Stable mRNP Formation and Export Require Cotranscriptional Recruitment of the mRNA Export Factors Yra1p and Sub2p by Hpr1p

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Yra1p/REF participates in mRNA export by recruiting the export receptor Mex67p to messenger ribonucleoprotein (mRNP) complexes. Yra1p also binds Sub2p, a DEAD box ATPase/RNA helicase implicated in splicing and required for mRNA export. We identified genetic and physical interactions between Yra1p, Sub2p, and Hpr1p, a protein involved in transcription elongation whose deletion leads to poly(A)$^+$ RNA accumulation in the nucleus. By chromatin immunoprecipitation (ChIP) experiments, we show that Hpr1p, Sub2p, and Yra1p become associated with active genes during transcription elongation and that Hpr1p is required for the efficient recruitment of Sub2p and Yra1p. The data indicate that transcription and export are functionally linked and that mRNA export defects may be due in part to inefficient loading of essential mRNA export factors on the growing mRNP. We also identified functional interactions between Yra1p and the exosome components Rrp45p and Rrp6p. We show that *yra1*, *sub2*, and Δ*hpr1* mutants all present defects in mRNA accumulation and that deletion of *RRP6* in *yra1* mutants restores normal mRNA levels. The data support the hypothesis that an exosome-dependent surveillance mechanism targets improperly assembled mRNPs for degradation.

mRNAs are exported from the nucleus as messenger ribonucleoprotein (mRNP) complexes which begin to be assembled during transcription. mRNA biogenesis requires multiple processing steps, including the addition of a 5′ cap, splicing, and 3′-end formation, which have to be completed before the mRNA can be exported. Fully mature mRNPs are subsequently recognized by the essential mRNA export receptor Mex67p (TAP in metazoans), which mediates the interaction between the mRNP and components of the nuclear pore complex (42, 51). The recruitment of Mex67p/TAP to the mRNP is facilitated by Yra1p (Aly in metazoans), an essential mRNA export factor which binds RNA and directly interacts with Mex67p (TAP) (47, 48, 52). Yra1p is a member of the evolutionary conserved REF family of RNA and export factor binding proteins, and multiple REF members exist in many organisms (48). In *Saccharomyces cerevisiae*, overexpression of the nonessential REF family member Yra2p can complement the otherwise lethal deletion of *YRA1*, indicating that these two REF proteins have redundant functions (47, 52).

Evidence has been accumulating that the different steps of gene expression, from transcription in the nucleus to translation and degradation in the cytoplasm, are intimately linked (37). Many mRNA processing factors are recruited to growing mRNPs through an interaction with the transcription machinery. For example, the yeast mRNA capping enzymes required for the addition of the m^{7}G cap structure associate with the C-terminal domain (CTD) of RNA polymerase II at an early stage of transcription (23). Similarly, components of the splicing or polyadenylation machinery associate with the CTD, thereby positioning them to mediate efficient RNA processing.

These interactions are required in vivo for maximum levels of splicing and polyadenylation, as well as for efficient transcription termination in vivo (2, 13, 17, 38).

In yeast, defects in 3′-end processing and/or polyadenylation prevent mRNA export and lead to the retention of transcripts at or close to their site of transcription, indicating a coupling between 3′-end formation and mRNA export in a step that involves the release of mRNA from nuclear foci (3, 20). Mutations in the nuclear exosome abolish the nuclear retention of incorrectly processed transcripts, suggesting that this exonuclease complex participates in a quality control mechanism that prevents the nuclear escape of improperly processed mRNAs and directs them to degradation (4, 16, 39).

A functional link between pre-mRNA splicing and mRNA export has also been established (34). In metazoan systems, splicing results in the deposition of the exon junction complex at a fixed position upstream of the splice junction, which marks the mRNA for subsequent steps, such as mRNA export or cytoplasmic turnover (21, 26, 27, 36). The exon junction complex contains the Yra1p homologue Aly/REF, as well as UAP56, a DECD box ATPase/RNA helicase implicated in an early step of spliceosome formation. UAP56 directly interacts with Aly/REF and was proposed to functionally link splicing to export by enhancing the recruitment of Aly/REF to spliced mRNAs, which would in turn facilitate the binding of the export receptor TAP (12, 14, 26, 32, 35, 54). Sub2p, the yeast functional homologue of UAP56, has also been implicated in splicing (22, 31, 53). Sub2p directly interacts with Yra1p, and both *sub2* and *yra1* mutants induce poly(A)$^+$ RNA export defects (19, 45). Although splicing is an essential step in the formation of virtually all metazoan mRNAs, only 5% of yeast genes have introns, excluding splicing as a major mode of recruitment for Yra1p. Accordingly, Sub2p/UAP56 is required for the

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TABLE 1. Yeast vectors and plasmids used in this study

Plasmid code	Plasmid name	Description	Reference and/or source
pFS2511	pCH1122-YRA1	*YRA1* gene +/−500 bp cloned as a *Bam*HI fragment into vector pCH1122 (*URA3 ADE3* CEN)	This study
pFS2576	Lac111-HPR1	*HPR1* gene +/−500 bp cloned as a *Bam*HI fragment into vector YCpLac111 (*LEU2* CEN)	This study
pFS2575	Lac111-RRP45	*RRP45* gene +/−500 bp cloned as a *Bam*HI fragment into YCpLac111 (*LEU2* CEN)	This study
pFS2525	Lac111-YRA1 Gen	*YRA1* gene +/−500 bp cloned as a *Bam*HI fragment into YCpLac111 (*LEU2* CEN)	This study
pFS2327	Lac111-HA-*yra1* ∆RBD	HA-tagged *YRA1* cDNA +/−500 bp, deleted for central RBD, subcloned as a *Bam*HI fragment from pFS2268 into YCpLac111 (*LEU2* CEN)	52
pFS2325	Lac111-HA-*yra1-77-227*	HA-*yra1-77-227* +/−500 bp*Bam*HI fragment from pFS2156 subcloned into YCpLac111 (*LEU2* CEN)	This study
pFS2328	Lac111-HA-*yra1-8*	*yra1-8* (*LEU2* CEN); for details, see plasmid and strain constructions	This study
pFS2554	Lac111-GFP-*yra1-8*	GFP–*yra1-8* (*LEU2* CEN); for details, see plasmid and strain constructions	This study
pFS2574	pFL36-SUB2	*SUB2* gene expressed from its own promoter (*LEU2* CEN)	19
pFS2655	Lac111-*sub2-201*	*sub2-201* (*LEU2* CEN); for details, see plasmid and strain constructions	This study
pFS2656	Lac111-*sub2-206*	*sub2-206* (*LEU2* CEN); for details, see plasmid and strain constructions	This study
pFS2625	pNOPProtA-SUB2	*SUB2* ORF cloned as a *Nco*I-*Bam*HI fragment into pNOPPATA1L (*LEU2* CEN)	This study, 15
pFS2653	pNOPProtA-*sub2-201*	*sub2-201* ORF cloned as a *Nco*I-*Bam*HI fragment into pNOPPATA1L (*LEU2* CEN)	This study
pFS2654	pNOPProtA-*sub2-206*	*sub2-206* ORF cloned as a *Nco*I-*Bam*HI fragment into pNOPPATA1L (*LEU2* CEN)	This study
pFS2015	pLGSD5	Galactose-inducible β-galactosidase reporter construct (*URA3*, 2μm)	25
pFS2672	GAL-YAT1	Galactose-inducible *YAT1* gene (*URA3*, 2μm)	8
pFS2642	pGex-HPR1	*HPR1* ORF PCR fragment cloned as a *Bam*HI fragment into pGex 4T-1 (Amersham Pharmacia)	This study
pFS1853	pGex-YRA1	*YRA1* ORF cloned into pGexCS	48
pFS2113	pGex-YRA2	*YRA2* ORF cloned into pGex4T-1	52
pFS2633	pET9d⁺-SUB2	*SUB2* ORF cloned as *Nco*I-*Bam*HI PCR fragment into pET9d⁺	This study
pFS2676	pProEX HT-SUB2	*SUB2* ORF cloned as *Bam*HI PCR fragment into pProEX HTb (Life Technologies)	This study

export of spliced mRNAs as well as of mRNAs derived from intronless genes. Sub2p/UAP56 may therefore play a general conserved role in recruiting Yra1p/Aly to mRNPs, irrespective of whether they contain an intron. In vitro experiments suggest that in a subsequent step, the binding of Mex67p to Yra1p induces the release of Sub2p from Yra1p prior to mRNA export (14, 19, 32, 35, 42, 45).

These observations raise the question of how Yra1p and Sub2p are directed to mRNAs derived from intronless genes. A recent study indicates that Yra1p/REF is associated with actively transcribed genes, suggesting that this essential mRNA export mediator is recruited to the mRNA during transcription (9, 28). A link between Sub2p and the transcription machinery was similarly suggested by the ability of high-copy-number *SUB2* to suppress the deletion of *HPR1*, a gene implicated in transcription elongation (11). Genetic interactions have also been described between Sub2p and Rad3p, a bona fide RNA polymerase II component (19).

To further characterize the role of Yra1p in mRNA biogenesis and define when and how Yra1p may be recruited to the newly synthesized transcripts, we have searched for mutations synthetically lethal with a *yra1* mutant. In this screen, we identified *HPR1* and demonstrated that Hpr1p, Sub2p, and Yra1p physically interact. Chromatin immunoprecipitation (ChIP) experiments show that Hpr1p, Sub2p, and Yra1p are recruited cotranscriptionally at a similar time during elongation and that Hpr1p is required for efficient recruitment of Yra1p and

Sub2p. Deletion or mutations in these components lead to diminished mRNA levels, suggesting that improper loading of mRNA export factors results in mRNP instability. These observations, together with those of genetic and physical interactions between Yra1p and the nuclear exosome, suggest that mRNPs undergo an exosome-dependent quality control step which competes with mRNP export.

MATERIALS AND METHODS

Plasmid and strain constructions. Plasmids and strains used in this study are summarized in Tables 1 and 2. The *yra1-8* temperature-sensitive allele was constructed by site-directed mutagenesis, which changed 2 amino acids within the conserved N-box ($D_{10}D_{11}$ to $K_{10}K_{11}$) of pGexCS-Yra1 (48), creating pGexCS-*yra1-8* (pFS1966). The *yra1-8* coding sequence was subsequently amplified by PCR and cloned as a *Sal*I fragment into YCplac33-Gal-GFP (pFS1846). The resulting GFP–*yra1-8* fusion was transferred as a *Xho*I PCR fragment into the YCpLac22HA-YRA1 +/−500 bp cassette, resulting in YCplac22-HA-GFP-*yra1-8* (pFS2557). To obtain YCpLac111-HA-GFP-*yra1-8* (pFS2554), the *Bam*HI GFP–*yra1-8* +/−500 bp fragment from YCpLac22-HA-GFP-*yra1-8* was transferred into YCpLac111. YCpLac111-HA-*yra1-8* (pFS2328) was obtained by inserting a *Sal*I PCR fragment of *yra1-8* into Ycplac22HA-YRA1 +/−500 and subsequent subcloning of a *Bam*HI HA–*yra1-8* +/−500 bp fragment in YCpLac111.

YCpLac111-*sub2-201* and *-206* (pFS2655 and 2656) were obtained by replacing the wild-type *SUB2* coding region by the *sub2-201* and *-206* mutant sequences into YCplac111-SUB2 +/−500 (OFS2624).

Genomic tagging of *HPR1*, *RRP45*, and *RRP6* was done as described previously (52). Deletions of *HPR1* and *RRP6* were obtained as described previously (33). ProtA-Sub2 wild-type and mutant strains were obtained by transforming plasmids pFS2625 (wild type), pFS2653 (*sub2-201*), and pFS2654 (*sub2-206*) into

TABLE 2. Yeast strains used in this study

Strain code	Strain name	Genotype	Reference(s) or source
CH1462 background			
CH1462	wild type	*MATα ade2 ade3 his3 leu2 ura3*	49, 50
FSY1371	SL screening strain	*MATα ade2 ade3 leu2 ura3 his3 yra2::KAN^r yra1ΔRBD* ⟨pCH1122-*URA3-ADE3*-YRA1, pFS2511⟩	This study
FSY1681	sl57	*MATα ade2 ade3 leu2 ura3 his3 hpr1-57 yra2::KAN^r yra1ΔRBD* ⟨pCH1122-*URA3-ADE3*-YRA1 Gen, pFS2511⟩	This study
FSY1682	sl5	*MATα ade2 ade3 leu2 ura3 his3 rrp45-1 yra2::KAN^r yra1ΔRBD* ⟨pCH1122-*URA3-ADE3*-YRA1 Gen, pFS2511⟩	This study
W303 background			
W303	wild type	*mat**a** ade2 his3 leu2 trp1 ura3*	
FSY1026	YRA1 shuffle	*mat**a** ade2 his3 leu2 trp1 ura3 yra1::HIS3* ⟨p*URA3*-YRA1 Gen, pFS1876⟩	48
FSY1568	GFP–*yra1-8*	*mat**a** ade2 his3 leu2 trp1 ura3 yra1::HIS3* ⟨p*LEU2*-GFP-*yra1-8*, pFS2554⟩	This study
FSY1655	YRA1 shuffle Δ*hpr1*	*mat**a** ade2 his3 leu2 trp1 ura3 hpr1::TRP1 yra1::HIS3* ⟨p*URA3*-YRA1 Gen, pFS1876⟩	This study
FSY1621	YRA1 shuffle Δ*rrp6*	*mat**a** ade2 his3 leu2 trp1 ura3 rrp6::KAN^r yra1::HIS3* ⟨p*URA3*-YRA1 Gen, pFS1876⟩	This study
FSY1624	Δ*hpr1*	*mat**a** ade2 his3 leu2 trp1 ura3 hpr1::TRP1*	This study
DLY23	SUB2 shuffle	*mat**a** ade2 his3 leu2 trp1 ura3 sub2::HIS3* ⟨p*URA3*-SUB2, pFS2574⟩	19
FSY1613	*sub2-201*	*mat**a** ade2 his3 leu2 trp1 ura3 sub2::HIS3* ⟨p*LEU2*-*sub2-201*, pFS2655⟩	This study
FSY1664	SUB2 shuffle Δ*hpr1*	*mat**a** ade2 his3 leu2 trp1 ura3 hpr1::TRP1 sub2::HIS3* ⟨p*URA3*-SUB2, pFS2574⟩	This study
FSY1473	ProtA-Sub2	*mat**a** ade2 his3 leu2 trp1 ura3 sub2::HIS3* ⟨p*LEU2*-ProtA-SUB2, pFS2625⟩	This study
FSY1615	ProtA-*sub2-201*	*mat**a** ade2 his3 leu2 trp1 ura3 sub2::HIS3* ⟨p*LEU2*-ProtA-*sub2-201*, pFS2653⟩	This study
FSY1616	ProtA-*sub2-206*	*mat**a** ade2 his3 leu2 trp1 ura3 sub2::HIS3* ⟨p*LEU2*-ProtA-*sub2-206*, pFS2654⟩	This study
FSY1656	ProtA-Sub2 Δ*hpr1*	*mat**a** ade2 his3 leu2 trp1 ura3 hpr1::TRP1 sub2::HIS3* ⟨p*LEU2*-ProtA-SUB2; pFS2625⟩	This study
FSY1525	Hpr1-ProtA	*mat**a** ade2 his3 leu2 trp1 ura3 sub2::HIS3* ⟨p*URA3*-SUB2⟩ *HPR1*-ProtA-*KAN^r*	This study, 19
FSY1476	Rrp45-ProtA	*mat**a** ade2 his3 leu2 trp1 ura3 sub2::HIS3* ⟨p*URA3*-SUB2⟩ *RRP45*-ProtA-*KAN^r*	This study, 19
FSY1657	Rrp6-ProtA	*mat**a** ade2 his3 leu2 trp1 ura3 sub2::HIS3* ⟨p*URA3*-SUB2⟩ *RRP6*-TAP-*tag-KAN^r*	This study, 19

the SUB2 shuffle stain (DLY23) or SUB2 shuffle strain Δ*hpr1* (FSY1664), followed by plasmid shuffling on 5-fluoroorotic acid (5-FOA). ProtA-*sub2-201* (FSY1615) and ProtA-*sub2-206* (FSY1616) showed a temperature-sensitive growth phenotype similar to that of the nontagged strains (data not shown).

Synthetic lethal screen. The Yra1 synthetic lethal screen was performed with the screening strain FSY1371 (*yra1ΔRBD yra2::KAN^r*) containing plasmid pCH1122-YRA1 (*URA3 ADE3*; pFS2511), encoding wild-type Yra1p. In brief, cells were mutagenized by UV irradiation to 10% survival, and surviving clones were analyzed for the absence of the red-white sectoring and the inability to grow on 5-FOA-containing medium, as described previously (49, 50). The screen was performed at 30, 34, or 37°C to increase the probability of selecting different synthetic lethal mutants. Mutants were transformed with a genomic library, and transformants were replicated on 5-FOA to select for complementing clones. Strains sl5 and sl57 were identified at 37 and 30°C, respectively.

Yeast extracts and affinity purifications of ProtA fusions. ProtA-tagged strains were grown in yeast extract-peptone-dextrose (YEPD) medium at 25°C to an optical density at 600 nm (OD$_{600}$) of 1.2. Cells were collected by centrifugation, washed once with 1× phosphate-buffered saline (PBS), resuspended in lysis buffer (20 mM HEPES-KOH [pH 7.0], 150 mM potassium acetate, 2 mM magnesium acetate, 10% glycerol, 0.1% Tween 20 5 mM β-mercaptoethanol, complete protease inhibitor mix [Roche]) at 80 OD$_{600}$ of cells/ml, and lysed with glass beads. The soluble fraction was recovered after centrifugation and used for binding on immunoglobulin G (IgG)-Sepharose for 90 min at 4°C. After extensive washing, associated proteins were eluted from the beads by addition of 2 M NaCl–20 mM HEPES-KOH (pH 7.5) for 20 min at 4°C and precipitated with

20% trichloroacetic acid. Typically, 1 of 100 input and 1 of 6 eluted samples were examined by Western blotting.

Antibodies and Western blotting. Western blotting was performed according to standard procedures. Anti-Yra1p and -Yra2p antibodies were obtained by injecting rabbits with purified glutathione S-transferase (GST) Yra1p and GST-Yra2p proteins. The obtained serum was used at dilutions of 1:3,000 for anti-Yra1p and 1:1,000 for anti-Yra2p in Western blotting. Anti-Hpr1p antibody (a gift from M. F. Christman) was used at 1:3,000. The protein signals were revealed with Super Signal West Pico substrate (Pierce).

Expression, purification of recombinant proteins, and in vitro bindings. All recombinant proteins were expressed in *Escherichia coli* BL21, except for His$_6$-tagged Sub2, which was expressed in *E. coli* DH5α. Cells were grown in Luria broth plus ampicillin (or ampicillin plus kanamycin for coexpressions) to an OD$_{600}$ of 0.6 to 0.8 and induced with 0.5 mM IPTG (isopropyl-β-D-thiogalacto-pyranoside) for 4 h at 37°C, except for GST-Hpr1, which was induced for 5.5 h at 28°C. Cells were collected by centrifugation and washed once with PBS, and the pellets were resuspended in universal buffer (20 mM HEPES-KOAc [pH 7.0], 100 mM KOAc, 2 mM MgOAc, 10% glycerol, complete protease inhibitor mix [Roche]) and lysed with a one-shot cell disrupter (Constant System Ltd.) set to 1.75 kPa. Triton X-100 was added to 0.2%, and lysates were incubated on a rotating wheel for 15 min at 4°C. Cell debris was removed by centrifugation, and total extracts were used for purification on either glutathione-Sepharose (Amersham Pharmacia) for GST fusions or Ni-nitrilotriacetic acid agarose (Qiagen) for His$_6$-Sub2. Bindings were performed for 90 min at 4°C on a rotating wheel. Bound proteins were eluted by addition of sodium dodecyl sulfate (SDS) sample

buffer, boiled, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining.

For binding of His$_6$-Sub2 to GST-Yra1 and -Yra2, an *E. coli* extract expressing His$_6$-Sub2 was incubated with purified GST-Yra1 and -Yra2 immobilized on glutathione beads and washed extensively, and associated proteins were analyzed as described above.

RNA extractions, primer extensions, and Northern blotting. RNA extractions and primer extensions were done as described previously (10). The following 5′ ^{32}P-labeled primers were used: OFS506 (5′ CACCAGTGAGACGGGC 3′) for β-galactosidase, OFS728B (5′ GCGCTCGTCCTGCAGGGGTT 3′) for *YAT1*, OFS751(5′ GCTCGGGCACTTTTCGGCCAA 3′) for *GAL1*, and OFS645 (5′ GGACTTCTTGATCTCCTCTG 3′) for U1 snRNA. Extension products were fractionated on a 6% polyacrylamide-urea gel, autoradiographed, quantified with an Instant Imager apparatus (Packard), and normalized to U1 snRNA levels to correct for loading. Northern blotting was performed according to standard procedures. *PMA1* mRNA levels were quantified with an Instant Imager apparatus and normalized to the 18S rRNA internal control.

In situ hybridizations. In situ hybridizations for poly(A)$^+$ RNA were done as described previously (44), except that a 3′ Cy3-labeled oligonucleotide dT$_{55}$ probe was used. *HSP104*-specific in situ hybridization was performed as described previously (20).

ChIPs. ChIPs were basically done as described previously (24). Cultures (250 ml) were grown at 25°C in YEPD medium to an OD$_{600}$ of 1.2, formaldehyde was added to a final concentration of 1%, and incubation continued for 25 min. When heat shock was performed, cells were incubated at 37°C for 20 min before addition of formaldehyde and were left at 37°C during the cross-linking reaction. Cross-linking was subsequently quenched by the addition of glycine to a concentration of 240 mM. Cells were collected, washed once with 1× PBS, and resuspended in 3 ml of FA lysis buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, complete protease inhibitor mix [Roche]). Cells were lysed with glass beads, and SDS was added to 0.5%. Extracts were then sonicated to obtain chromatin fragments with an average size between 250 and 450 bp. Insoluble material was removed by centrifugation for 20 min at 12,000 × *g* in a tabletop centrifuge, and the soluble material was used as input material. For immunoprecipitation, 200 μl of input material was mixed with 800 μl of FA lysis buffer, 30 μl of protein A Sepharose (Amersham Pharmacia), and the appropriate antibodies and incubated overnight at 4°C on a rotating wheel. Six microliters of anti-Yra1p and anti-Yra2p, 5 μl of anti-Cbp80p (gift from Dirk Görhlich), 5 μl of anti-TATA-binding protein (anti-TBP) (gift from M. Collart), and 9 μl of anti-CTD (Babco 8WG16) were used. For strains containing ProtA fusions, 30 μl of packed IgG-Sepharose (Amersham Pharmacia) was used. Beads were then washed (1 ml each time) once with FA lysis buffer, once with buffer FA500 (50 mM HEPES-KOH [pH 7.5], 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once with buffer III (10 mM Tris HCl [pH 8.0], 1 mM EDTA, 250 mM LiCl, 1% NP-40, 1% sodium deoxycholate), and once with 1× Tris-EDTA. Immunoprecipitated material was eluted from the beads by heating for 15 min at 65°C in 200 μl of elution buffer (50 mM Tris HCl [pH 7.5], 1% SDS, 10 mM EDTA). Cross-links were reversed by incubating the eluted fragments for 5 h at 65°C in the presence of 0.75 mg of proteinase K/ml, and DNA was subsequently purified on a QIAquick PCR purification column (Qiagen).

Real-time PCR. Quantification of the immunoprecipitated DNA was done by quantitative real-time PCR using a Eurogentec qPCR core kit for SYBR Green I and an Applied Biosystems 7000 or 7700 sequence detection system. Immunoprecipitated DNA and different dilutions of sonicated and de-cross-linked input DNA were used for quantification and correction for different PCR efficiencies. PCRs were performed in a volume of 25 μl. Standard PCR conditions were used, i.e., 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The following primer pairs were used: for the *PMA1* promoter, OFS739 (5′ TCCTATCATTATCGTCTAACATCTAAT 3′) and OFS740 (5′ AAATTTATCAACGAGGTTGATAGAAAA 3′); for *PMA1* 5′, OFS705 (5′ TCAGCTCATCAGCCAACTCAAG 3′) and OFS706 (5′ CGTCGACACCGT GATTAGATTG 3′); for PMA1 middle, OFS741 (5′ TTGCCAGCTGTCGTT ACCAC 3′) and OFS742 (5′ TCGACACCAGCCAAGGATTC 3′); for *PMA1* 3′, OFS708 (5′ TACTGTCGTCCGTGTCTGGATCT 3′) and OFS709 (5′ CCT TCATTGGCTTACCGTTCA 3′); for the *PMA1* 3′ untranslated region (3′ UTR), OFS759 (5′ TCTCTGGATGGTACTTTTTCTTTCTTG 3′) and OFS760 (5′ TGCGTGTTGTGAATTGTGCTC 3′); and for the intergenic region, OFS710 (5′ CGCATTACCAGACGGAGATGT 3′) and OFS711 (5′ CAAGC AAGCCTTGTGCATAAGA 3′). The intergenic region lies on the right arm of chromosome IV (nucleotides 1516119 to 1516229). Primers were used at a final concentration of 100 nM, except OFS739 and OFS740, which were used at 300 nM. Each PCR was run in duplicate to control for PCR validation, and all

immunoprecipitations were repeated at least twice with different chromatin extracts. To express the severalfold increase in signal of the different *PMA1* regions compared to that of the nontranscribed intergenic region, the absolute quantities obtained by the quantitative PCR were divided by the quantity of intergenic region immunoprecipitating with the different proteins.

RESULTS

Mutations in Yra1p are synthetically lethal with a component of the transcription machinery. To further characterize Yra1p function, we performed a synthetic lethal screen with strain FSY1371 (*yra1ΔRBD yra2::KAN*^{r}, pCH1122-YRA1), in which the endogenous *YRA1* gene was replaced by the mutant *yra1ΔRBD* gene encoding a protein lacking the central RNA binding domain (RBD). The deletion of the central RBD is viable and induces only a mild growth phenotype at 37°C. To increase the stringency of the screen, we also deleted the redundant, nonessential *YRA2* gene (52).

Many synthetic mutants were identified, most of which were found only once, indicating that the screen was not saturated. The synthetic lethal phenotype of mutant sl57 was rescued by plasmids containing the *HPR1* gene (Fig. 1A). *HPR1* encodes a protein with a proposed role in transcription elongation (1). The *HPR1* mutation in strain sl57 has not been determined, but the protein could not be detected by Western blotting with a polyclonal antibody against Hpr1p, indicating that at least a part of the protein is deleted (data not shown). Since *HPR1* is not essential, we tested whether the complete disruption of *HPR1* would be lethal in combination with different *yra1* mutations. The YRA1 shuffle or YRA1 shuffle Δ*hpr1* strain was transformed with wild-type or mutant *YRA1* constructs (*LEU2* CEN) and tested for growth on 5-FOA (Fig. 1B). Three *yra1* mutants were tested in addition to *yra1ΔRBD*. One was the deletion mutant *yra1-77-227*, which lacks the N-terminal domain of Yra1p but has only a mild growth phenotype (52). The second, *yra1-8*, contains two amino acid substitutions within the conserved N-terminal box (D$_{10}$D$_{11}$ to K$_{10}$K$_{11}$) and has no obvious growth phenotype on its own. However, this mutant becomes temperature sensitive at 37°C when GFP is fused to its N terminus (GFP–*yra1-8*). The growth phenotypes of all these *yra1* mutants were substantially enhanced in the absence of Hpr1p, which is consistent with a synthetic interaction between *yra1* mutants and Δ*hpr1*.

Since Sub2p directly interacts with Yra1p and acts as a high-copy-number suppressor of Δ*hpr1*, synthetic lethality between *sub2* mutants and Δ*hpr1* was also investigated. The growth of two described temperature-sensitive mutants, *sub2-201* and *sub2-206* (31), was examined in the presence or absence of Hpr1p (Fig. 1C). The phenotype of both s*ub2* mutants was enhanced at 25°C in the absence of Hpr1p, indicating a synthetic lethal relationship between *sub2* mutants and Δ*hpr1*.

Physical interactions between Hpr1p, Sub2p, and Yra1p and Yra2p. To further characterize the functional relationship between Yra1p, Sub2p, and Hpr1p, we looked for physical interactions among these proteins (Fig. 2A). Strains were constructed in which the endogenous *SUB2* gene was deleted and replaced by plasmids expressing N-terminally ProtA-tagged wild-type (ProtA-Sub2p) or mutant (ProtA–*sub2-201* and ProtA–*sub2-206*) Sub2p proteins. A strain expressing ProtA-Sub2 in a Δ*hpr1* background was also engineered (ProtA-Sub2p, Δ*hpr1*). Finally, a strain expressing Hpr1-ProtA was
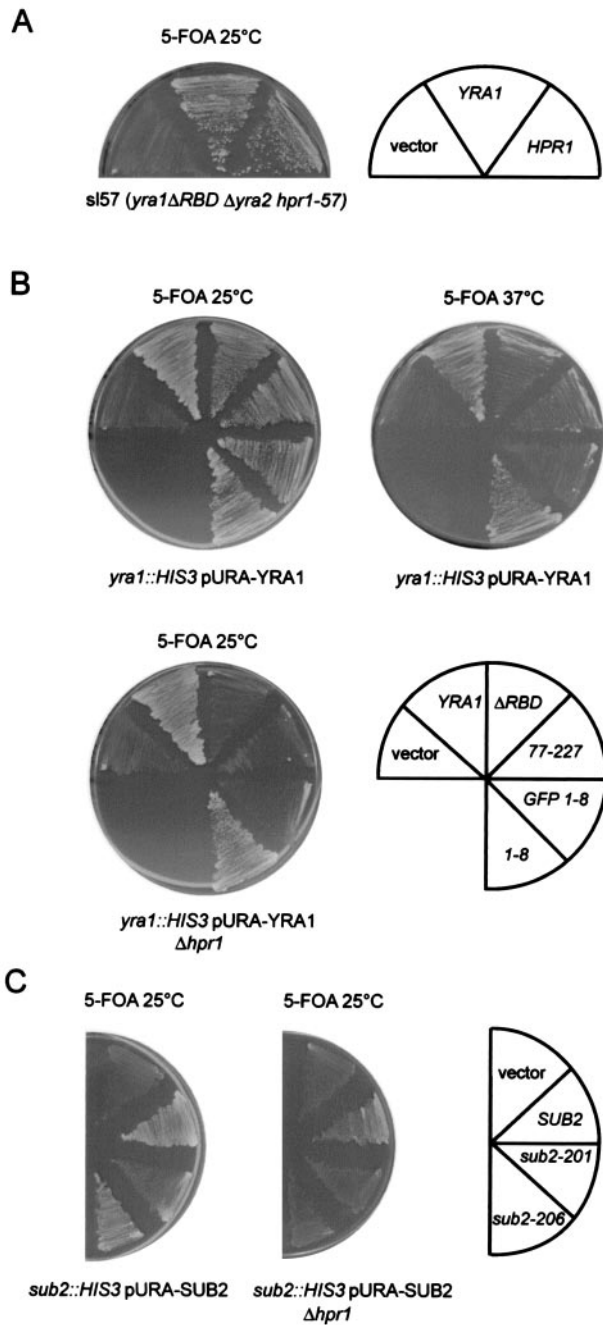
FIG. 1. (A) *yra1ΔRBD* is synthetically lethal with a mutation in *HPR1*. Strain sl57 (*yra1ΔRBD hpr1-57* ⟨pCH1122 *YRA1 URA ADE3*⟩) was transformed with empty vector or *YRA1* and *HPR1* gene constructs (pFS2525 and pFS2576). Transformants were streaked on 5-FOA plates to select against wild-type pCH1122-*YRA1* and examine rescue of the synthetic phenotype. (B) Various *yra1* mutants are synthetically lethal with Δ*hpr1*. The YRA1 shuffle (FSY1026) or YRA1 shuffle Δ*hpr1* (FSY1655) strains were transformed with vector or plasmids encoding wild-type or mutant Yra1p (*LEU2* CEN). Transformants were streaked on 5-FOA plates and incubated at 25 or 37°C for 4 days. (C) *sub2* mutants are synthetically lethal with Δ*hpr1*. The SUB2 shuffle (DLY23) or the SUB2 shuffle Δ*hpr*1 (FSY1664) strains were transformed with empty vector or plasmids encoding wild-type or mutant Sub2p (*LEU2* CEN). Transformants were analyzed as described for panel B.

obtained by genomically tagging the 3′ end of *HPR1* with ProtA (Hpr1-ProtA). Total extracts were prepared from the five ProtA-tagged strains and affinity purified on IgG beads. Copurifying proteins eluted with high levels of salt were analyzed by Western blotting for the presence of Yra1p, Yra2p, and Hpr1p. Hpr1p very efficiently copurified with ProtA-Sub2, and this interaction was insensitive to an RNase A treatment (Fig. 2A, lane 9, and data not shown). ProtA-Sub2 also selected Yra1p, which is consistent with the direct interaction described between these two proteins (45). Similarly, Yra2p purified with ProtA-Sub2, confirming the Yra1p and Yra2p functional redundancy. Yra1p and Yra2p were also selected by Hpr1-ProtA (Fig. 2A, lane 11), suggesting that Hpr1p, Sub2p, and Yra1p/Yra2p may be part of a common complex. Mutations in Sub2p (*sub2-201* and *sub2-206*) strongly decreased its ability to interact with Hpr1p and Yra1p and Yra2p (Fig. 2A, lanes 7 and 8). The association of Sub2p with Yra1p and Yra2p was not affected by the deletion of *HPR1*, and even larger amounts of Yra1p and Yra2p were associated with ProtA-Sub2 in the absence of Hpr1p (Fig. 2A, compare lanes 9 and 10). This observation may indicate a regulatory role for Hpr1p in the interaction of Yra1p with Sub2p.

In vitro interactions between Hpr1p, Sub2p, and Yra1p. To further characterize the physical interactions identified in vivo, Hpr1p, Sub2p, and Yra1p were expressed as recombinant proteins in *E. coli* and examined for direct interaction in vitro (Fig. 2B). First, plasmids encoding GST-Hpr1 or GST alone were coexpressed in *E. coli* with a construct encoding nontagged Sub2p. Total extracts from *E. coli* strains expressing the GST fusions in the presence or absence of Sub2p were purified on glutathione beads. Affinity-purified proteins were fractionated on SDS-PAGE gels and Coomassie stained (Fig. 2B). Sub2p efficiently copurified with GST-Hpr1p but not with GST alone (Fig. 2B, compare lanes 4 and 6), indicating a direct interaction between Hpr1p and Sub2p. Next we examined the ability of GST-Yra1p and GST-Yra2p immobilized on beads to select a His$_6$-Sub2-tagged protein from an *E. coli* extract (Fig. 2C). His$_6$-Sub2 specifically copurified with Yra1- and Yra2-GST fusions but not GST alone (Fig. 2C, lanes 7 to 9). These observations show that Yra2p, like Yra1p (45), directly interacts with Sub2p.

A direct interaction between Hpr1p and Yra1p recombinant proteins could not be examined because of tag incompatibilities. In vitro-translated Yra1p or Yra2p showed no specific binding to GST-Hpr1p immobilized on beads (data not shown), suggesting that Yra1p and Yra2p do not directly interact with Hpr1p. For these reasons, it is likely that the interaction between Hpr1p and Yra1p detected in vivo (Fig. 2A) is bridged by Sub2p.

Hpr1p, Sub2p, and Yra1p are recruited during transcription elongation. Hpr1p has a proposed role in transcription elongation and is therefore expected to associate with transcriptionally active genes. Earlier ChIP experiments have shown that the recruitment of Yra1p to the mRNP occurs during transcription (28). These observations, together with those of the genetic and physical interactions between Hpr1p, Sub2p, and Yra1p, strongly suggested that Hpr1p could aid the cotranscriptional recruitment of Sub2p and Yra1p to growing mRNP complexes. We have used a similar ChIP approach to analyze the association of Hpr1p, Sub2p, and Yra1p with an
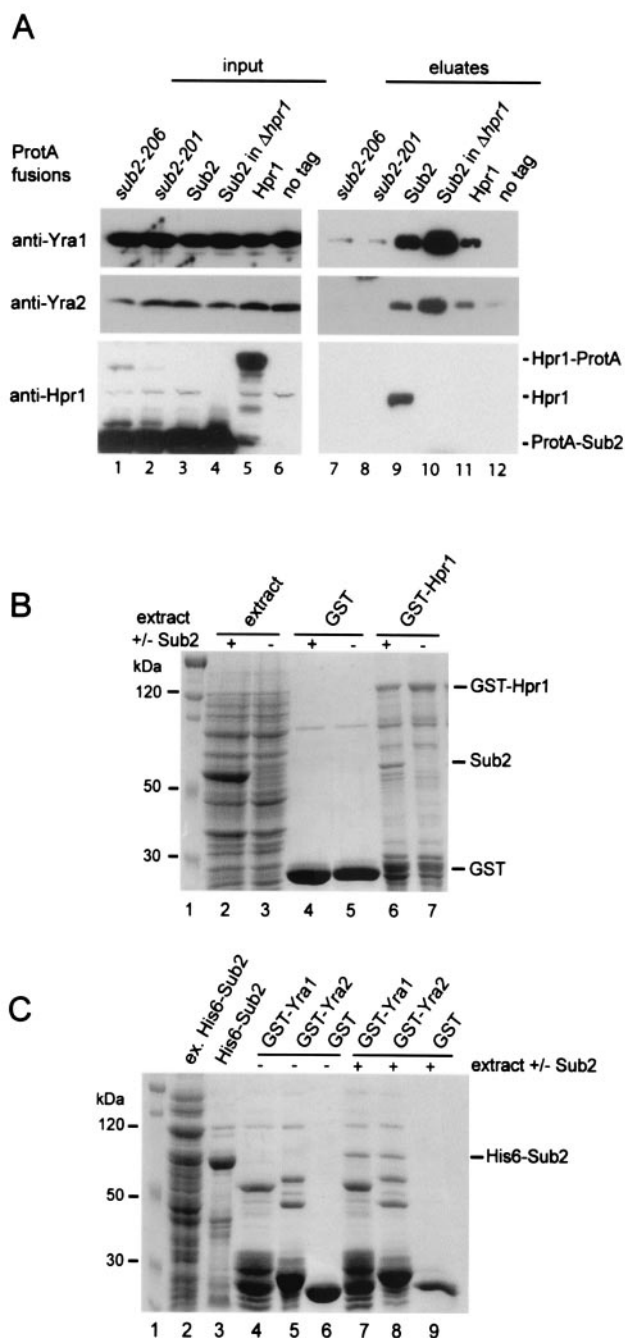
FIG. 2. (A) Hpr1p, Sub2p, and Yra1p physically interact in vivo. Total extracts were prepared from the indicated ProtA-tagged strains and purified on IgG beads. Copurifying proteins were eluted with high salt levels. Total extracts (input; lanes 1 to 6) or eluted proteins (eluates; lanes 7 to 12) were examined by Western blotting with antibodies against Yra1p, Yra2p, or Hpr1p. A nontagged (no tag) strain was used as a control for nonspecific binding to IgG beads. The antibodies against Hpr1p also recognize the ProtA-Sub2 and Hpr1-ProtA fusions present in input samples. (B) Sub2p interacts with GST-Hpr1p. GST alone or GST-Hpr1p (pFS2642) was coexpressed in E. coli with plasmid pFS2633 encoding nontagged Sub2p (lanes 4 and 6) or an empty vector (lanes 5 and 7). Total extracts were purified on glutathione beads, and the selected material was fractionated by SDS-PAGE (lanes 4 to 7) and Coomassie stained. A size marker (lane 1) and aliquots from total lysates expressing GST-Hpr1p plus Sub2p (lane 2) or GST-Hpr1p alone (lane 3) were run in parallel. (C) Sub2p interacts

actively transcribed gene and to define whether the recruitment of Sub2p and Yra1p depends on Hpr1p. In these experiments, we examined the association of these proteins with different regions of the constitutively expressed 3-kb-long PMA1 gene. PMA1 was chosen because analysis of this gene would allow for comparison with earlier published studies (23, 28).

To validate our experimental conditions, we first analyzed three proteins whose distribution on the PMA1 gene is known or can be anticipated. These included the TBP, RNA polymerase II, and the cap binding protein Cbp80p. Cross-linked and sonicated extracts prepared from wild-type cells grown at 25°C were immunoprecipitated with antibodies specific for TBP, the CTD of RNA polymerase II, or Cbp80p. Coprecipitating DNA segments were amplified with a quantitative real-time PCR assay using primer pairs specific for the promoter (pro) and the 5', middle, and 3' coding regions, as well as the 3' UTR, of the PMA1 gene (Fig. 3A). The precipitated DNA samples were also amplified with primers specific for an intergenic, nontranscribed DNA segment, which is devoid of an open reading frame (ORF) and which served as an internal control for the background. The relative amounts of each fragment in the coprecipitated DNA were defined as described above (see Materials and Methods) and were expressed as a severalfold increase with respect to the intergenic control region, whose value was arbitrarily set to 1.

As shown earlier, TBP was uniquely detected at the PMA1 promoter (23, 28). The absence of TBP within the coding region indicates that the conditions used allow good resolution between the PMA1 gene segments (Fig. 3B). Except for a slight increase at the 5' end of the gene, RNA polymerase II (CTD) was evenly distributed over the PMA1 promoter, coding regions, and 3' UTR (Fig. 3C). The slight increase at the 5' end may correspond to stalling of the polymerase at the transition from transcription initiation to elongation (23, 41). Cbp80p, in complex with Cbp20p, binds the m^7GpppN cap structure added to mRNA 5' ends at an early time point during transcription (23, 29). Consistently, we found Cbp80p mainly associated with the 5' coding region of the PMA1 gene (Fig. 3D), which indicates cotranscriptional recruitment of Cbp80p to the mRNA at a very early stage of transcription, perhaps through an initial interaction with the transcription complex. The weaker cross-linking of Cbp80p to more downstream PMA1 regions may reflect the increasing distance between the mRNA 5' end and the DNA template.

Next, we examined the association of Yra1p, Sub2p, and Hpr1p with the PMA1 gene. The distribution of Yra1p was analyzed in wild-type extracts with a polyclonal antibody specific for Yra1p, whereas the association of Hpr1p or Sub2p was determined in strains expressing these two proteins as Hpr1-

with Yra1p and Yra2p in E. coli. GST-Yra1p (pFS1853), GST-Yra2p (pFS2113), or GST alone was immobilized on beads and incubated with extracts from E. coli transformed with vector (lanes 4 to 6) or plasmid pFS2676 expressing a His$_6$-Sub2p-tagged protein (lanes 7 to 9). Material associated with the beads (lanes 4 to 9) and aliquots of His$_6$-Sub2p extract (lane 2) or His$_6$-Sub2p purified on Ni-nitrilotriacetic acid beads (lane 3) were fractionated by SDS-PAGE followed by Coomassie staining.
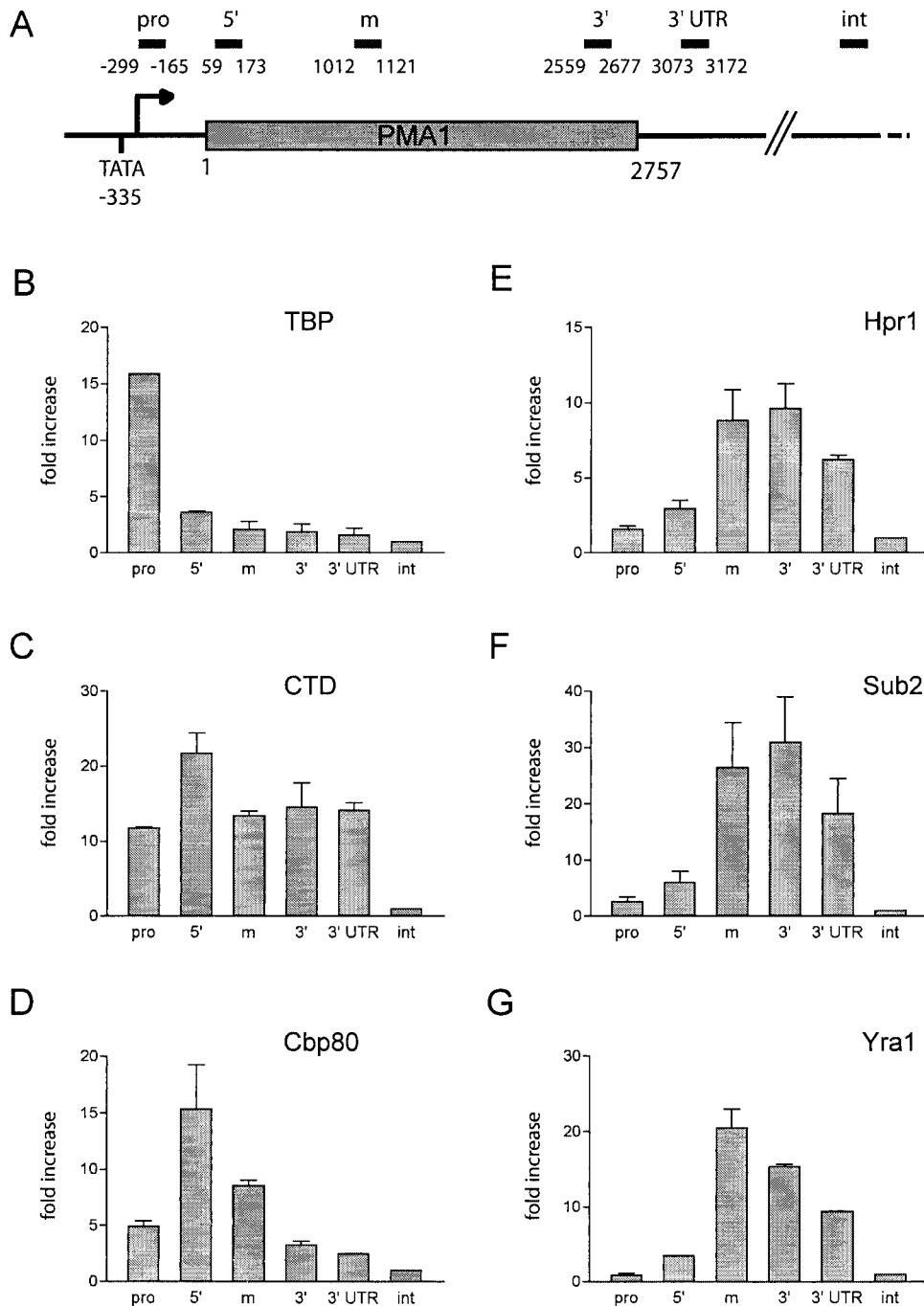
FIG. 3. Hpr1p, Sub2p, and Yra1p associate with the *PMA1* gene during transcription elongation. (A) Diagram of the constitutively expressed *PMA1* gene. Primer sets were designed to amplify 100- to 130-bp-long fragments corresponding to the *PMA1* promoter (pro), to the 5′, middle (m), and 3′ coding regions, or to the 3′ UTR. An additional primer pair specific for a nontranscribed intergenic region (int) devoid of an ORF served as the internal background control. As shown in panels B, C, D, and G, a cross-linked and sonicated extract from wild-type cells grown at 25°C was immunoprecipitated with antibodies specific for TBP, the CTD of RNA polymerase II, the 80-kDa cap binding protein (Cbp80), and Yra1p, respectively. Coprecipitating DNA was amplified by quantitative real-time PCR with the primers indicated in panel A, as described in Materials and Methods. The relative abundance of the different *PMA1* gene segments in each immunoprecipitate was expressed as a severalfold increase with respect to the nontranscribed intergenic region value, arbitrarily set to 1. The indicated values correspond to the means of at least two independent experiments. (E and F) Cross-linked and sonicated extracts were prepared from strains expressing Hpr1-ProtA (FSY1525) or ProtA-Sub2p (FSY1473) and affinity purified on IgG beads. Copurifying DNA fragments were quantified as described above.

ProtA or ProtA-Sub2 fusions (Fig. 3E, F, and G). Hpr1p, Sub2p, and Yra1p showed very similar distribution patterns over the *PMA1* gene. None of these proteins was detectable at the promoter. The association of the three proteins with the 5′ coding region was very weak but clearly strengthened in the middle and the 3′ end of the gene. The association pattern of Yra2p, as defined by immunoprecipitation with a polyclonal antibody against Yra2p, was comparable to that of Yra1p (data not shown). These profiles are consistent with a parallel recruitment of Hpr1p, Sub2p, and Yra1p and Yra2p to the *PMA1* gene during transcription elongation. Cross-linking of both Hpr1p and Sub2p started to decrease in the *PMA1* 3′ UTR, whereas the interaction of Yra1p and Yra2p with the *PMA1* gene weakened within the 3′ coding region earlier than that of Sub2p and Hpr1p. The reduced binding at the 3′ end of the gene may reflect mRNP remodeling or changes in the interactions of these proteins with the transcription machinery.

Hpr1 is required for efficient association of Sub2p and Yra1p to the transcribing *PMA1* gene. To define whether Hpr1p influences the cotranscriptional recruitment of Sub2p and Yra1p to the *PMA1* gene, we compared the association of these two proteins with *PMA1* in the presence or absence of Hpr1p (Fig. 4). The binding of ProtA-Sub2p to *PMA1* was substantially decreased in a Δ*hpr1* strain background, indicating that Hpr1p is important for efficient recruitment of Sub2p to the growing mRNP (Fig. 4A). The association of Yra1p with *PMA1* in the absence of Hpr1p was lowered to a comparable extent (Fig. 4B). Deletion of *HPR1* did not affect the levels of ProtA-Sub2p or Yra1p in the extracts, nor did it modify the level of association of TBP with the *PMA1* gene (data not shown). Furthermore, the decreased association of Sub2p with the *PMA1* gene in Δ*hpr1* cells grown at 25°C (Fig. 4A) cannot be due to a lower transcription rate, since the *PMA1* transcript levels are comparable to those of the wild type in these cells (see below, Fig. 7A). All these observations support the hypothesis that the decreased cross-linking of Yra1p and Sub2p to *PMA1* represents a direct effect of *HPR1* deletion.

The temperature-sensitive GFP–*yra1-8* mutant protein is badly recruited to the *PMA1* gene and leads to the accumulation of newly synthesized *HSP104* transcripts in nuclear foci. To further investigate the importance of the cotranscriptional recruitment of Yra1p for mRNA export, we next examined the association of the temperature-sensitive GFP–*yra1-8* mutant protein with the *PMA1* gene after a short heat shock treatment at 37°C (Fig. 5A). Extremely low levels of *PMA1* gene fragments were pulled down by anti-Yra1p antibodies, although the mutant protein is recognized by these antibodies in standard immunoprecipitation experiments and is present in amounts similar to those of the wild type (data not shown). These observations show that the cotranscriptional recruitment of this mutant Yra1p protein is severely compromised.

Various mRNA 3′ processing and polyadenylation and export mutants induce nuclear retention of bulk poly(A)$^+$ RNA and sequester newly synthesized transcripts in nuclear foci at or close to the site of transcription (20). To determine whether the GFP–*yra1-8* mutant strain induces these phenotypes, we examined the distribution of poly(A)$^+$ RNA or *HSP104* mRNA in these cells grown at 25°C or shifted to 37°C or 42°C (Fig. 5B). GFP–*yra1-8* mutant cells exhibit a poly(A)$^+$ RNA export defect at 37°C, and the nuclear poly(A)$^+$ signal be-
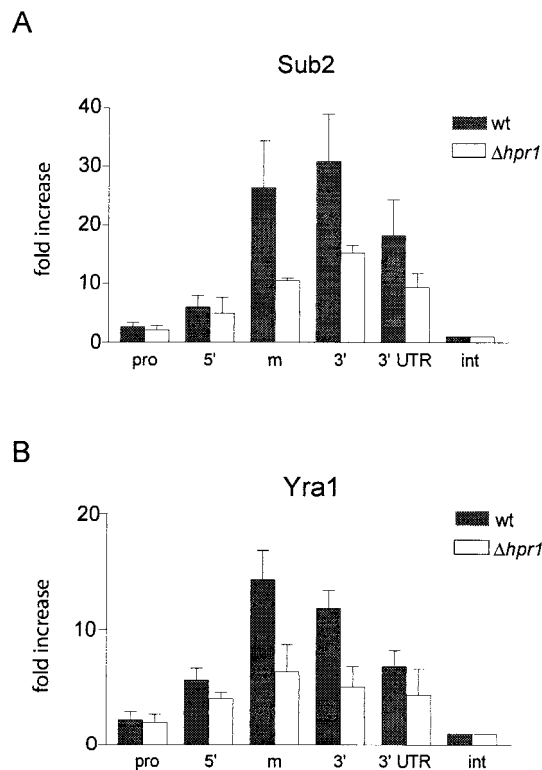

FIG. 4. The efficient recruitment of Sub2p and Yra1p to the transcribing *PMA1* gene requires Hpr1p. (A) Strains expressing ProtA-Sub2p in the presence (FSY1473) or absence (FSY1656) of Hpr1p were grown at 25°C and analyzed as described for Fig. 3F. (B) W303 wild-type and Δ*hpr1* (FSY1624) strains were shifted to 37°C for 20 min prior to cross-linking. Sonicated extracts were immunoprecipitated with antibodies against Yra1p, and coprecipitating DNA fragments were analyzed as described for Fig. 3G.

comes even stronger at 42°C, probably reflecting the strong production of heat shock mRNAs and an even more severe export block. As described for *sub2-201* and other mRNA export mutants, GFP–*yra1-8* cells retain *HSP104* mRNAs induced at 42°C in a nuclear dot. Taken together, these data suggest that the mRNA export defects may be due, at least in part, to poor cotranscriptional recruitment of the mutant Yra1p protein, probably resulting in reduced binding of Mex67p to the mRNP.

Yra1p is synthetically lethal with components of the exosome. Our synthetic lethal screen identified different genes implicated in mRNA metabolism (data not shown). The synthetic lethal clone of strain sl5 was complemented by *RRP45*, an essential component of both the cytoplasmic and the nuclear exosome. The exosome is a multiprotein complex of 3′-to-5′ exonucleases involved in the processing and degradation of various cellular transcripts (4). Earlier studies have shown that the phenotype of *sub2* mutant strains is enhanced in the absence of Rrp6p, an exonuclease specific for the nuclear exosome (4, 19). To define whether *yra1* mutants exhibit a similar synthetic behavior, the YRA1 shuffle or YRA1 shuffle Δ*rrp6* strains were transformed with wild-type or mutant *YRA1* plasmids, as described for Fig. 1B, and tested for growth on 5-FOA (Fig. 6A). The absence of Rrp6p exacerbated the
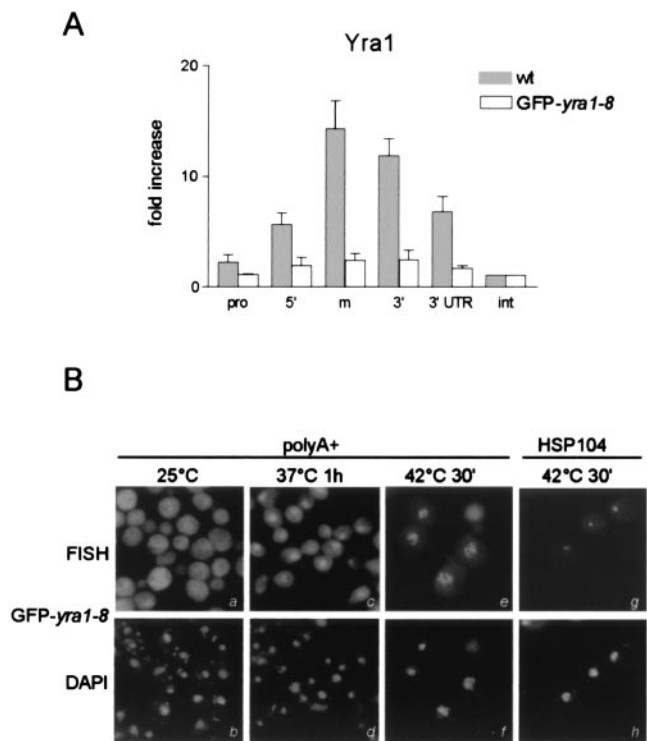
FIG. 5. GFP–*yra1-8* is not recruited to the *PMA1* gene, exhibits a poly(A)[+] RNA export defect, and sequesters *HSP104* transcripts within nuclear foci. (A) W303 wild-type and GFP–*yra1-8* strains were shifted to 37°C for 20 min prior to cross-linking. Sonicated extracts were immunoprecipitated with antibodies against Yra1p, and coprecipitating DNA fragments were analyzed as described for Fig. 3G. (B) The GFP–*yra1-8* mutant strain was grown at 25°C and shifted to 37°C for 1 h or to 42°C for 30 min. After fixation, the distributions of poly(A)[+] RNA or *HSP104* mRNAs were examined by in situ hybridization with Cy3-labeled oligo(dT)$_{55}$ or *HSP104*-specific oligonucleotide probes, as indicated. DAPI (4′,6′-diamidino-2-phenylindole) staining was used to verify the nuclear localization of the *HSP104* and poly(A)[+] RNA signals.

growth phenotypes of all *yra1* mutants examined, indicating a strong functional relationship between *yra1* mutants and Δ*rrp6*.

Yra1p physically interacts with the exosome component Rrp45p. To further analyze the relationship between Yra1p and the exosome, we examined their physical interaction in vivo (Fig. 6B). Extracts were prepared from strains in which *RRP45* or *RRP6* had been genomically tagged with protein A and purified as described for Fig. 2A. Copurifying proteins were analyzed by Western blotting for the presence of Yra1p and Hpr1p. Substantial amounts of Yra1p copurified with Rrp45, but we were unable to detect a physical connection between Yra1p and Rrp6p, perhaps because of a lack of sensitivity. Hpr1p was detectable in neither the Rrp45-ProtA nor the Rrp6-ProtA eluate. These observations indicate that at least one component of the exosome is stably associated with Yra1p or the mRNP.

Hpr1, Sub2p, and Yra1p mutants exhibit defects in transcript accumulation. Because the deletion of *HPR1* affects the expression levels of β-galactosidase and *YAT1* or *LYS2* transcripts, it was proposed that Hpr1p is preferentially required for transcription through long or G+C-rich DNA sequences
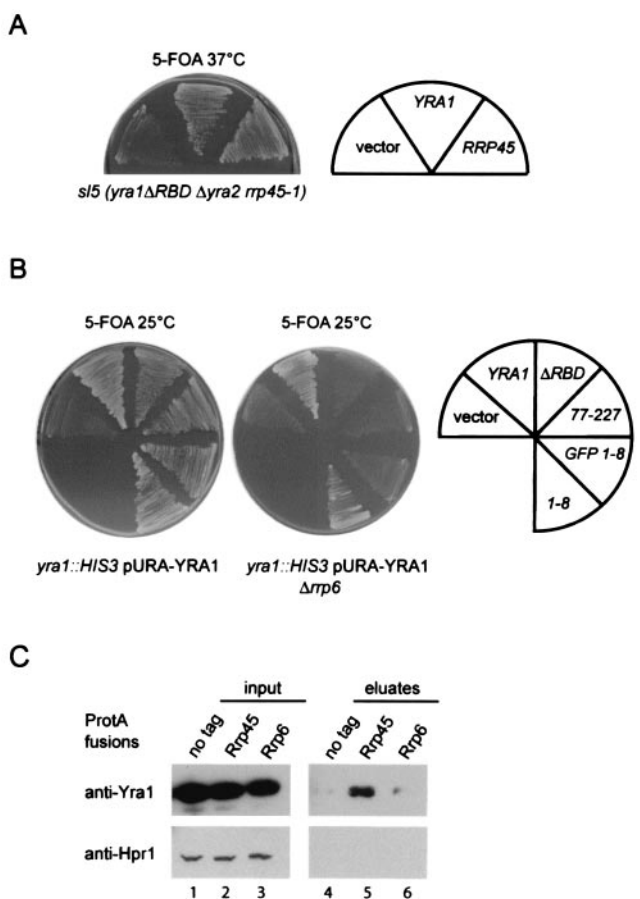

FIG. 6. Yrap1 genetically and physically interacts with components of the exosome. (A) *yra1*Δ*RBD* is synthetically lethal with a mutation in *RRP45*. Strain sl5 (*yra1*Δ*RBD rrp45-1* ⟨pCH1122-*YRA1 URA ADE3*⟩) was transformed with empty vector or *YRA1* and *RRP45* gene constructs (pFS2525 and pFS2575). Transformants were streaked on 5-FOA as described for Fig. 1A. (B) Mutations in *YRA1* are synthetically lethal with Δ*rrp6*. The YRA1 shuffle (FSY1026) or YRA1 shuffle Δ*rrp6* (FSY1621) strains were transformed with vector or plasmids encoding wild-type or mutant Yra1p (*LEU2* CEN). Transformants were streaked on 5-FOA plates and analyzed as described for panel A. (C) Yra1p physically interacts with components of the exosome. Total extracts were prepared from a nontagged strain (no tag) or strains expressing Rrp45-ProtA (FSY1476) or Rrp6-ProtA (FSY1657) and purified on IgG beads (lanes 1 to 6). Copurifying proteins were eluted with high levels of salt. Total extracts (input; lanes 1 to 3) or eluted proteins (eluates; lanes 4 to 6) were examined by Western blotting with antibodies against Yra1p and Hpr1p.

(8). Based on the genetic and physical interactions between Hpr1p, Sub2p, and Yra1p, we asked whether mutations in Sub2p and Yra1p would have effects on transcript accumulation similar to those observed in Δ*hpr1* strains (Fig. 7). First we examined the steady-state levels of the endogenous *PMA1* transcripts. The wild-type, GFP–*yra1-8*, *sub2-201*, or Δ*hpr1* strain was grown to mid-log phase in YEPD medium at 25°C and either kept at 25°C or shifted to 37°C for 45 min. Total RNAs were extracted and analyzed by Northern blotting with probes specific for *PMA1* or 18S rRNA as an internal control for loading (Fig. 7A). *PMA1* transcript levels were comparable in all four strains grown at 25°C but were substantially reduced in the three mutant strains after a shift to 37°C. Next, the levels
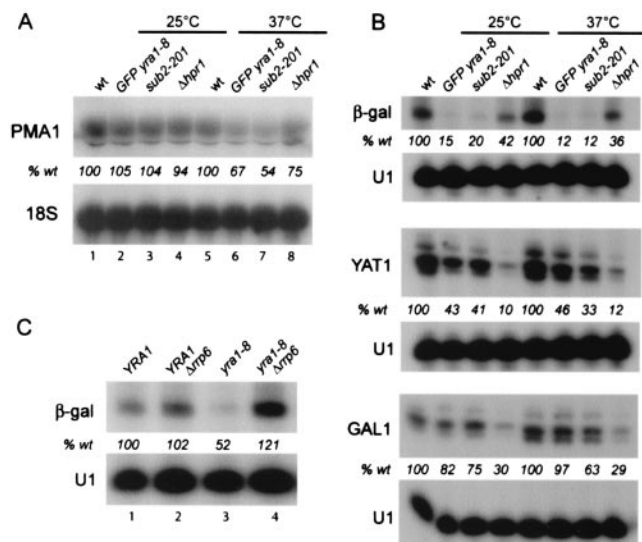
FIG. 7. *yra1*, *sub2*, and Δ*hpr1* mutants induce defects in mRNA accumulation. (A) Northern blot analysis of *PMA1* mRNAs in wild-type or GFP–*yra1-8*, *sub2-201*, and Δ*hpr1* mutant strains grown at 25°C (lanes 1 to 4) or shifted to 37°C for 45 min (lanes 5 to 8). The *PMA1* signals were normalized to 18S rRNA and expressed as a percentage of that of the wild type, as indicated (% wt). (B) Wild-type or GFP–*yra1-8* (FSY1568), *sub2-201* (FSY1613), and Δ*hpr1* (FSY1624) mutant strains, transformed with plasmids (2μm URA3) containing the β-galactosidase or *YAT1* genes driven by a galactose-inducible promoter, were grown to mid-log phase in selective medium containing 2% lactate–2% glycerol–0.05% glucose and induced with 3% galactose–1% raffinose for 150 min at 25 or 37°C, as shown above the lanes. Total RNA was analyzed by primer extension with ^{32}P-labeled oligonucleotides specific for β-galactosidase, *YAT1*, or the endogenous *GAL1* gene transcripts, as indicated. A primer specific for U1 snRNA was added to the reactions as an internal control for loading. The primer-extended bands were quantified with an Instant Imager apparatus and normalized to U1 snRNA. The transcript levels in mutant strains were expressed as a percentage of that of the wild type, as indicated at the bottom of each gel (% wt). (C) The YRA1 (FSY1026) or YRA1 Δ*rrp6* (FSY1621) strain was shuffled, as described for Fig. 6B, with plasmid Lac111-YRA1 Gen (pFS2525; lanes 1 and 2) or Lac111-*yra1-8* (pFS2328; lanes 3 and 4) and transformed with the galactose-inducible β-galactosidase reporter construct (pLGSD5). Transformants were grown, and RNAs were analyzed as for panel B, except that the galactose induction was performed at 30°C.

of β-galactosidase or *YAT1* transcripts were examined in a wild-type (W303) strain or in mutant GFP–*yra1-8*, *sub2-201*, and Δ*hpr1* strains transformed with high-copy-number plasmids expressing these genes from a galactose-inducible promoter (8, 25). The strains were grown to mid-log phase at 25°C in selective medium and induced for 150 min with galactose at 25°C or 37°C. Total RNA was extracted and subjected to primer extension analysis with primers specific for β-galactosidase and *YAT1* transcripts (Fig. 7B). Endogenous constitutively expressed *YAT1* mRNAs were not detectable in this assay (data not shown). As previously reported, the levels of galactose-induced β-galactosidase and *YAT1* mRNAs were clearly lower in the absence of Hpr1p compared to those for the wild type. The accumulation of these transcripts was also substantially affected in the GFP–*yra1-8* and *sub2-201* mutant strains. Because the levels of endogenous induced *GAL1* transcripts were moderately affected in the absence of Hpr1p, as

described previously (8), and appeared mildly altered in GFP–*yra1-8* and *sub2-201*, we conclude that the decrease of β-galactosidase and *YAT1* mRNA levels in these mutant strains is not due to a loss in galactose inducibility. These observations, taken together, indicate that the functionally related Hpr1p, Sub2p, and Yra1p proteins are required for the efficient accumulation of certain transcripts. These experiments did not determine, however, whether mutations in these proteins affect mRNA production, mRNA stability, or both.

The genetic and physical interactions between Yra1p and the nuclear exosome indicate that they participate in a common process. This raises the question of whether the mRNA accumulation defect in the GFP–*yra1-8* mutant strain is linked to an exosome-dependent RNA degradation mechanism which recognizes and degrades defective mRNPs resulting from inefficient loading of export factors. We therefore tested whether the low mRNA levels detected in the *yra1* mutant strain could be restored by deleting *RRP6*. The GFP–*yra1-8* mutant strain could not be used in this experiment, because it is unviable in the absence of *RRP6*. We therefore used the milder *yra1-8* mutant, which grows when *RRP6* is deleted (Fig. 6B). The *yra1-8* mutant grows nearly as well as the wild type and shows only weak nuclear accumulation of poly(A)$^+$ RNA at 37°C, and retention of *HSP104* transcripts at 42°C in nuclear foci could not be detected (data not shown). However, as for GFP–*yra1-8*, the β-galactosidase mRNA levels are significantly decreased in *yra1-8* and accumulate only to 50% of the amounts present in the wild-type strain. Deletion of *RRP6* restored the levels of these transcripts to those present in the wild type (Fig. 7C). Deletion of *RRP6* in the wild-type background did not substantially affect the β-galactosidase mRNA levels. These data show that the defect in mRNA accumulation in the *yra1-8* mutant strain is not due to reduced mRNA production but rather to the degradation of the transcripts by the nuclear exosome.

DISCUSSION

We have used a synthetic lethal approach to search for additional partners of the essential mRNA export factor Yra1p. We identified *HPR1*, a gene encoding a protein with a proposed role in transcription elongation (8) and whose deletion induces poly(A)$^+$ RNA export defects (43). Deletion of Hpr1p is synthetically lethal with mutations in Yra1p as well as in Sub2p, and pull-down experiments show that Hpr1p, Sub2p, and Yra1p and Yra2p physically interact in vivo (Fig. 1 and 2A). In vitro binding experiments demonstrate that these interactions are direct (Fig. 2B and C). Finally, ChIP assays show that Hpr1p, Sub2p, and Yra1p are recruited cotranscriptionally and that efficient association of Sub2p and Yra1p with a transcribing gene is dependent on the presence of Hpr1p (Fig. 3 and 4). These results establish a functional link between transcription and mRNA export and indicate that the mRNA export defects in *hpr1*, *sub2*, and *yra1* mutants are due, at least in part, to poor loading of export factors onto nascent mRNAs. These improperly assembled mRNPs are unstable and are degraded through an exosome-dependent surveillance mechanism (Fig. 5, 6, and 7) (30).

Hpr1p is part of the THO complex, which contains three other proteins, Tho2, Mft1, and Thp2p. These four compo-

nents behave as a functional entity, as deletion of any of them results in poor growth at 37°C and induces similar gene expression defects (7, 40). Recent studies published while this work was in preparation show that Yra1p, Sub2p, and the THO components can be purified as a complex (18, 46). Synthetic lethality of *sub2* and *yra1* mutants with all four THO subunits confirms the functional relevance of these interactions. Finally, the poly(A)$^+$ RNA export defect exhibited by *yra1*, *sub2*, and THO complex mutants further supports the idea of a role of these proteins in a common function (30, 46).

THO components have been implicated in transcription elongation, but the exact role of the complex in this process has not been defined (6–8, 40). Hpr1p and the other THO component, Tho2p, have been shown to be recruited to active genes (46). Whereas this latter study proposed a transcription-dependent association of Hpr1p and Tho2p over the entire coding region, our ChIP and quantitative PCR analyses allowed us to identify a preferential binding of Hpr1p towards the middle and 3′ end of the *PMA1* gene. Genetic and physical interactions functionally link Hpr1p to the Cdc73p/Paf1p/Ccr4p complex, proposed to function in transcription through an association with RNA polymerase II (5). Since Paf1p/Cdc73p complexes are present at the promoter and on coding regions of different classes of genes (41), they could mediate the recruitment of Hpr1p/THO to active genes during transcription elongation.

Our data further show that the association profiles of Sub2p and Yra1p with *PMA1* parallel the distribution of Hpr1p, which is consistent with a synchronous recruitment of these factors during elongation. The specific association of Yra1p with *PMA1* was proposed earlier to occur preferentially towards the 3′ end of the gene (28). Our observations are consistent with these data. However, the utilization of additional primer sets in the present study shows that the peak of Yra1p association occurs farther upstream, in the middle of the *PMA1* coding region, a distribution similar to that observed on the *GAL10* transcription unit (28). Importantly, the binding of Sub2p and Yra1p to *PMA1* is substantially decreased in the absence of Hpr1p. This reduced association with *PMA1* in the absence of Hpr1p is unlikely due to a reduced transcription rate, since the levels of *PMA1* transcripts at 25°C are not affected in Δ*hpr1* (Fig. 4A and 7A). The data therefore support that Hpr1p is required for efficient recruitment of Sub2p and Yra1p to the growing mRNP. It is not possible so far to distinguish whether Sub2p and Yra1p first become associated with the transcription machinery through an interaction with Hpr1p/THO and subsequently bind the mRNP or whether Hpr1p/THO and the nascent transcript stimulate the binding of Yra1p and Sub2p directly to the mRNP. The residual binding of Sub2p and Yra1p in the absence of Hpr1p is consistent with the viable phenotype of Δ*hpr1* mutants and indicates the existence of alternate binding sites, perhaps provided by the other THO subunits or the mRNA itself. Sub2p was earlier identified as a high-copy-number suppressor of Δ*hpr1* and becomes associated with an RNA polymerase II subunit in the absence of Hpr1p (11). It will be interesting to determine whether overexpression of Sub2p in Δ*hpr1* restores optimal recruitment of Yra1p to the nascent mRNP.

The association of Yra1p, Sub2p, and Hpr1p to *PMA1* decreases towards the 3′ end of the gene. The reduction in Yra1p

and Sub2p cross-linking may reflect remodeling of the mRNP or a decrease in the proximity of the mRNP with respect to the DNA template. Alternatively, it may correspond to the transfer of Yra1p and Sub2p from the transcription complex to the mRNP. Since THO components exhibit RNA binding activity (1), Hpr1p/THO may also become associated with the mRNP, perhaps marking the mRNA for a subsequent step in mRNP biogenesis. Examining the distribution of Yra1p, Sub2p, and Hpr1p/THO on other genes, those of different sizes and with or without introns, will be necessary to better understand the mechanisms underlying the cotranscriptional assembly of export-competent mRNPs.

We found that the phenotypes of *yra1* mutants are enhanced or become lethal in combination with *rrp45* and *rrp6* exosome mutants (Fig. 6A and B). The phenotypes of *sub2*, Δ*hpr1*, and other THO mutants are similarly exacerbated by the deletion of *RRP6*, and these synthetic interactions represent an additional common feature of Yra1p, Sub2p, and THO components (19, 30). Recent evidence suggests that the nuclear exosome functions in an mRNA surveillance system that degrades transcripts in response to defects in mRNA processing and export (4). Interestingly, the expression levels of several mRNAs are substantially reduced in *yra1*, *sub2*, and Δ*hpr1* mutant backgrounds (Fig. 7). Importantly, the β-galactosidase mRNA levels, which are very low in the *yra1-8* mutant strain, are restored to wild-type levels when Rrp6p is deleted (Fig. 7C). Similarly, the levels of *HSP104* transcripts induced at high temperature are severely reduced in *sub2* and Δ*hpr1* and other THO mutants but rescued when Rrp6p is deleted (19, 30). Taken together, these data support the hypothesis that mRNPs assembled in all these mutant backgrounds are unstable and subject to an exosome-dependent quality control function which targets these transcripts to degradation. These observations also suggest that THO components may have a primary role in stable mRNP formation rather than in transcription elongation.

Different RNA processing and export mutants, including *sub2*, Δ*hpr1*, and THO mutants, sequester newly made heat shock *HSP104* transcripts within nuclear dots at or close to the transcription site. These foci were suggested to be involved in exosome-dependent quality control, since they disappear in the absence of Rrp6p (16, 19, 20, 30). Mutations in Yra1p also induce the accumulation of heat-shock transcripts within nuclear dots (Fig. 5B). However, we were not able to show that these foci disappear upon deletion of *RRP6*, since the double mutants are unviable (Fig. 6B and data not shown). Using ChIP experiments, we could not detect an association of Rrp45p or Rrp6p with the *PMA1* gene (data not shown), suggesting that mRNPs are recognized by the surveillance machinery at a posttranscriptional step, perhaps in the nuclear foci. The observed physical interaction between Yra1p and Rrp45p (Fig. 6C) may therefore correspond to a step subsequent to mRNP release from the template.

Although the possibility cannot be excluded that Yra1p, Sub2p, and Hpr1p contribute to the overall integrity and activity of the transcription-elongating complex, our results and those of Libri et al. (30) support the idea that normal levels of full-length transcripts are produced in *yra1*, *sub2*, and *hpr1* backgrounds but that these transcripts are subsequently degraded by an Rrp6p-dependent process. mRNPs produced in

these mutant strains are likely to be malformed and recognized by a surveillance mechanism which competes with other mRNP biogenesis steps, including mRNA export. It will be interesting to identify which features of these incorrectly assembled mRNP complexes are recognized by this exosome-dependent quality control.

ACKNOWLEDGMENTS

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