1), compared with the wild-type. The latter result is in reasonable accord with the 14% reduction in 40 S ribosomal subunits in the hcr1Δ mutant calculated from the data in Fig. 1B. Taken together, the data in Fig. 3 (A and B) indicate that eIF3j is required for a wild-type rate of processing of 20 S pre-rRNA to mature 18 S rRNA.

Considering the possibility that eIF3j might participate directly in processing 20 S pre-rRNA, we investigated whether recombinant eIF3j/Hcr1p can bind to 18 S rRNA. Toward this end, a full-length GST-Hcr1p fusion was tested for binding to...
biotinylated full-length 18 S rRNA in a Northwestern assay. As shown in Fig. 3C (lane 2), GST-Hcr1p bound to biotinylated 18 S rRNA, whereas GST alone did not (lane 2). Under similar conditions, GST-Hcr1p interacted to a much lesser extent with an equivalent amount of biotinylated H9252-globin mRNA. We conclude that eIF3j is an RNA binding protein that may show a preference for double-stranded RNA. Thus, it is conceivable that eIF3j/Hcr1p binds to 20 S pre-rRNA in the nascent 40 S subunit and directly influences the efficiency of cleavage at the D processing site.

Combining hcr1/H9004 with drs2/H9004 or rps0a/H9004 Produces a Synthetic Growth Defect—It is known that mutations in RPS0a and DRS2 impair the processing of 20 S pre-rRNA to 18 S rRNA (19, 20). The processing defect we observed in hcr1Δ cells is comparable to that reported previously for a drs2Δ mutant (19) but less severe than that seen in rps0aΔ cells (20). If eIF3j is involved specifically in this aspect of 40 S ribosome biogenesis, we would expect that combining hcr1Δ with a mutation in one of these genes would exacerbate the 20 S pre-rRNA processing and the attendant growth defect. To test this prediction, we obtained mutant strains with deletions of RPS0A or DRS2, both nonessential genes, and crossed them with an hcr1Δ strain to produce the appropriate double mutants (see “Experimental Procedures” for details). We observed that the single deletion of RPS0A led to a modest reduction in the growth rate, whereas the drs2Δ cells grew like the wild-type (Fig. 3D), as reported previously (19, 21). Importantly, combining either drs2Δ or rps0aΔ with hcr1Δ exacerbated the Slg− phenotype conferred by the hcr1Δ single mutation, in accordance with our prediction (Fig. 3D). These data provide a genetic link between HCR1, RPS0A, and DRS2, and they support the idea that eIF3j/Hcr1p is a member of the post-nucleolar 40 S ribosome biogenesis pathway.

Human eIF3j/p35 Binds to Yeast eIF3 and Partially Complements the Slg− Phenotype of hcr1Δ—eIF3j/Hcr1p is 26% identical and 42% similar in sequence to its human ortholog, eIF3j/p35 (5, 7). None of the mammalian eIF3 subunits tested thus far could functionally substitute for their yeast counterparts in vivo (reviewed in Ref. 22). Nevertheless, we decided to test whether heIF3j could provide either of the functions of eIF3j in translation initiation or 40 S ribosome biogenesis. Toward this end, we placed the heIF3j cDNA behind the methionine-repressible yeast MET3 promoter on a high copy (hc) plasmid (hcPMET-p35) and tested it for complementation of the Slg− phenotype of hcr1Δ mutant when the transformant was grown on SD medium (17) lacking methionine. By comparison, a single copy (sc) construct containing HCR1 under the control of the MET3 promoter (scPMET-HCR1) fully complemented the hcr1Δ Slg− phenotype (Fig. 4A).

The fact that hcPMET-p35 only partially complemented the hcr1Δ Slg− phenotype might indicate that heIF3j can provide...
only one of the two functions of eIF3j in yeast. A prerequisite for heIF3j to substitute for eIF3j in translation initiation would be the ability to associate with the yeast eIF3 complex. To test this possibility, we prepared whole cell extracts (WCEs) from the hcr1/H9004 strain transformed with the hcP MET-p35 construct grown in SC medium (17) containing, or lacking, 2 mM methionine. We used antibodies against eIF3a/Tif32p to immunoprecipitate eIF3 and probed the immune complexes with antibodies against heIF3j, eIF3 subunits eIF3a/Tif32p and eIF3b/Prt1p, eIF2/H9253, eIF1, and eIF5. As observed previously, the eIF3a antibodies immunoprecipitated 60–70% of eIF3a and eIF3b, 20% of the eIF2 and eIF5, and 10% of the eIF1 from both extracts (Fig. 4B, lanes 2 and 5), consistent with the recovery of the multifactor complex (MFC) containing these initiation fac-
tors. As expected, heIF3j was detected only in the WCE from cells grown in the absence of methionine where P MET-p35 expression is turned on (Fig. 4A, lane 1 versus 4). Importantly, a fraction of the heIF3j in this extract coimmunoprecipitated with eIF3a. Taking into account the efficiency of immunoprecipitaiting eIF3a, we estimate that ~10% of the heIF3j was associated with eIF3a. A somewhat greater proportion of native eIF3j/Hcr1p was coimmunoprecipitated with eIF3a in our previous experiments (8). These results indicate that human eIF3j/p35 can interact with yeast eIF3 in vivo.

We next investigated whether heIF3j/p35 can interact directly with the eIF3a and eIF3b subunits, as eIF3j does. Moreover, we asked whether heIF3j can bind to the cognate subunits in human eIF3, hIF3a and hIF3b. A GST-p35 fusion protein and GST alone were expressed in Escherichia coli, purified on glutathione-Sepharose beads, and incubated with 35S-labeled subunits of human or yeast eIF3 synthesized in a reticulocyte lysate. As shown in Fig. 4C, GST-p35 interacted strongly with [35S]hIF3a/Tf32p (~35% bound) and [35S]eIF3a/Prt1p (~20% bound) but weakly with [35S]eIF3a/Tf32p and [35S]hIF3b/Prt1p. It showed no interaction with [35S]eIF3a/Tf34p that we examined as a negative control. Note that the heIF3j-heIF3a interaction was described previously (5). Interestingly, we reported that eIF3b was the major binding partner in yeast eIF3 for eIF3j (8), as observed here for human eIF3j/p35 expressed in yeast. In contrast, heIF3j interacted much more strongly with hIF3a than with hIF3b, suggesting that the major eIF3 binding site for eIF3j (Hcr1p/p35) varies between eIF3b (Prt1p/hPrt1p) and eIF3a (Tf32p/hTf32p) in budding yeast and humans. We also obtained evidence that the RNA binding activity of eIF3j is conserved in heIF3j, by showing that GST-p35 binds to biotinylated 18 S rRNA in a North-western assay (Fig. 4C, lane 3). Thus, it seems likely that heIF3j/Hcr1p and heIF3j/p35 interact with the analogous subunits in yeast and human eIF3 complexes, and both proteins may carry out a conserved function in eIF3 that involves RNA binding.

**heIF3j/p35 Does Not Substitute for eIF3j/Hcr1p in 40 S Ribosome Biogenesis**—We wished to determine what function of eIF3j was complemented by expression of heIF3j in yeast. Toward that end, we first compared the polysome profile of the hcr1Δ strain expressing heIF3j from the strong constitutive GPD promoter (P GPD-p35) with those of the wild-type HCR1 and hcr1Δ mutant strains. The P GPD-p35 construct complemented the Slg phenotype of hcr1Δ to the same extent as the hpm MET-p35 construct on medium lacking methionine (Fig. 5B and 4A). The results in Fig. 5A showed clearly that heIF3j did not correct the 40 S ribosome deficiency in the hcr1Δ strain, the excess of free 60 S subunits in the hcr1Δ strain was not reduced by expression of heIF3j. This result was subsequently confirmed under the conditions in which 80 S ribosomes are dissociated to 40 S and 60 S subunits (absence of cycloheximide and MgCl2), where expression of heIF3j had no significant effect on the 60 S/40 S ratio in hcr1Δ cells (data not shown). Consistently, the P GPD-p35 construct did not reduce the paromomycin sensitivity of the hcr1Δ strain (Fig. 5B). Thus, heIF3j cannot substitute for eIF3j in 40 S ribosome biogenesis. This finding implies that heIF3j expression in-
several mutants in 40 S ribosomal proteins or proteins involved was localized entirely in the cytoplasm (Fig. 2). There are some biogenesis pathway. Consistently, we found that eIF3j is dispensable for the nucleolar phase of the 40 S ribosome (23), suggesting that eIF3j has a dual function in translation initiation and the assembly or stability of 40 S ribosomes, suggesting that eIF3j has a stimulatory destabilization of the MFC, and reduction in the rate of translation initiation (7, 8), indicating that eIF3j has a stimulatory increase in the polysome/monosome (P/M) ratio of 3.71 ± 0.12 measured for the wild-type HCR1 strain (Fig. 5A).

discussion
eIF3j/Hcr1p Has a Role in 40 S Ribosome Biogenesis—eIF3j/Hcr1p, the yeast ortholog of the p35 subunit of the human eIF3, was shown to be a loosely associated, substoichiometric subunit of S. cerevisiae eIF3. It displayed specific genetic and physical interactions with the eIF3a/Tif32p and eIF3b/Prt1p subunits of eIF3 and was found to bind 40 S ribosomes, most likely as a part of the MFC (8). Deletion of HCR1 led to a Slg" phenotype, destabilization of the MFC, and reduction in the rate of translation initiation (7, 8), indicating that eIF3j has a stimulatory role in translation initiation. Unexpectedly, the hcr1Δ mutation also produced a significant reduction in 40 S ribosomal subunits, suggesting that eIF3j has a dual function in translation initiation and the assembly or stability of 40 S ribosomes (8).

Here we showed that eIF3j is required for rapid processing of the 20 S rRNA precursor to mature 18 S rRNA, one of the final steps of 40 S ribosome biogenesis that render the 40 S subunit competent for translation (3). As in other mutants defective in this step of 40 S biogenesis, including rps6Δ and drs2Δ (19, 20), the 20 S rRNA precursor accumulated in the hcr1Δ strain, as judged by pulse-chase and Northern blotting experiments (Fig. 3). This processing step involves an endonucleolytic cleavage (called cleavage D) of 209 nucleotides from the 3′-end of the 20 S rRNA precursor (23) that occurs in the cytoplasm (4). We did not detect any nucleolar rRNA cleavage intermediates such as 35 S, 32 S, and 23 S (for review see Ref. 1), suggesting that eIF3j is dispensable for the nucleolar phase of the 40 S ribosome biogenesis pathway. Consistently, we found that eIF3j was localized entirely in the cytoplasm (Fig. 2). There are several mutants in 40 S ribosomal proteins or proteins involved in 40 S ribosome biogenesis that are hypersensitive to the aminoglycoside antibiotic paromomycin (16, 24, 25). This antibiotic interferes with translational fidelity by targeting 18 S rRNA in the context of the 40 S ribosomal subunit (16, 26). It is believed that the sensitivity of these mutants arises from improper assembly of the 40 S subunits, leaving the ribosome more accessible to, or more strongly affected by, paromomycin (27). The fact that hcr1Δ cells are hypersensitive to paromomycin further supports the involvement of eIF3j in promoting 40 S subunit biogenesis.

Combining hcr1Δ with a deletion of RPS0A or DRS2 resulted in a synthetic growth defect (Fig. 3D), indicating that eIF3j functionally interacts with these proteins in 40 S biogenesis. RPS0A encodes ribosomal protein S0, and DRS2 codes for a putative membrane-associated Ca2+ ATPase. The function of these proteins in 20 S pre-rRNA processing is unknown. The ability of eIF3j to bind 40 S ribosomes independently of eIF3 (10) and the current finding that GST-Hcr1p has RNA binding activity (Fig. 3C) are consistent with a direct role for eIF3j in stimulating 40 S biogenesis. One possibility is that eIF3j promotes incorporation of the “late” ribosomal proteins into the 40 S pre-ribosome, although, according to Kruiswijk et al. (28), the 40 S preribosomal particle already contains all small ribosomal proteins when imported to the cytoplasm. Another possibility is that eIF3j helps to recruit the unknown endonuclease responsible for the D cleavage of 20 S pre-rRNA to the 18 S rRNA. A screen for specific interacting partners of eIF3j should shed more light on its role in 40 S subunit assembly.

The slower rate of 20 S to 18 S processing in the hcr1Δ mutant may account completely for the −14% reduction in the steady-state amount of 40 S subunits; however, given that eIF3j/Hcr1p can bind to 40 S ribosomes in the absence of eIF3 (10), it is possible that 40 S subunits also turn over more rapidly in hcr1Δ cells. The modest effect of deleting HCR1 on the abundance of 40 S ribosomes is in accordance with the fact that the slow growth phenotype of the hcr1Δ mutant is not severe. Furthermore, it was partially suppressed by expressing human eIF3j/p35, even though the latter did not correct the
defect in 40 S ribosome biogenesis. Thus, the loss of Hcr1p function in ribosome biogenesis is responsible for only a portion of the growth defect in hcr1/H9004 cells. Presumably, the contribution of Hcr1p to 40 S biogenesis is more critical in mutant strains lacking other nonessential proteins that enhance this process, such as Drs2p and Rps0ap.

Evolutionary Conservation between eIF3j/Hcr1p and Its Human Ortholog heIF3j/p35—None of the human eIF3 subunits tested so far are able to replace its yeast ortholog and support cell growth (22), despite the fact that the yeast eIF3 complex can replace mammalian eIF3 in an in vitro methionyl-puromycin synthesis assay that measures first peptide bond synthesis (29). eIF3j/Hcr1p is 26% identical and 42% similar in amino acid sequence to heIF3j/p35, a somewhat lower conservation of sequence than that observed for other eIF3 subunits found in yeast and humans (22). In addition, there are no obvious regions of striking similarity between the two proteins (7). Thus, it was surprising to find that heIF3j/p35 could partially complement the growth defect of an her1\Delta mutant (Fig. 4A). We show that expression of heIF3j did not suppress the deficit in 40 S ribosomal subunits or the paromomycin sensitivity conferred by her1\Delta (Fig. 5, A and B). It is not known whether heIF3j/p35 has a role in ribosome biogenesis in human cells. Although the major steps in ribosome synthesis appear to be well conserved throughout the eukaryotic kingdom, far more information is available for yeast than for other eukaryotes (1, 30). Either heIF3j does not carry out the function of eIF3j in 40 S biogenesis in humans or it cannot interact with the yeast 20 S pre-rRNA processing machinery.

On the other hand, we found that heIF3j could associate with yeast eIF3 and 43 S initiation complexes in vivo (Fig. 4B and data not shown). Moreover, GST-p35 interacted strongly with yeast eIF3b/Prt1p and weakly with yeast eIF3a/Tif32p in vitro (Fig. 4C), the two binding partners for eIF3j/Hcr1p in yeast eIF3 and the orthologs of heIF3j/p35 binding partners in human eIF3 (Fig. 4C). Thus, it seems quite likely that eIF3j/Hcr1p and heIF3j/p35 interact with the analogous subunits in yeast and human eIF3 and that the binding domains for these eIF3 subunits are conserved between eIF3j/Hcr1p and heIF3j/p35. We suggest that the ability of heIF3j/p35 to interact appropriately with yeast eIF3 can account for its ability to partially suppress the translation initiation defect in her1\Delta cells. Consistently, expression of heIF3j produced a small but significant increase in the polysome content in the her1\Delta strain (Fig. 6).
5A). One possibility is that eIF3j can replace eIF3j in stabilizing the MFC and in promoting its binding to the 40 S ribosomes. However, we were unable to detect any influence of p35 expression on the stability of the MFC in her1Δ cells (data not shown). Either our assay for MFC stability is not sensitive enough to detect a modest stimulation by p35 or there is another unknown stimulatory function of eIF3j in translation initiation that can be supported by heIF3j.

How Does eIF3j/Hcr1p Perform Its Two Distinct Functions?—Our current understanding of eIF3j function is summarized in the model shown in Fig. 6. We postulate that eIF3j is strictly a cytoplasmic protein that participates in two independent processes involving the 40 S ribosome. The immature 40 S ribosome containing the 20 S rRNA precursor is exported from the nucleolus to the cytoplasm. eIF3j, free of eIF3, interacts directly with the nascent 40 S subunit and enhances the D cleavage, producing mature 18 S rRNA. eIF3j either dissociates from the mature 40 S subunit or remains ribosome-bound to participate in its second function in the initiation pathway.

Free eIF3j can also associate with eIF3, and it promotes or stabilizes formation of the MFC-containing eIFs 1, 2, 3, 5, and the Met-tRNAiMet (8, 9). eIF3j seems to be particularly important for proper association of eIF1 and eIF5 with other factors in the MFC. Its absence in her1Δ cells delays a step in the initiation pathway following association of eIFs 1, 3, 5 and the TC with the 40 S ribosome (8). Presumably, eIF3j is ejected along with other components of the MFC upon joining of the 60 S subunit.

To our knowledge, eIF6 is the only other nonribosomal protein postulated to have a dual function in ribosome biogenesis and translation initiation. It was initially reported that eIF6 possessed ribosomal subunit anti-association activity (31); however, its requirement in translation of mRNAs has never been defined (32). In fact, it was reported that eIF6 was dispensable for mRNA translation in yeast cell extracts. In addition, analysis of polysome profiles in eIF6-depleted cells showed decreased levels of 60 S and 80 S ribosomes but no defects characteristic of an inactive translation initiation factor (32). Hence, these authors concluded that eIF6 does not function as a bona fide translation initiation factor. More recently, eIF6 was shown to play a role in 60 S ribosomal subunit biogenesis (33). Thus, at present, eIF3j/Hcr1p is a unique dual function protein that enhances ribosome biogenesis and translation initiation.

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