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Suppression of Yeast RNA Polymerase III Mutations by *FHL1*, a Gene Coding for a *fork head* Protein Involved in rRNA Processing

SYLVIE HERMANN-LE DENMAT,† MICHEL WERNER,* ANDRÉ SENTENAC, AND PIERRE THURIAUX

Service de Biochimie et Génétique Moléculaire, Département de Biologie Moléculaire et Cellulaire, Centre d'Etudes de Saclay, F-91191 Gif-sur-Yvette Cedex, France

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The *FHL1* gene was isolated by screening for high-copy-number suppressors of conditional RNA polymerase III mutations. This gene is unique on the yeast genome and was located close to *RPC40* and *PRE2* on the right arm of chromosome XVI. It codes for a 936-amino-acid protein containing a domain similar to the *fork head* DNA-binding domain, initially found in the developmental *fork head* protein of *Drosophila melanogaster* and in the HNF-3 family of hepatocyte mammalian transcription factors. Null mutations caused a severe reduction in growth rate and a lower rRNA content that resulted from defective rRNA processing. There was no detectable effect on mRNA splicing. Thus, the Fhl1p protein plays a key role in the control of rRNA processing, presumably by acting as a transcriptional regulator of genes specifically involved in that process. Moreover, mutants carrying the RNA polymerase III mutations were slightly defective in rRNA processing. This accounts for the isolation of *FHL1* as a dosage-dependent suppressor and suggests that rRNA processing depends on a still-unidentified RNA polymerase III transcript.

RNA polymerase III is one of the most complex enzymes of eukaryotic cells (12, 13). Its heteromultimeric organization was biochemically and genetically investigated in *Saccharomyces cerevisiae* (see references 46 and 51 for reviews), revealing at least 13 distinct polypeptides. This number includes two large subunits related to the β′ and β subunits of the bacterial enzyme and to homologous polypeptides of RNA polymerase I and II, five subunits common to all three enzymes, two subunits shared by RNA polymerases I and III, and at least four enzyme-specific subunits (17). The corresponding genes were cloned, sequenced, and inactivated by null or conditional mutations (2, 7, 10, 14, 19, 25, 29, 35, 49, 53, 58, 60). Several of these conditional mutations were screened for extragenic suppression in the presence of a yeast genomic library borne on a high-copy-number vector (48). A similar study revealed dosage-dependent interactions between distinct RNA polymerase III subunits, pointing to specific subunit-subunit association patterns within the quaternary structure of the enzyme (25). Using the same approach, we also isolated multicopy suppressor genes that operate specifically on RNA polymerase III mutations but do not code for a structural component of that enzyme. The properties of one of these suppressors, *FHL1*, are described in the present report.

MATERIALS AND METHODS

Strains and media. The strains used are listed in Table 1. Yeast genetic techniques and media were as described by Sherman (47), Boeke et al. (4), and Mosrin et al. (35). *Escherichia coli* DH5αF′ I^q [*endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* φ80d*lacZ*ΔM15 Δ(*lacZYAargF*) *U169*/F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*5*(Km^r)] was obtained from Bethesda Research Laboratories, Inc.

Selection of multicopy suppressors. Multicopy suppressors were isolated by transformation of the temperature-sensitive *rpc160-112* mutant MW657 by a wild-type genomic library of *S. cerevisiae* FL100 (48) on the multicopy vector pFL44L. After one night at the permissive temperature (25°C), the plates were incubated at the restrictive temperature (37°C) and observed for 11 days. A first set of 11 fast-growing transformants were able to lose the plasmid-borne *rpc160-112* allele in a plasmid shuffle assay (58), indicating that they contained a wild-type *RPC160* gene complementing the deleted chromosomal copy. This was confirmed by plasmid extraction (16) and restriction analysis of some inserts. The remaining transformants corresponded to weak suppressors that grew slowly at 37°C. Plasmid pRPS13, which harbors a 171-amino-acid C-terminal deletion of *FHL1*, was obtained from the same genomic library in a separate experiment (6a).

Sequencing of *FHL1*. The 2.7-kb *Bam*HI fragment and the 2.8-kb *Pst*I fragment (Fig. 1) were cloned in both orientations on the Bluescript plasmid pBSSK(+) (Stratagene), yielding plasmids pSHB16 and -17 for the *Bam*HI fragment and plasmids pSHB22 and -23 for the *Pst*I fragment. In each case, deletions were produced by exonuclease III-mung bean nuclease treatment as described by the kit manufacturer (Stratagene). Deletant plasmids were sequenced by the dideoxynucleotide method with modified T7 DNA polymerase (Sequenase; United States Biochemical Corp.). When necessary, synthetic oligonucleotides were used to complete the sequence. Sequences were analyzed with DNA Strider (31) software. Homology searches of data bases were performed on the EMBL (release 34) and SWISS-PROT (release 24) data banks with the FASTA program (39). The sequence was assigned accession number Z28348.

***FHL1* gene disruption.** Plasmid pSHB64 was constructed by

* Corresponding author. Mailing address: Service de Biochimie et de Génétique Moléculaire, Département de Biologie Moléculaire et Cellulaire, Bâtiment 142, Centre d'Etudes de Saclay, F-91191 Gif-sur-Yvette Cedex, France. Phone: 33.1. 69 08 93 42. Fax: 33.1. 69 08 47 12. Electronic mail address: werner@jonas.saclay.cea.fr.

† Present address: I.G.D., Université de Paris-Sud, F-91405 Orsay, France.

TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Reference
YNN281	*MAT***a** *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200*	15
YNN282	*MAT*α *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200*	15
MW420	*MAT*α *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc160-Δ1::HIS3*(pC160-6 [*URA3 CEN4 RPC160*])	58
MW657	*MAT*α *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc160-Δ1::HIS3*(pC160-112 [*TRP1 CEN4 rpc160-112*])	15a
MW658	*MAT*α *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc160-Δ1::HIS3*(pC160-209 [*TRP1 CEN4 rpc160-209*])	15a
MW659	*MAT*α *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc160-Δ1::HIS3*(pC160-112 [*TRP1 CEN4 rpc160-112*]) (pSHB4 [*URA3* 2μmD *FHL1*])	This work
MW661	*MAT*α *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc160-Δ1::HIS3*(pC160-209 [*TRP1 CEN4 rpc160-209*]) (pSHB4 [*URA3* 2μmD *FHL1*])	This work
MW663	*MAT***a**/*MAT*α *ade2-101/ade2-101 ura3-52/ura3-52 trp1-Δ1/trp1-Δ1 lys2-801/lys2-801 his3-Δ200/his3-Δ200 fhl1::HIS3/FHL1*	This work
MW664	*MAT*α *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 fhl1::HIS3*	This work
MW665	*MAT***a**/*MAT*α *ade2-101/ade2-101 ura3-52/ura3-52 trp1-Δ1/trp1-Δ1 lys2-801/lys2-801 his3-Δ200/his3-Δ200 fhl1-Δ1::HIS3/FHL1*	This work
MW666	*MAT***a** *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 fhl1-Δ1::HIS3*	This work
MW667	*MAT*α *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc160-Δ1::HIS3*(pC160-206 [*TRP1 CEN4 rpc160-206*])	15a
D48-1c	*MAT***a** *ade2-101 ura3-52 lys2-801 rpc160-41*	14
C236	*MAT***a** *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc31-236*	48
SC91	*MAT***a** *ade2-101 ura3-52 lys2-801 his3-Δ200 leu2-1Δ trp1::rpc53-Δ254/424 rpc53-Δ::TRP1*	30
DLY202	*MAT***a** *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc19-Δ::HIS3*(p3519/G73D [*TRP1 rpc19-G73D*])	25
DLY7C	*MAT***a** *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc40-V78R*	25
CMY237	*MAT***a** *can1 ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc40-Δ::HIS3*(CPM235 [*TRP1 rpc40-ts4*])	29
SC116	*MAT*α *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 leu2-1Δ rpc82-Δ::HIS3*(pRS314/C82-6 [*TRP1 rpc82-6*])	7
21-Δ104	*MAT***a** *can1-100 ade2-101 ura3-1 trp1-Δ1 lys2-801 his3-11,15 leu2-3,112 rpo21-Δ104*	1
Z316	*MAT***a** *ura3-52 his3-Δ200 leu2-3,112 rpb1-Δ187::HIS3*(pRP1-17 [*LEU2 rpb1-17*])	36
NOY259	*MAT*α *trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can*^{R} *rpa190-1*	59
Cy103	*MAT*α *ura3-52 trp1-289 leu2-3,112 his3-Δ1 cyh*^{R} *prp9-1*	6

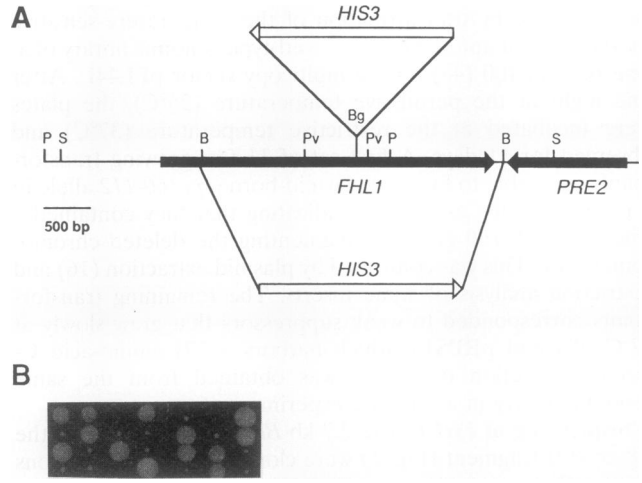

FIG. 1. Inactivation of the *FHL1* gene. (A) Restriction map of the *FHL1-PRE2* region. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I. The *FHL1* and *PRE2* open reading frames are represented by solid arrows. The region displayed corresponds to the overlapping DNA sequences of *FHL1* (EMBL accession number Z28348) and *PRE2* (GenBank accession number X68662). The structure of the *fhl1::HIS3* allele made by inserting the 1.7-kb *Bam*HI *HIS3* cassette at the *Bgl*II site of the *FHL1* gene is shown above the restriction map. The *HIS3* reading frame is oriented opposite to that of *FHL1*. The *fhl1-Δ1::HIS3* deletion allele, constructed by replacement of the 2.7-kb *FHL1 Bam*HI fragment with the *HIS3* cassette, is shown below the restriction map. The *HIS3* and *FHL1* open reading frames are in the same orientation. (B) Tetrad analysis of *fhl1-Δ1::HIS3/FHL1* diploid strain MW665. The small colonies are invariably associated with the HIS$^+$ phenotype. The same results were obtained from tetrad analysis of *fhl1::HIS3/FHL1* diploid strain MW663.

inserting the 4.3-kb *Sal*I fragment (Fig. 1) at the corresponding site of pSHB29, a Bluescript plasmid derivative from which the polylinker *Bam*HI site had been removed with exonuclease III. The null allele *fhl1::HIS3* was obtained by inserting the 1.7-kb *Bam*HI *HIS3* fragment from pSZ63 (37) into the *Bgl*II site of pSHB64 (Fig. 1). In this pSHB66 derivative of pSHB64, the *HIS3* open reading frame is oriented opposite to the *FHL1* gene. The *fhl1-Δ1::HIS3* deletion was constructed by replacing the 2.7-kb *Bam*HI *FHL1* fragment with the 1.7-kb *Bam*HI *HIS3* fragment, yielding plasmid pSHB68. These plasmids were digested with *Apa*I and *Sac*I and transformed into the YNN281 × YNN282 diploid strain to generate the heterozygous *fhl1::HIS3/FHL1* and *fhl1-Δ1::HIS3/FHL1* strains MW663 and MW665, respectively (Table 1). Their genetic structure was confirmed by Southern analysis with the 762-bp *Pvu*II *FHL1* internal fragment (Fig. 1) and the 1.7-kb *HIS3* fragment as probes (data not shown).

Southern analysis and chromosomal mapping. Genomic DNA was isolated as described by Hoffman and Winston (16). After electrophoresis, gels were soaked in 0.25 M HCl, rinsed in water, and transferred to positively charged nylon membranes (Boehringer Mannheim) by vacuum transfer with a Milliblot-V system (Millipore) in 0.4 N NaOH as the transfer buffer. DNA was bound to the membranes by baking at 80°C for 30 min. Southern hybridizations were revealed by using a digoxigenin luminescent detection kit (Boehringer Mannheim). A commercial chromoblot supplied by Clontech was used to determine the chromosomal location of the *FHL1* gene. Hybridization was performed with the 2.8-kb *Pst*I *FHL1* digoxigenin-labeled fragment as the probe and confirmed by using the 3.7-kb *Bam*HI *GAL4* fragment from pG525 (26) as a specific marker for chromosome XVI. Chromosomal mapping of *FHL1* was done on yeast prime clone grid filters, kindly provided by L. Riles and M. V. Olson.

Northern (RNA blot) analysis. Cells were grown in casamino acids medium supplemented with the appropriate requirements. RNA extractions were done as described by Köhrer and Domdey (22) except that cells were resuspended in 0.5 M NaCl–0.2 M Tris-HCl (pH 7.5)–10 mM EDTA–1% sodium dodecyl sulfate (SDS). Only one hot phenol extraction step was done in the presence of glass beads, after which the aqueous phase was extracted once with phenol-dichloromethane-isoamyl alcohol (25:24:1). For U3 and 7-2/MRP analysis, the oligonucleotides used were 5′-GTTATGGGAGACTTCAAC CC-3′ (3) and 5′-TTGGTGGGAGACTTTCAACCC-3′ (45). Both oligonucleotides were kinased with ^{32}P-labeled ATP and used as probes in Northern blotting against total RNA extracts.

Polyadenylated RNA was isolated with an mRNA purification kit (Pharmacia) and separated on 1% agarose gels after denaturation with glyoxal and dimethyl sulfoxide as described by Sambrook et al. (43). RNA was transferred to a positively charged nylon membrane (Boehringer) by capillary elution with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as the transfer buffer and baked for 30 min at 80°C. Prehybridization and hybridization were performed at 65°C in 0.5 M sodium phosphate (pH 7.2)–10 mM EDTA–7% SDS. Probes were labeled with a random-priming DNA labeling kit supplied by Boehringer, denatured, and added to membranes without purification. After overnight hybridization, filters were washed three times for 15 min each at 65°C in 40 mM sodium phosphate (pH 7.2)–1% SDS, dried, and autoradiographed at −70°C.

In vivo labeling. Cells were grown to an optical density at 600 nm of 0.3 to 0.4 at 30°C in casamino acids medium supplemented with adenine (20 μg/ml) and tryptophan (20 μg/ml). In kinetic experiments, 900 μCi of [5,6-^{3}H]uracil (400 mCi/mg; Amersham Inc.) was added to 60 ml of culture for various periods of time (see the legend to Fig. 5). In pulse-chase experiments, 2.7 mCi of [5,6-^{3}H]uracil was added to 90 ml of culture; after 2.5 min of labeling, a 320-fold excess (24 μg/ml, final concentration) of cold uracil was added, and incubation was continued for various periods of time (see the legends to Fig. 5 and 6). In both experiments, cells were harvested by mixing 15-ml aliquots with 1 volume of ice-cold water, pelleted by centrifugation at 4°C, and rinsed with 1 ml of cold water. RNA was extracted as described by Schmitt et al. (44) with some modifications. Cells were resuspended in 300 μl of 50 mM sodium acetate (pH 5.3)–10 mM EDTA–30 μl of 10% SDS, mixed with about 150 μl of acid-washed glass beads and 300 μl of warm phenol (satured with the same buffer), vortexed vigorously, and incubated for 5 min at 65°C with intermittent vortexing. This stringent extraction procedure is required to ensure a reproducible yield of large rRNAs. RNA was precipitated with 1 M LiCl and 2.5 volumes of ethanol at −20°C overnight.

Small RNAs were separated on a 6% acrylamide gel containing 7 M urea. After electrophoresis (250 V for 4 h), the gel was stained with ethidium bromide (10 μg/ml) and photographed. RNA was fixed for 15 min with methanol-water-acetic acid (5:5:1) and incubated for 30 min in Amplify (Amersham Inc.), after which the gel was dried and autoradiographed at −70°C. High-molecular-weight RNAs were analyzed on 1.2% agarose gels after denaturation with glyoxal and dimethyl sulfoxide as described by Sambrook et al. (43). After electrophoresis, RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) by capillary elution with 10× SSC as the transfer buffer. The membrane was baked for 30 min at 80°C, sprayed with En^{3}Hance (DuPont NEN), and autoradiographed at −70°C.

In vivo splicing assay. The growth and assay conditions have been described previously (6, 27) except that the strains were grown at 30°C and the values given were obtained after 3 h of induction in galactose and are expressed as a percentage of the activity found in the wild-type strain YNN281. β-Galactosidase activity was measured in cell extracts prepared as described by Werner et al. (57) instead of in permeabilized cells.

TABLE 2. Suppressibility of conditional RNA polymerase I, II, or III mutations by overexpression of *FHL1*

Strain	Relevant genotype[a]	Subunit affected	Growth (generation time, min) on rich medium at 37°C[b]	
			−FHL1	+FHL1
MW657	*rpc160-112**	C160	−	+ (580)
MW658	*rpc160-209**	C160	−	+ (370)
MW667	*rpc160-206**	C160	(−)	(−)
D48-1c	*rpc160-41**	C160	−	−
SC116	*rpc82-6**	C82	(−)	(−)
SC91	*rpc53-254/424**	C53	−	−
C236	*rpc31-236**	C31	−	+ (650)
CMY237	*rpc40-ts4*	AC40	−	−
DLY7C1	*rpc40-V78R*	AC40	−	−
DLY202	*rpc19-G73D*	AC19	−	−
Z316	*rpb1-17*	B220	(−)	(−)
21-Δ104	*rpo21-Δ104*	B220	(−)	(−)
NOY259	*rpa190-1*	A190	−	−

[a] Mutations affecting subunits specific to RNA polymerase III are marked (*).
[b] Symbols: −, no growth; (−), weak growth after 5 days; +, growth after 5 days. Generation time was measured in liquid YPD rich medium at 37°C. Wild-type generation time was 90 min.

RESULTS

Cloning of *FHL1* as a multicopy suppressor of RNA polymerase III mutations. The amino acid sequence motif (Y/F) NADFDGDEMN belongs to a conserved domain (domain d) of the largest subunits of DNA-dependent RNA polymerases (32) and is almost invariant in all DNA-dependent RNA polymerases tested so far. The motif corresponds to positions 508 to 518 in the sequence of C160, the largest subunit of yeast RNA polymerase III. Systematic oligonucleotide-directed mutagenesis of this region (15a) yielded two temperature-sensitive alleles, *rpc160-112* (corresponding to a T506I N509Y double substitution) and *rpc160-209* (an N518Q substitution). Starting from *rpc160-112*, we isolated multicopy suppressors at 37°C by transforming the *rpc160-112* mutant strain MW657 with a wild-type genomic library borne on the multicopy vector pFL44L (48) and selecting colonies that grew at the restrictive temperature. For each transformant, we checked that plasmid extraction and retransformation of the original mutant strain suppressed its temperature-sensitive phenotype and that removing the suppressor plasmid restored the mutant phenotype.

A first class of 11 transformants had a wild-type growth rate at 37°C and harbored plasmids with *RPC160* inserts, as shown by plasmid shuffling (see Materials and Methods) and restriction mapping analysis. The remaining transformant plasmids only partially restored growth at 37°C (Table 2). Restriction mapping showed that two of them, pSHB4 and pSHB5, harbored inserts of 11.7 and 7 kb that overlapped for a length of 5.3 kb. By subcloning smaller fragments in the pFL44L vector and testing them for suppression of *rpc160-112*, we could ascribe the suppressor phenotype to a 4.3-kb *Sal*I fragment.

Sequence analysis, mRNA transcription, and genetic mapping of *FHL1*. Sequencing the 4,366-bp *Sal*I fragment (Fig. 1)

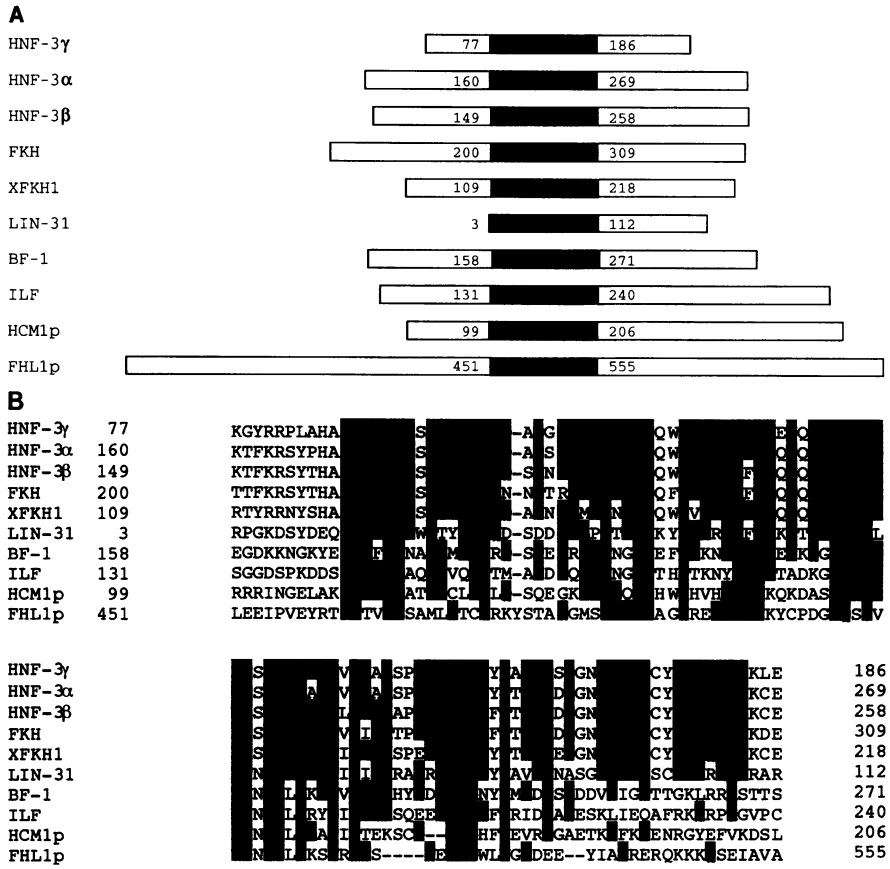

FIG. 2. Homology of Fhl1p to the *fork head* protein family. (A) Schematic diagram of the protein family, with location of the *fork head* DNA-binding domain. The homologous region of about 110 residues is indicated by a solid box. The positions of residues at the N and C termini of the *fkh* domain are given. The proteins (translated from the DNA sequences; EMBL accession numbers in parentheses) examined were: HNF-3α (X55955), -3β (L09647), and 3γ (L09648), rat hepatocyte nuclear factors; FKH (J03177), *D. melanogaster fork head* protein; XFKH1 (S93559), activin-inducible *fork head*-like *X. laevis* protein; LIN-31 (L11148), product of *C. elegans* cell lineage gene *lin-31*; BF-1 (M87634), rat brain factor 1; ILF (X60787), human interleukin binding factor; Hcm1p (L08252), high-copy-number suppressor of *S. cerevisiae* calmodulin mutation; Fhl1p (Z28348), *fork head*-like *S. cerevisiae* protein. (B) Amino acid sequence of the proteins in the 110-amino-acid homologous region. Residues that are identical in at least six of the proteins are shaded. A more extensive homology can be noted for the HNF-3 fork head family.

on both strands revealed a unique 2,808-bp open reading frame, coding for a 936-amino-acid protein with a predicted molecular mass of 103 kDa. Inspection of current data banks revealed significant homology to the *fork head* family of proteins (Fig. 2). This homology was restricted to the *fork head* DNA-binding domain itself, originally identified as a 110-amino-acid motif strongly conserved between the product of the *Drosophila melanogaster* homeotic gene *fork head* (55, 56), the three rat hepatocyte HNF-3 transcription factors (23, 24), and the *Xenopus laevis XFKH1* gene, which is also expressed in a tissue-specific manner (11). As summarized in Fig. 2, the *fork head* domain is strongly conserved among these five proteins (which are otherwise not sequence related), less so in the ILF1 (28), LIN-31 (33), and BF-1 (50) proteins, and even less so in the products of the two yeast genes *FHL1* (this work) and *HCM1* (61). *HCM1*, isolated as a multicopy suppressor of a conditional calmodulin mutation, has no sequence homology to *FHL1* beyond the *fork head* domain itself.

Fhl1p differs from all other known *fork head* proteins by its high content of charged residues. These residues are distributed in clusters (Fig. 3), with a stretch of basic amino acids immediately upstream of the *fork head* motif and two large acidic domains at the N and C termini. The C terminus also contains stretches of serine residues, in keeping with the rather high serine content of *fork head* proteins. As mentioned below, removing this carboxy-terminal region did not markedly affect cell growth.

Northern analysis with the 762-bp *Pvu*II intragenic fragment as the probe revealed a single 3.4-kb mRNA that was strongly overexpressed in strains containing the *FHL1* multicopy plasmid (Fig. 4). *FHL1* overexpression, however, did not affect the level of the *RPC160* transcript itself (data not shown), indicating that suppression was not due to increased transcription of the latter gene. The 5′-flanking sequence of the gene contains an ABF-I box (9) at positions −401 to −389 that might be relevant to gene regulation.

An open reading frame located on the 3′ side of *FHL1* and transcribed in the opposite orientation (Fig. 1) was identified as *PRE2*, encoding a yeast proteosomal subunit (15). Southern hybridization against yeast chromosomal blot and prime clone grid filters (see Materials and Methods) showed that the *FHL1* gene is unique and located on the right arm of chromosome XVI. Tetrad analysis indicated a very tight linkage (18 parental ditypes among 18 asci) with the *RPC40* gene, encoding the 40-kDa subunit common to RNA polymerase I and III (29). The precise order of the linked *FHL1*, *PRE2*, and *RPC40* genes on chromosome XVI remains to be determined.

Suppressor effect of *FHL1* on other conditional mutations

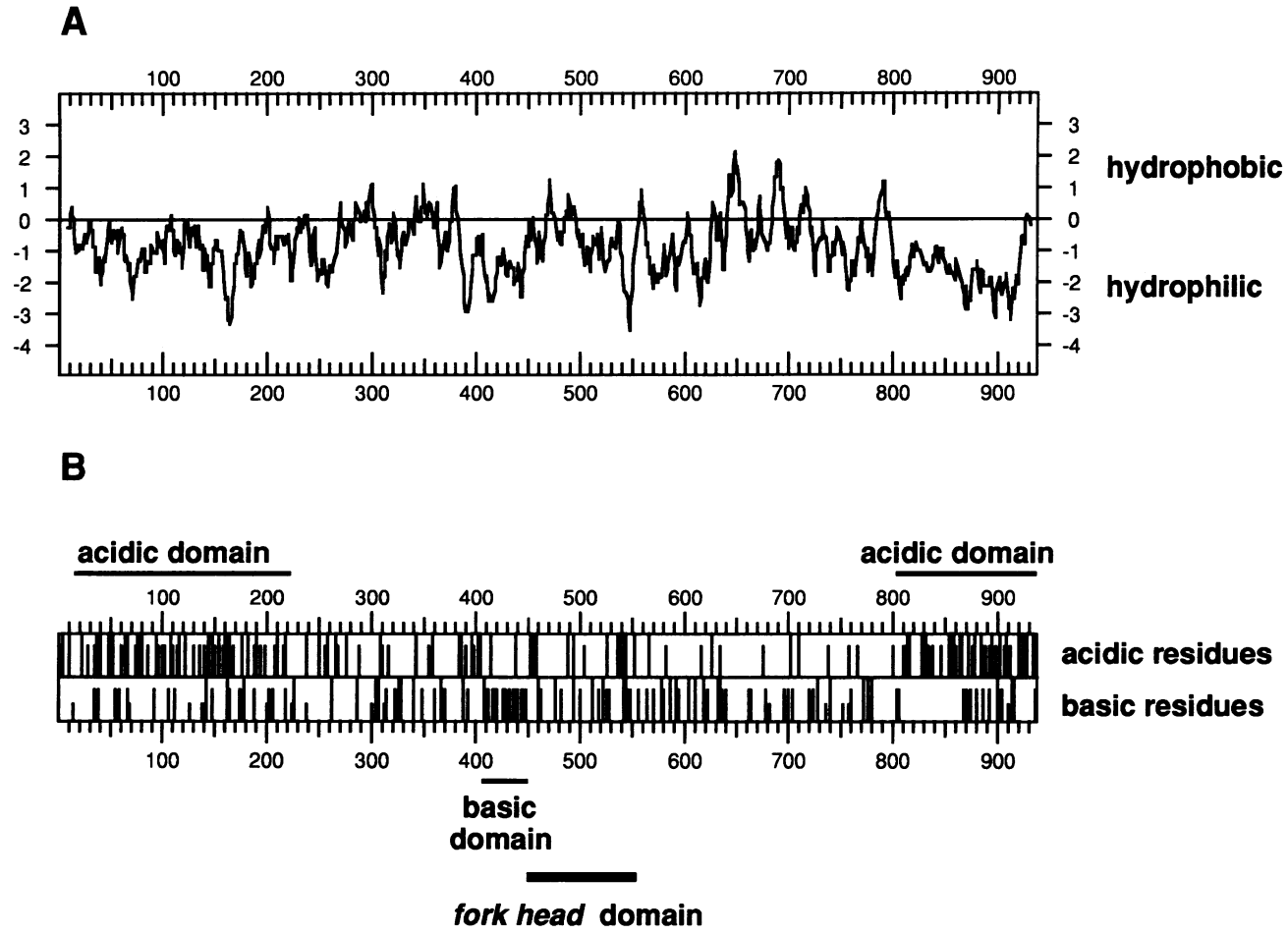

FIG. 3. Sequence analysis of Fhl1p. (A) Hydrophobicity plot of Fhl1p amino acid sequence. (B) Distribution of acidic and basic residues in Fhl1p amino acid sequence. Top box, acidic residues. Full bars indicate glutamic acid, and partial bars indicate aspartic acid. Bottom box, basic residues. Full bars indicate arginine, intermediate bars indicate lysine, and small bars indicate histidine.

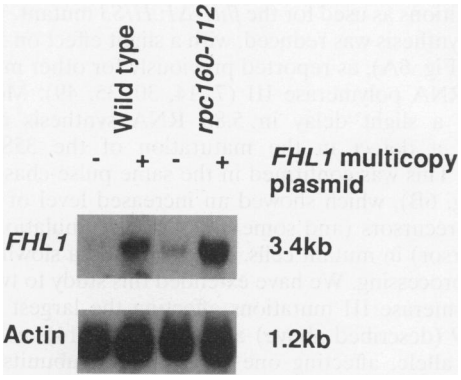

FIG. 4. Northern blot analysis of *FHL1* transcription. Yeast polyadenylated RNA from wild-type YNN281 and mutant MW657 (*rpc160-112*) strains, carrying the pSHB4 (*FHL1*) multicopy plasmid or not, as indicated, were separated on a 1% agarose gel. After transfer, the nylon membrane was hybridized with the 600-bp *Eco*RI-*Hin*dIII actin fragment from pSPACT (38) and the 762-bp *Pvu*II *FHL1* internal fragment (Fig. 1). The mRNAs for the actin and *FHL1* genes are 1.2 and 3.4 kb long, respectively. A denatured commercial RNA ladder (Bethesda Research Laboratories) was used as size markers.

affecting RNA polymerase I, II, or III. *FHL1* was tested for its ability to suppress other conditional mutations causing defects in RNA polymerase I, II, or III subunits (Table 2). There was no effect on the RNA polymerase I and II mutations tested, but three of seven RNA polymerase III mutations were suppressed: *rpc160-112*, *rpc160-209*, and *rpc31-236*. The last affects the C31-specific subunit of RNA polymerase III (48), indicating that suppression was not subunit or domain specific. There was allele specificity in the suppressibility of mutations affecting the largest subunit, since *rpc160-41* (14) and *rpc160-206* (located in the same conserved motif as *rpc160-112* and *rpc160-209* [15a]) were not suppressed. As discussed below, the three suppressible mutations partly affect rRNA processing, but this is also the case for the nonsuppressible *rpc160-41* allele. The suppression specificity may reflect the somewhat stronger effect of *rpc160-41* on rRNA processing (see Fig. 6B). However, *rpc160-41* may also differ from the three other mutations in chiefly affecting enzyme assembly rather than enzyme function (14, 28a).

Properties of *fhl1* null alleles. As shown in Fig. 1, *FHL1* was inactivated in the diploid strain YNN281 × YNN282 by insertion of the *HIS3* marker in the middle of the open reading frame (*fhl1*::*HIS3*) or by deletion of the 2.7-kb *Bam*HI fragment, which removes all of the coding sequence except for the first 128 codons (*fhl1*-Δ*1*::*HIS3*). Tetrad analysis showed that

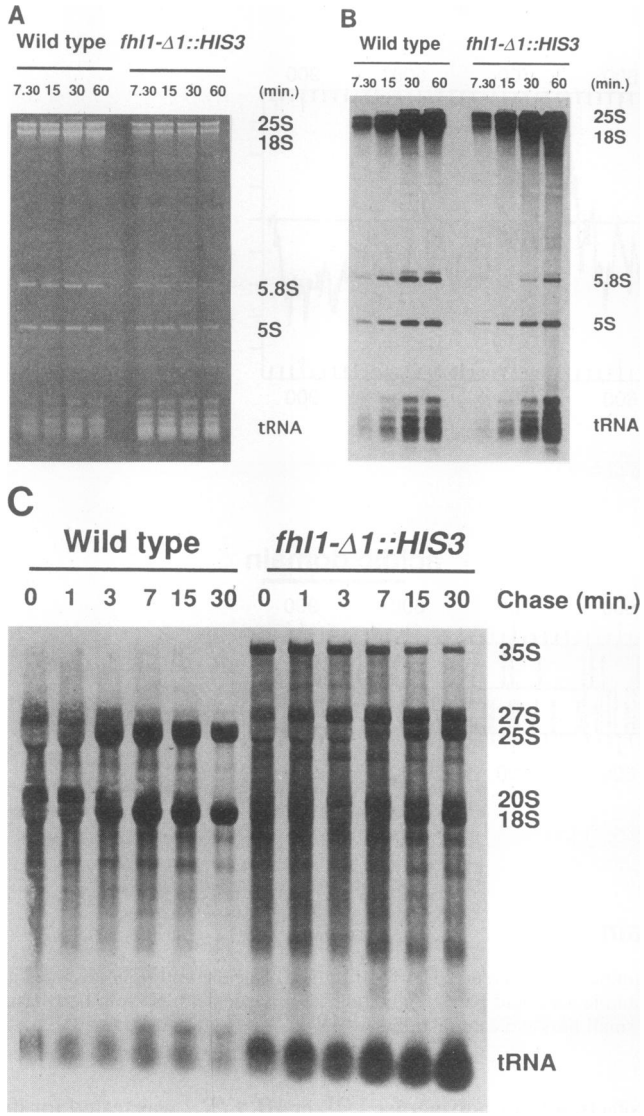

FIG. 5. RNA labeling in wild-type and *fhl1-Δ1::HIS3* deleted strains. RNAs were labeled and prepared as described in Materials and Methods. Samples were separated on a 7 M urea–6% polyacrylamide gel (A and B) or on a 1.2% agarose gel (C). (A) Ethidium bromide staining of RNA species labeled for various periods of time, as shown above the lanes. Each lane contained 3 μg of total RNA. (B) Autoradiography of the dried polyacrylamide gel shown in panel A. (C) Autoradiography of pulse-chase-labeled RNAs after agarose gel separation and membrane blotting. Cells were labeled for 2.5 min and chased with a 320-fold excess of cold uracil. Each lane contained about 20,000 cpm of total RNAs. rRNA precursors (35S, 27S, and 20S) and mature products (25S and 18S) are indicated.

both alleles were viable in the haploid state (Fig. 1) but led to a reduced growth rate at all temperatures tested. These two mutations are phenotypically indistinguishable, and all subsequent work was done with the deletion. In YPD rich medium at 30°C, the *fhl1-Δ1::HIS3* haploid strain MW666 had a doubling time of 330 min versus 90 min for the wild-type strain YNN281. Complementation by the *FHL1* gene borne on plasmid pSHB84 restored the wild-type phenotype. Thus, *FHL1* is a nonessential gene, but its inactivation greatly impairs the growth of the deleted strain. The truncated allele *fhl1-Δ2*, in which the codons for the last 171 amino acids of Fhl1p are removed, also restored full growth when transferred to MW666, implying that the corresponding C-terminal region is not essential for the biological activity of Fhl1p (6a).

Cell extracts from an *fhl1-Δ1::HIS3* mutant culture yielded about five times less RNA than a wild-type culture, essentially because of reduced rRNA accumulation, since the rRNA/tRNA ratio was five times lower in the mutant extract (Fig. 5A). We examined the de novo synthesis of tRNAs and rRNAs in the *fhl1-Δ1::HIS3* mutant strain MW666 by in vivo labeling at 30°C with ^{3}H-labeled uracil. Figure 5B compares the relative labeling of the small rRNA species synthesized by RNA polymerases I (5.8S) and III (5S rRNA and tRNA). There is a marked delay in the labeling of the 5.8S rRNA in the mutant context. The 5S and 5.8S rRNAs accumulate at equimolar ratios in both the wild-type and mutant strains (Fig. 5A) and are synthesized at similar rates in the wild-type strain, yet there is a two- to threefold decrease in the rate of 5.8S accumulation in the *fhl1-Δ1::HIS3* mutant (Fig. 5B). This suggests a specific degradation of excess 5S rRNA in the *fhl1-Δ1::HIS3* mutant.

The slower rate of 5.8S rRNA synthesis could be due to defective transcription of the 35S rRNA precursor or to defective processing of the precursor into the mature 5.8S, 18S, and 25S rRNA species. To distinguish between these two possibilities, we monitored rRNA processing after a short pulse (2.5 min) in tritiated uracil. As shown in Fig. 5C, there was a marked accumulation of the 35S, 27S, and 20S rRNA precursors in the *fhl1-Δ1::HIS3* mutant strain. In addition, since the synthesis of the large rRNA precursor in the mutant is comparable to that in the wild type (Fig. 5B) and the amounts of accumulated rRNAs (25S and 18S rRNA) are much lower in the mutant than in the wild type (Fig. 5A), a fraction of the large precursor rRNAs are likely to be degraded. Taken together, our data are consistent with a defect in rRNA maturation associated with the genetic inactivation of *FHL1*, leading to a lower cellular rRNA content and thus to a slower growth rate.

Effect of temperature-sensitive RNA polymerase III mutations on rRNA processing. Since *FHL1* was cloned as a multicopy suppressor of RNA polymerase III mutations, we wondered whether the latter themselves affected rRNA maturation, in addition to their known effects on tRNA synthesis. The synthesis of small RNAs in the *rpc160-112* mutant at 30°C was monitored after in vivo labeling with uracil, under the same conditions as used for the *fhl1-Δ1::HIS3* mutant. The rate of tRNA synthesis was reduced, with a slight effect on 5S RNA synthesis (Fig. 6A), as reported previously for other mutations affecting RNA polymerase III (7, 14, 30, 35, 49). Moreover, there was a slight delay in 5.8S RNA synthesis at 30°C, suggesting a defect in the maturation of the 35S rRNA precursor. This was confirmed in the same pulse-chase experiment (Fig. 6B), which showed an increased level of the 35S and 27S precursors (and some effect on accumulation of the 20S precursor) in mutant cells. This indicated a slowing down of rRNA processing. We have extended this study to two other RNA polymerase III mutations affecting the largest subunit, *rpc160-209* (described above) and *rpc160-41* (14), and to the *rpc31-236* allele, affecting one of the small subunits of that enzyme (48), grown at the semipermissive temperature of 30°C. All showed an accumulation of pre-rRNA precursors that was similar to or even stronger than that for *rpc160-112* (Fig. 6B and data not shown).

We examined the level of tRNA synthesis and pre-rRNA processing of the *rpc160-112* mutant in the presence of the *FHL1* multicopy suppressor plasmid in steady-state growth at the semipermissive temperature (30°C). Since the synthesis of RNAs was not affected by the presence of *FHL1* under these

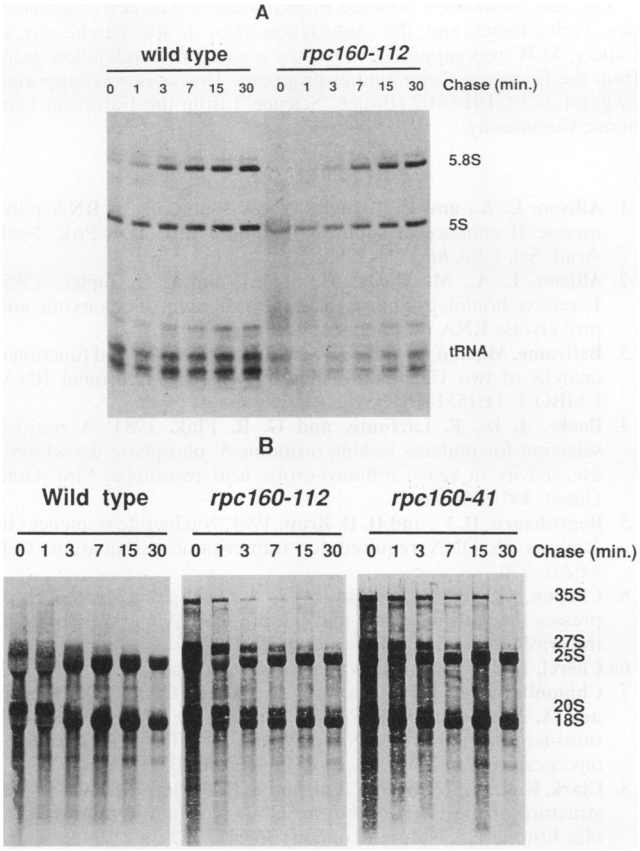

FIG. 6. Pulse-chase labeling of RNAs in wild-type and *rpc160-112* and *rpc160-41* mutant strains. Cells were labeled as described in the legend to Fig. 5C. RNAs were separated either on 7 M urea–6% polyacrylamide gel (A) or on a 1.2% agarose gel (B). (A) Autoradiogram of dried polyacrylamide gel, showing 5.8S RNA, 5S RNA, and tRNA species. A total of 60,000 cpm was loaded in each lane. (B) Autoradiogram of transfer membrane of agarose gels, showing 35S, 27S, and 20S rRNA precursors and 25S and 18S final products. The radioactivity loaded on the gel and the times of exposure were 40,000, 5,000, and 10,000 cpm and 3, 15, and 15 days for the wild-type and *rpc160-112* and *rpc160-41* mutant strains, respectively, to avoid gel overloading.

growth conditions (data not shown), we shifted the temperature to 37°C for 0, 1, 4, and 8.5 h. The synthesis of rRNA was partly restored by the suppressor, but tRNA synthesis was also enhanced (data not shown). Increasing the *FHL1* copy number therefore generally improved RNA synthesis in an *rpc160-112* context, making it difficult to discriminate between direct suppression effects and indirect effects due to the improved growth rate of the suppressed mutant.

mRNA splicing in *rpc160-112* and *fhl1-Δ1::HIS3* mutants. A deficiency of ribosomal protein synthesis has been found to affect rRNA maturation (41, 54). Many ribosomal protein genes contain an intron. A defect in splicing of mRNAs might then affect rRNA maturation because the synthesis of ribosomal proteins would be reduced. Decreased splicing in an *rpc160-112* mutant could be brought about by reduced levels of the spliceosomal U6 RNA, which is an RNA polymerase III transcript (34). We thus investigated the splicing efficiency of mRNA in *rpc160-112* and *fhl1-Δ1::HIS3* mutants by the method of Legrain and Rosbash (27). In this method, an intron is inserted into the β-galactosidase coding sequence of a *GAL1::lacZ* gene fusion. The intron has to be spliced out for β-galactosidase expression. Splicing can thus be monitored simply by measuring β-galactosidase production by mutants transformed with this construction. The results presented in Table 3 indicate that splicing is not significantly affected in mutants carrying the *rpc160-112* or *fhl1-Δ1::HIS3* mutation as it is in a bona fide splicing mutant carrying *prp9-1*, which abolishes splicing at the restrictive temperature (6). We also examined the accumulation of precursor and mature mRNAs of actin and ribosomal protein 51 (rp51) by Northern blotting in the *fhl1-Δ1::HIS3* mutant. There was no difference in the level of either species between mutant and wild-type strains (data not shown), confirming the absence of a splicing defect and suggesting that *FHL1* does not control rp51 synthesis.

TABLE 3. In vivo assay of splicing in the *rpc160* and *fhl1* mutant strains

Strain	Relevant genotype	Growth temp (°C)	β-Galactosidase activity (% of wild-type activity)
YNN281	Wild type	30	100
MW657	*rpc160-112*	30	134
MW666	*fhl1-Δ1::HIS3*	30	159
Cy103	*prp9-1*	25	21
		37	1

DISCUSSION

The *fork head* protein family was initially defined by the ~110-amino-acid *fork head* motif that is highly conserved between the product of the developmental gene *fkh* of *D. melanogaster* (56) and the mammalian tissue-specific transcription factors HNF-3α, HNF-3β, and HNF-3γ (23, 24). It also includes the product of the cell lineage gene *lin-31* (33), the human transcription factor ILF (28), the product of the human brain-specific BF-1 cDNA (50), and the tissue-specific protein encoded by *XFKH1* in *X. laevis* (11). Except for the *fork head* domain itself, these proteins are not sequence related. The HNF-3α and ILF transcription factors have a well-demonstrated DNA-binding activity. Deletion analysis strongly suggested that this activity resides on the *fork head* domain (23, 28), and two mutations of the *fkh* domain of the *D. melanogaster* gene resulted in amorphic phenotypes (56). In addition, the cocrystal structure of the HNF3/*fork head* DNA recognition motif has now been determined (8).

The cloning of *FHL1* and of another yeast *fork head* gene, *HCM1* (61), shows that *fork head* proteins are not restricted to tissue-specific or developmental regulation in animals but are also present in unicellular eukaryotes. The *fork head* domain of these two gene products is distantly related to the canonic motif, which contrasts with the very strong conservation observed between the mammalian HNF-3 factors, the *X. laevis XFKH1* gene product, and the *fork head* protein of *D. melanogaster* (the mammalian *fork head* proteins ILF and BF-1 show an intermediate degree of similarity to the canonical sequence). Therefore, Fhl1p belongs to a superfamily of *fork head* proteins. It also differs from other *fork head* proteins by the abundance of charged residues, with a basic domain located immediately upstream of the *fork head* motif and two large acidic regions at the N and C termini of the polypeptide. Removal of the latter by partial truncation of Fhl1p has no effect on cell growth.

Our data show that Fhl1p is important for optimal growth and plays a key role in the control of rRNA processing, leading to transient accumulation of the 35S, 27S, and 20S rRNA

precursors and a reduced steady-state level of mature 25S, 18S, and 5.8S rRNAs in an *fhl1* deletant strain. A similar multistep defect in rRNA processing was observed in mutants depleted in small nucleolar RNAs (snoRNAs) or proteins involved in pre-rRNA processing (40, 42, 52). The presence of a *fork head* DNA-binding domain implies that Fhl1p, rather than being itself a component of the RNA processing machinery, presumably operates as a transcription factor, regulating (among other possible targets) the synthesis of some rate-limiting RNA(s) or protein(s) of the rRNA processing machinery. As discussed below, the cloning of *FHL1* as a suppressor of RNA polymerase III mutations need not imply that Fhl1p belongs to the RNA polymerase III transcriptional apparatus rather than to the RNA polymerase II transcription system. The potential effects of Fhl1p on the RNA polymerase II or III machinery will ultimately be clarified by the identification of its presumptive target genes.

RNA polymerase III-defective mutants are mildly defective in pre-rRNA processing, which, along with its primary effect on tRNA synthesis, may contribute to the reduced growth rate of these mutants. The increased gene dosage of *FHL1* might therefore have a weak suppressor effect simply by alleviating the pre-rRNA processing defect. Interestingly, *FHL1* was recently recloned as a multicopy suppressor of a mutation causing defects in the RNA polymerase III transcription factor TFIIIC. Moreover, the *NOP1* gene, which encodes the snoRNA-binding nucleolar protein fibrillarin, was recovered in the same screen (26a). Strikingly, depleting the yeast cell of fibrillarin provokes an rRNA maturation defect very similar to that caused by the *fhl1* mutation (52). These observations strengthen the link between RNA polymerase III transcription and rRNA maturation. For *rpc160-112* and the other *FHL1*-suppressible mutations causing defects in TFIIIC or RNA polymerase III, the rRNA maturation defect might be due to the depletion of an RNA polymerase III transcript that is rate limiting for pre-rRNA processing. An obvious candidate is 5S rRNA: its total pool is hardly affected in *rpc160-112* mutants (see references 14 and 49), but pre-rRNA maturation may conceivably depend on a minor pool of ribosome-free 5S rRNA. Similarly, the level of U6 small nuclear RNA (a proven RNA polymerase III transcript [34]) may affect the accumulation of ribosomal proteins by preventing the splicing of the corresponding mRNAs, impairing the maturation of the rRNA. This possibility is, however, very unlikely, since *rpc160-112* had no detectable effect on mRNA splicing. Finally, two small nucleolar RNA species, U3 and 7-2/MRP, are RNA polymerase III transcripts in some metazoan species (20, 21) but probably not in *S. cerevisiae*, since the 5′ m_3G cap of the yeast U3 suggests that it is an RNA polymerase II transcript (18), whereas the *NME1* gene (encoding the yeast 7-2/MRP RNA [45]) contains an internal stretch of 8 T's that is a strong RNA polymerase III terminator signal (5). Moreover, *rpc160-112* had no effect on the level of the corresponding yeast RNA species in Northern hybridization experiments (data not shown). The identity of the RNA polymerase III transcript involved in the pre-rRNA processing defect of RNA polymerase III-defective mutants therefore remains to be established.

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