RNA (1999), *5*:1055–1070. Cambridge University Press. Printed in the USA.

Overexpression of truncated Nmd3p inhibits protein synthesis in yeast

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ABSTRACT

The yeast *NMD3* gene was identified in a two-hybrid screen using the nonsense-mediated mRNA decay factor, Upf1p, as bait. *NMD3* was shown to encode an essential, highly conserved protein that associated principally with free 60S ribosomal subunits. Overexpression of a truncated form of Nmd3p, lacking 100 C-terminal amino acids and most of its Upf1p-interacting domain, had dominant-negative effects on both cell growth and protein synthesis and promoted the formation of polyribosome half-mers. These effects were eliminated by truncation of an additional 100 amino acids from Nmd3p. Overexpression of the *nmd3Δ100* allele also led to increased synthesis and destabilization of some ribosomal protein mRNAs, and increased synthesis and altered processing of 35S pre-rRNA. Our data suggest that Nmd3p has a role in the formation, function, or maintenance of the 60S ribosomal subunit and may provide a link for Upf1p to 80S monosomes.

Keywords: mRNA; nonsense-mediated mRNA decay; ribosome; rRNA processing

INTRODUCTION

In yeast, nonsense-mediated mRNA decay (NMD), the degradation of mRNAs containing a premature translation termination codon, occurs by deadenylation-independent decapping followed by 5′ → 3′ exoribonucleolytic decay (Decker & Parker, 1993, 1994; Muhlrad & Parker, 1994; Muhlrad et al., 1994, 1995). Two major steps of this turnover pathway are catalyzed by the products of *DCP1* (Beelman et al., 1996; LaGrandeur & Parker, 1998; Stevens, 1988) and *XRN1* (Hsu & Stevens, 1993), and regulated by the product of *UPF1* (Leeds et al., 1991, 1992; Peltz et al., 1993a; Weng et al., 1996; Czaplinski et al., 1998; He F & Jacobson A, in prep.; Maderazo A, He F, Mangus D, Jacobson A, in prep.). Deletion or mutation of *UPF1* results in the stabilization and accumulation of nonsense-containing mRNAs without a concurrent increase in the abundance or stability of wild-type transcripts (Leeds et al., 1992; He et al., 1993; Czaplinski et al., 1995). A two-hybrid screen for *UPF1* interactors identified six genes that passed genetic tests for specificity and thus encoded potential Upf1p-interacting proteins (He & Jacobson, 1995). Two of the genes identified in this screen, *NMD2/UPF2* and *DBP2*, have been characterized further and shown to be additional components of the nonsense-mediated mRNA decay pathway (He & Jacobson, 1995; Cui et al., 1995; Bond A, He F, Mangus D, Jacobson A, in prep.). A subsequent two-hybrid screen with *NMD2*, and an independent genetic screen, identified the *UPF3* gene, which, when disrupted, also leads to selective stabilization of nonsense-containing mRNAs (Lee & Culbertson, 1995; He et al., 1997). Further studies of the respective gene products have revealed that Upf1p, Nmd2p, and Upf3p either interact sequentially or function as a complex to regulate translation termination and promote mRNA decay (He et al., 1997; Czaplinski et al., 1998).

Consistent with these roles, all three proteins have been shown to colocalize with polyribosomes and 80S ribosomes (Peltz et al., 1993b; Atkin et al., 1995, 1997; Mangus & Jacobson, 1999). However, several different experimental approaches have failed to demonstrate an association between any of these proteins and specific ribosomal subunits (Atkin et al., 1995, 1997; Mangus & Jacobson, 1999). Indeed, it has been suggested that such an association may not be manifested if the activity of these proteins depends on 40S/60S joining (Mangus & Jacobson, 1999).

Here, we present the characterization of *NMD3,* a highly conserved, essential gene also identified in the two-hybrid screen for Upf1p-interacting proteins. Full-

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length Nmd3p was shown to associate with the 60S ribosomal subunit, and overexpression of a truncated version of the protein inhibited translation, promoted the formation of aberrant polyribosomes, and caused a generalized disruption of the normal course of ribosome biogenesis. These results suggest that Nmd3p has a role in ribosome biogenesis or function and may be a contact site for Upf1p on the 80S ribosome.

RESULTS

Identification of Nmd3p as a Upf1p-interacting protein

We used the yeast two-hybrid system to screen for genes encoding putative Upf1p-interacting proteins (He & Jacobson, 1995). Using a fusion of full-length Upf1p to the Gal4p DNA-binding (DB) domain as bait, we identified six candidate genes, including *NMD3*, that activated transcription of the *GAL1-lacZ* reporter gene. Cells expressing a *GAL4* activation domain (AD) fusion to a C-terminal segment of *NMD3* (encoding the last 120 amino acids of the protein) demonstrated β-galactosidase activity when cotransformed with the *GAL4-DB-UPF1* fusion (data not shown). The specificity of this interaction was indicated by the observation that the same *GAL4-AD-NMD3* fusion promoted no detectable β-galactosidase activity when cotransformed with plasmids encoding only the *GAL4-DB*, a *GAL4-DB-CEP1* fusion, or a *GAL4-DB-LAM5* fusion (data not shown).

Molecular cloning of the *NMD3* gene

To isolate the complete *NMD3* gene, a *Cla*I-*Hin*dIII fragment of the *GAL4-AD-NMD3* two-hybrid fusion plasmid was used to screen a YCp50 genomic DNA library. Clones encompassing the entire gene were identified and sequenced (GenBank accession no. U31376). This analysis identified a 1,557-nt open reading frame that encoded a 518-amino-acid protein (Nmd3p) with a theoretical molecular mass of 59 kDa and a predicted pI of 5.33. Analysis of the *NMD3* transcript was consistent with the predicted open reading frame. Northern analysis of total mRNA isolated from cells harboring either one or two copies of the *NMD3* gene identified a transcript of approximately 1.8 kb (Fig. 1A, lanes 1 and 2). Using primer extension analysis, this mRNA was shown to have three major 5′ ends (at nt −40, −42, and −48), as well as several minor 5′ ends (at nt −56, −73, −79, and −86; Fig. 1B, lanes 1 and 2).

Nmd3p has highly conserved homologs in several species, including (see Fig. 2) *Caenorhabditis elegans* (full-length protein: 43% identical/58% similar), *Drosophila melanogaster* (full-length protein: 48% identical/61% similar), and *Homo sapiens* (full-length protein: 47% identical/62% similar). Within Nmd3p, three domains have significant homologies to those in other proteins: (1) amino acids 30–39 and 53–62 are highly homologous to canonical zinc fingers (MacKay & Crossley, 1998), (2) amino acids 207–239 are homologous to a segment of ribosomal protein S13 in numerous organisms, and (3) amino acids 380–480, a region that includes at least part of the Upf1p-interacting domain (see Fig. 3A), are homologous to domains within approximately 25 other proteins.

NMD3 is essential for cell viability

To determine whether the *NMD3* gene is essential for cell viability, we performed a gene disruption in which a 1.2-kb *Cla*I-*Sac*I fragment of the *NMD3* gene was deleted and replaced by an ~1.8-kb fragment of the *HIS3* gene (Figs. 4A and 4B). The disruption was targeted to the *NMD3* locus in diploid W303 cells by homologous recombination (Thomas & Rothstein, 1989) and integration was verified by Southern blot analysis. Figure 4C (lanes P1 and P2) shows that DNA from the disrupted cells contains two *Xba*I fragments that hybridize to the *NMD3* probe: a 1.2-kb fragment harboring the disruption and a 1.4-kb fragment from the endogenous *NMD3* gene. DNA from the untransformed cells contained only the latter fragment. Sporulation and subsequent tetrad analysis of diploid cells harboring the *NMD3* disruption yielded only two viable his^- spores from each of 10 tetrads. Southern analysis of genomic DNA from the two viable spores in a single tetrad revealed only the 1.4-kb wild-type fragment (Fig. 4C, lanes 1A and 1B), indicating that the *NMD3* gene is essential for cell viability.

High level expression of truncated Nmd3p has a dominant-negative effect on cell growth

In an effort to obtain insight into the function of the *NMD3* gene, we sought to construct a conditional lethal allele. Recognizing that truncated polypeptides are often potent inhibitors of the activity of macromolecular complexes in which they function (He & Jacobson, 1995; Eisinger et al., 1997), we examined the consequences of overexpression of *NMD3* constructs that lacked the C-terminal Upf1p-interacting domain. A unique restriction site was inserted immediately downstream of the *NMD3* stop codon and used to generate C-terminal truncations that resulted in the deletion of 100 or 200 amino acids from Nmd3p. The resulting constructs, as well as full-length (FL) *NMD3*, were cloned into pRS316 under the control of the inducible *GAL1* promoter (Fig. 3A), transformed into W303 haploid cells, and assayed for the ability to grow on media containing glucose or galactose. On galactose-containing medium, cells harboring the pRS316-*GAL-nmd3*Δ*100* plasmid were unable to grow, whereas strains harboring the

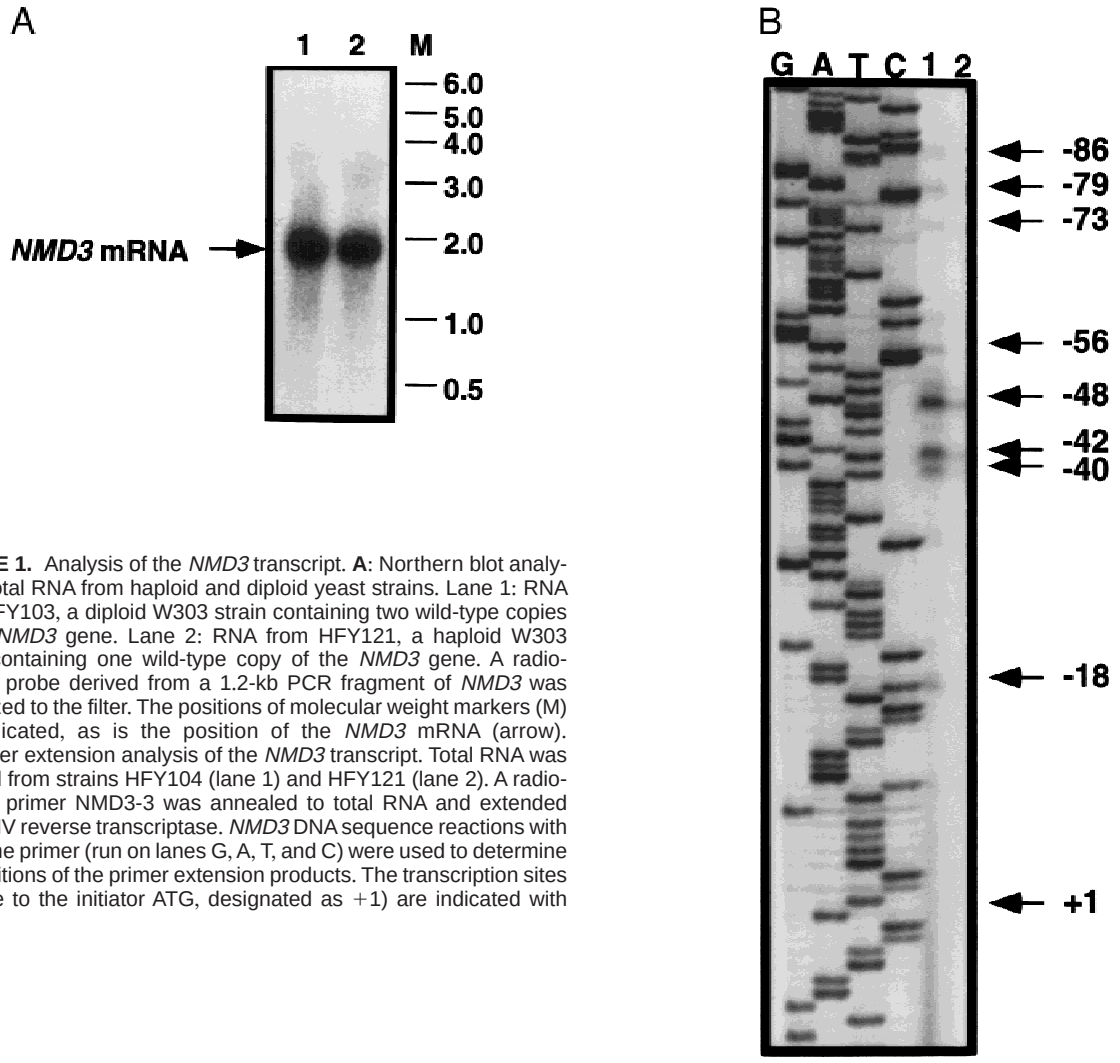

FIGURE 1. Analysis of the *NMD3* transcript. **A**: Northern blot analysis of total RNA from haploid and diploid yeast strains. Lane 1: RNA from HFY103, a diploid W303 strain containing two wild-type copies of the *NMD3* gene. Lane 2: RNA from HFY121, a haploid W303 strain containing one wild-type copy of the *NMD3* gene. A radiolabeled probe derived from a 1.2-kb PCR fragment of *NMD3* was hybridized to the filter. The positions of molecular weight markers (M) are indicated, as is the position of the *NMD3* mRNA (arrow). **B**: Primer extension analysis of the *NMD3* transcript. Total RNA was isolated from strains HFY104 (lane 1) and HFY121 (lane 2). A radiolabeled primer NMD3-3 was annealed to total RNA and extended with AMV reverse transcriptase. *NMD3* DNA sequence reactions with the same primer (run on lanes G, A, T, and C) were used to determine the positions of the primer extension products. The transcription sites (relative to the initiator ATG, designated as +1) are indicated with arrows.

pRS316-*GAL-NMD3FL*, the pRS316-*GAL-nmd3Δ200*, or the pRS316 control plasmid were viable (Fig. 3B). Because all four strains grew with comparable efficiency on glucose-containing medium, and because all strains contain an intact endogenous *NMD3* gene, high level expression of the *nmd3* allele lacking 100 C-terminal codons must inhibit cell growth in a dominant-negative manner. The absence of a dominant-negative phenotype in cells expressing the pRS316-*GAL-nmd3Δ200* construct suggests that the Nmd3p domain responsible for growth inhibition resides within amino acids 318–418 or that the presence of this segment of the protein allows the formation of a specific inhibitory structure.

The dominant-negative effect of Nmd3pΔ100 expression is targeted to mRNA translation

Given the well documented links between nonsense-mediated mRNA decay and translation (Jacobson & Peltz, 1996), we investigated whether the lethal phenotype exhibited by cells expressing the pRS316-*GAL-nmd3Δ100* plasmid was a consequence of the inhibition of protein synthesis. First, sucrose-gradient analysis was used to determine whether expression of truncated Nmd3p caused any specific alterations in cellular polyribosome profiles. Cells containing pRS316-*GAL-NMD3FL* and pRS316-*GAL-nmd3Δ100* were grown in medium containing raffinose as a carbon source and subsequently induced for 2 h in galactose-containing medium. Cytoplasmic extracts were prepared and fractionated on sucrose gradients. Figures 5A and 5B show that cells harboring the plasmid expressing full-length Nmd3p displayed a wild-type polyribosome profile after 2 h of galactose induction, whereas cells expressing the truncated protein exhibited a profile indicative of a translation defect. More specifically, polyribosome peaks from the latter cells all showed discontinuities characteristic of half-mer formation, that is, polyribosomes that lacked stoichiometric amounts of both ribosomal subunits (Helser et al., 1981; Fried et al., 1985; Rotenberg et al., 1988; Moritz et al., 1991). This effect was a specific consequence of overexpression of Nmd3pΔ100,

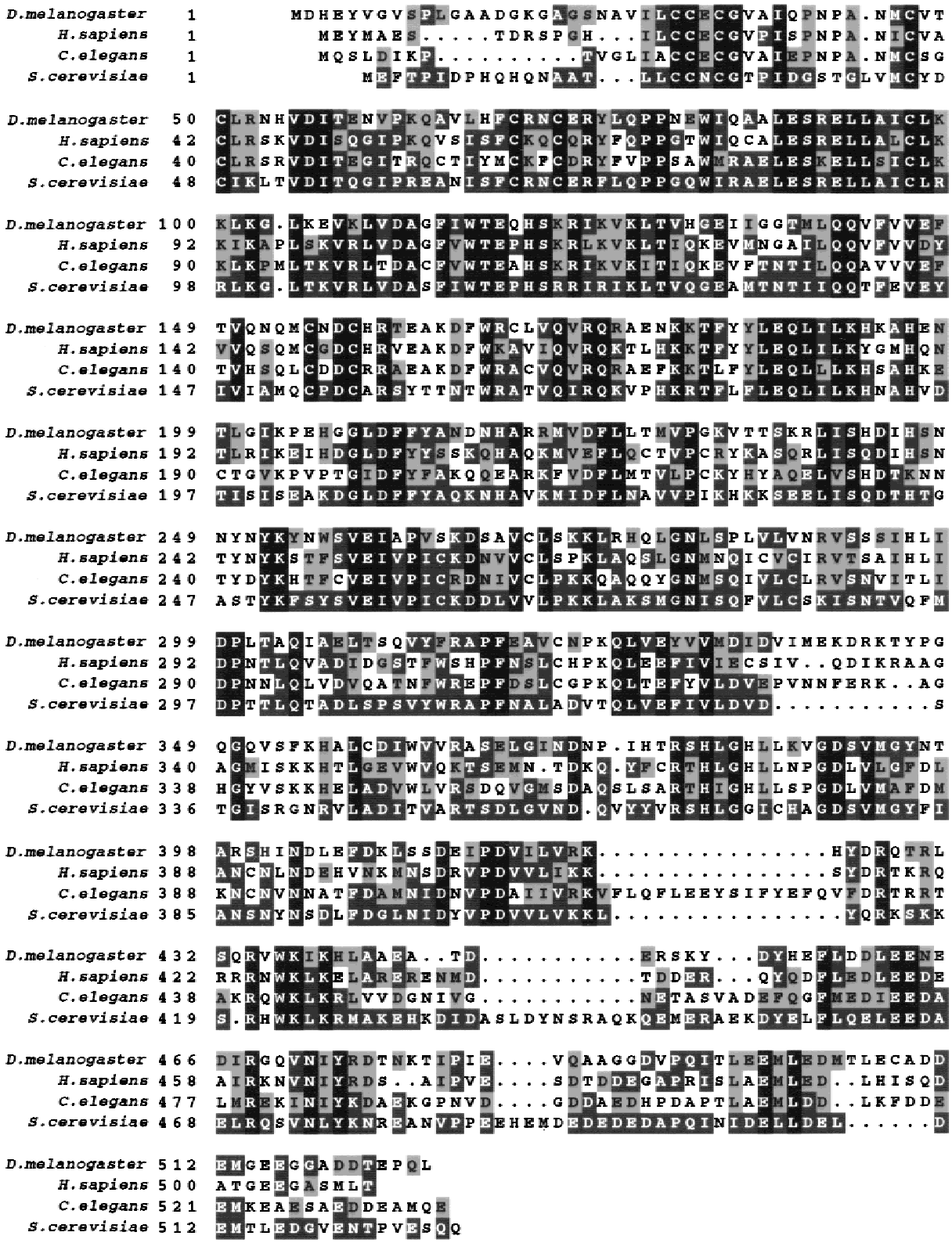

FIGURE 2. Alignment of the *S. cerevisiae NMD3* protein sequence with those from *D. melanogaster*, *C. elegans*, and *H. sapiens.* Light grey shaded boxes with black lettering indicate amino acids that are similar relative to the *S. cerevisiae* sequence, dark grey shaded boxes with white lettering indicate amino acids that are conserved relative to the *S. cerevisiae* sequence, and black boxes with white lettering indicate amino acids that are conserved among all three sequences. Sequence alignment was generated using the pileup feature of the Wisconsin Package Version 9.1 of the GCG sequence analysis program.

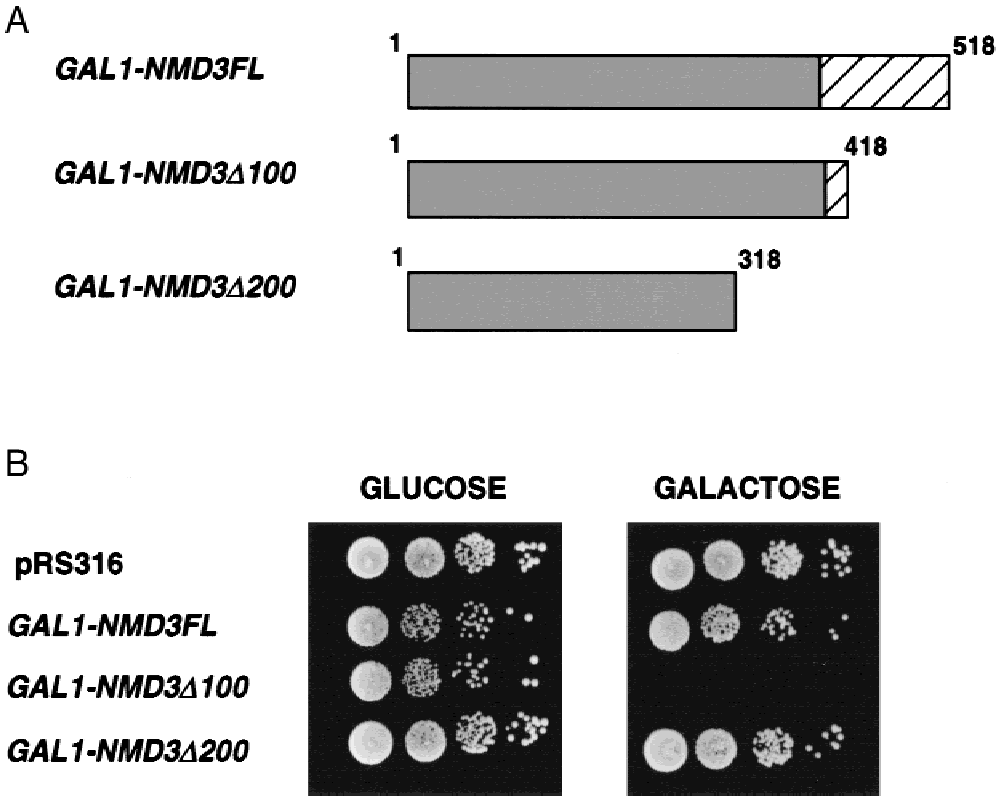

FIGURE 3. A truncation allele of the *NMD3* gene exhibits a dominant-negative growth phenotype. **A**: Schematic of *NMD3* coding region segments placed under the control of the *GAL1* promoter and cloned into pRS316. The hatched area indicates the Upf1p interacting region of Nmd3p, defined by its recovery in a two-hybrid screen. **B**: Induction of the *nmd3Δ100* allele inhibits cell growth. The constructs shown in **A** and the empty vector pRS316 were transformed into HFY121, which contains a wild-type copy of the *NMD3* gene. Individual transformants were selected and then serially diluted onto SC −ura plates with either glucose or galactose as the sole carbon source.

as galactose-grown cells that harbored the plasmids pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ200* and glucose grown cells that harbored the plasmids pRS316-*GAL-NMD3FL*, pRS316-*GAL-nmd3Δ100*, or pRS316-*GAL-nmd3Δ200* all displayed wild-type polyribosome profiles (data not shown). The inhibitory activity of Nmd3pΔ100 was: (1) not due to preferential stability of this form of the protein, as western blotting experiments showed that the *Δ100*, *Δ200*, and *FL* forms all reached comparable levels in the same amount of time (data not shown) and (2) a direct consequence of the extent of induction of Nmd3pΔ100, as polyribosome analysis of cells in which galactose induction of pRS316-*GAL-nmd3Δ100* was continued to 4 or 6 h exhibited higher levels of half-mers (data not shown).

To confirm the lack of ribosome subunit stoichiometry detected when Nmd3pΔ100 was overexpressed, we used high-salt sucrose gradients to evaluate the relative levels of 60S and 40S subunits. Wild-type and truncated Nmd3p were induced as shown in Figures 5A and 5B, but extracts and sucrose gradients contained 0.7 M NaCl to disrupt polyribosomes and monosomes into individual ribosomal subunits. Analysis of the relative amounts of 60S to 40S ribosomal subunits revealed a shift from a ratio of 2.15 in the strain expressing pRS316-*GAL-NMD3FL* to a ratio of 1.59 in the strain expressing pRS316-*GAL-nmd3Δ100* (Figs. 5C and 5D). This analysis thus confirmed the disruption of normal 60S/40S stoichiometry suggested by the formation of half-mers in Figure 5B.

To investigate the apparent translation defect in a more quantitative manner, we measured the ability of cells to incorporate ^{35}S-labeled amino acids after galactose induction of the full-length and *nmd3Δ100* alleles. Figure 6 shows that, for the first 2 h after induction, cells expressing either construct had comparable abilities to incorporate labeled amino acids. Pulse-labeling of cells at later times indicated that the truncated Nmd3p inhibited protein synthesis, that is, by 6 h post-induction, cells expressing pRS316-*GAL-nmd3Δ100* had only 50% of the incorporation activity of cells expressing pRS316-*GAL-NMD3FL*.

Nmd3p cofractionates with the 60S ribosomal subunit

The experiments of Figures 5 and 6 indicated that the dominant-negative effect of truncated Nmd3p was tar-

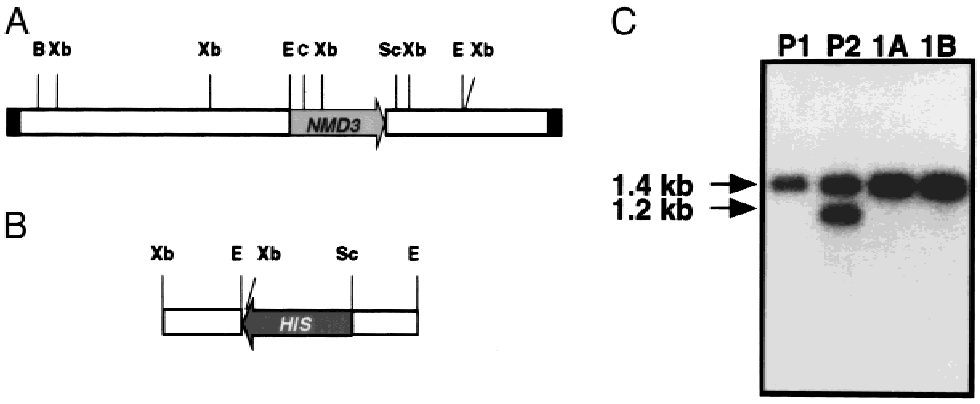

FIGURE 4. Southern blot analysis of the *NMD3* disruption strain. **A**: A restriction map of the 8.0-kb genomic DNA fragment, isolated from a YCp50 library, that contains the *NMD3* gene. The *NMD3* open reading frame and direction of transcription are indicated by the shaded arrow. The solid box represents a segment of the cloning vector YCp50, and restriction site abbreviations are B: *Bam*HI; c: *Cla*I; E: *Eco*RI; Sc: *Sac*I; and Xb: *Xba*I. **B**: A restriction map of the *nmd3::HIS3* disruption allele. The *Cla*I-*Sac*I fragment of the *NMD3* gene, containing ~1.2 kb of the *NMD3* coding region, was deleted and replaced with the yeast *HIS3* gene. The shaded arrow represents the *HIS3* gene and indicates the direction of transcription. **C**: Southern analysis of the *NMD3* gene disruption. A restriction fragment carrying the *nmd3::HIS3* allele was used to transform the yeast diploid strain W303. His+ transformants were sporulated and tetrads were dissected. Two viable spores were obtained from each tetrad and were subsequently analyzed. Genomic DNA from wild-type and disrupted diploid strains, as well as progeny haploid strains, were isolated, digested with *Xba*I, and analyzed by Southern blotting with a radiolabeled probe derived from the 5′ UTR and coding region of *NMD3*. Lane P1: DNA isolated from the homozygous *NMD3/NMD3* diploid strain HFY103; lane P2: DNA isolated from a diploid His+ transformant of HFY103 (HFY91); lanes 1A and 1B: DNA isolated from the progeny of the two viable spores dissected from the same tetrad (HFY121 and HFY122). The 1.4-kb and 1.2-kb fragments represent the wild-type and disrupted alleles of *NMD3,* respectively.

geted to the translation apparatus, thus suggesting that wild-type Nmd3p had a role in protein synthesis. To test this possibility further, we sought to determine whether Nmd3p was normally associated with ribosomes. We constructed a plasmid-borne, HA-epitope-tagged allele of *NMD3* (pRS316-3xHA-*NMD3*; see Table 1) that was deemed functional by virtue of its ability to restore viability to an *nmd3::his3* disruption strain (data not shown). Cytoplasmic extracts from cells harboring this allele were fractionated on sucrose gradients, and western blotting was used to identify the fractions containing HA-Nmd3p. These experiments indicated that HA-Nmd3p cofractionated with polyribosomes and with either 60S or 40S ribosomal subunits (Fig. 7A). To localize HA-Nmd3p more specifically, higher resolution sucrose gradients were utilized. These experiments demonstrated that the majority of HA-Nmd3p cosedimented with the 60S ribosomal subunit (Fig. 7B). Confirmation that the peak designated 60S was indeed the large ribosomal subunit was obtained by simultaneously monitoring the sedimentation of Tcm1p, the large ribosomal subunit protein L3. The majority of Tcm1p was shown to cosediment with the 80s and 60s peaks of the sucrose gradient (Fig. 7B), thus validating the 60S assignment of Nmd3p. Interestingly, the principal ribosomal peak with which Upf1p was associated in this gradient was that of the 80S monosome. This suggests that Upf1p interaction with Nmd3p bound to the 60S subunit may be dependent on 40S/60S joining.

Overexpression of Nmd3pΔ100 increases the abundance and decreases the stability of a subset of ribosomal protein mRNAs

Because Nmd3p was identified as a two-hybrid interactor with Upf1p, we sought to determine whether cells expressing pRS316-*GAL-nmd3Δ100* would stabilize nonsense-containing mRNAs. RNA was isolated from cells harboring pRS316, pRS316-*GAL-nmd3Δ100*, pRS316-*GAL-nmd3Δ200*, or pRS316-*GAL-NMD3FL* at different times after galactose induction and analyzed by Northern blotting for the relative levels of the *CYH2* mRNA and pre-mRNA. These transcripts were chosen for analysis because the *CYH2* pre-mRNA is an endogenous substrate of the nonsense-mediated mRNA decay pathway and its abundance is a direct indicator of the degree of inhibition of the pathway (He et al., 1993, 1996, 1997; He & Jacobson, 1995). The results of these experiments paralleled those analyzing translational inhibition, that is, the abundance of the *CYH2* transcripts was altered only in those cells in which pRS316-*GAL-nmd3Δ100* had been induced for 2–6 h (Fig. 8). In galactose-induced cells containing the other plasmids, the levels of the *CYH2* transcripts diminished versus time of induction, such that by 6 h post-induction the abundance of the *CYH2* mRNA and pre-mRNA had decreased 10–15-fold and 2–3-fold, respectively (Fig. 8 and Table 2). In contrast, cells expressing pRS316-*GAL-nmd3Δ100* maintained time zero (t_0)

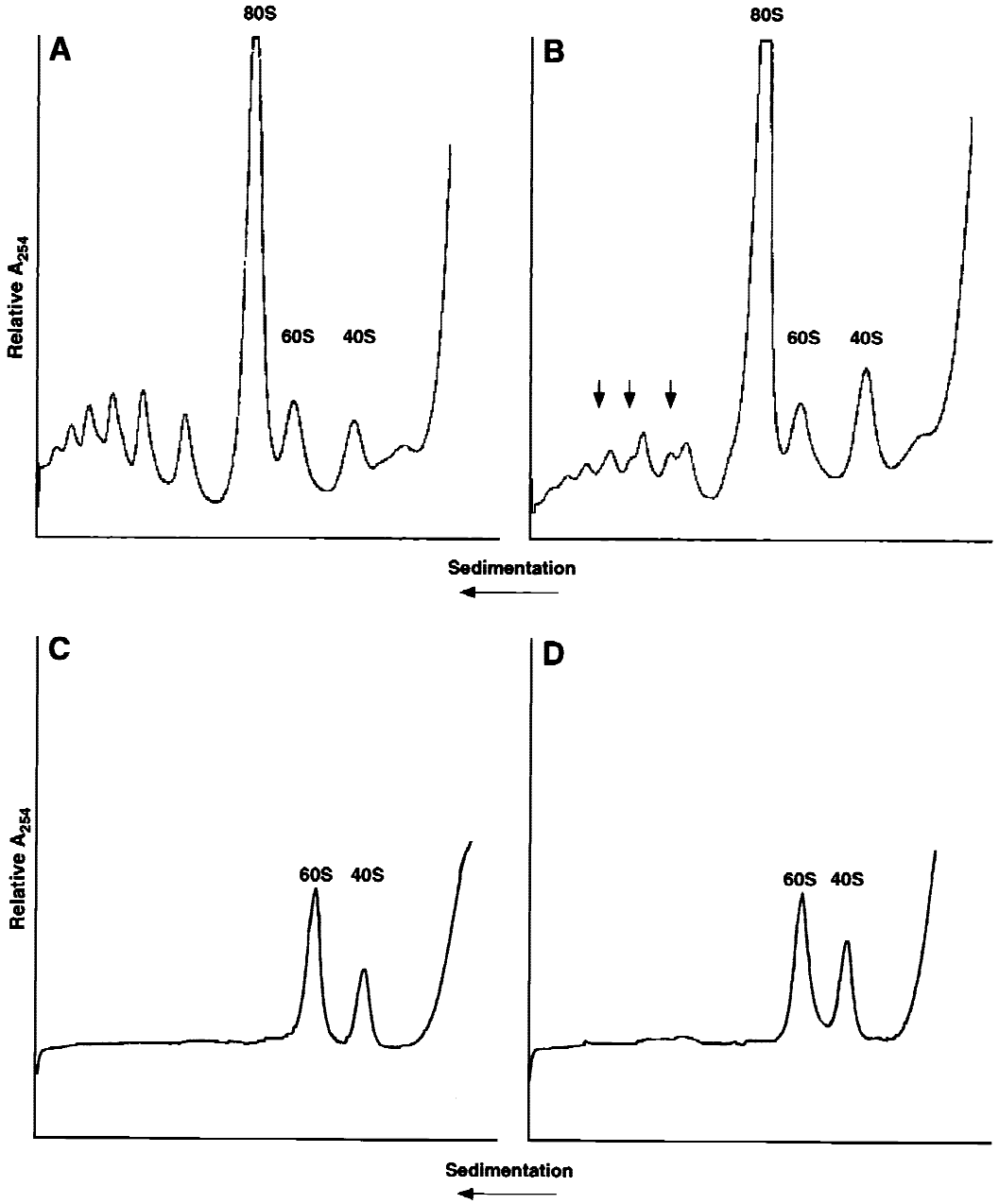

FIGURE 5. Induction of pRS316-*GAL-nmd3Δ100* promotes the formation of polyribosome half-mers. Cytoplasmic extracts were prepared from cells after 2 h of galactose induction. Extracts were fractionated on 7–47% sucrose gradients lacking (**A** and **B**) or containing (**C** and **D**) 0.7 M NaCl. The OD traces of the ribosome profiles are shown, with half-mer polyribosome peaks indicated by arrows and the peaks of the 40S subunits, 60S subunits, and 80S monosomes labeled as such. **A** and **C**: Cells harboring pRS316-*GAL-NMD3FL*. **B** and **D**: Cells harboring pRS316-*GAL-nmd3Δ100*.

levels of the *CYH2* mRNA and increased the level of the pre-mRNA approximately threefold (Fig. 8 and Table 2). Analyses of *STE2* mRNA abundance in the same set of cells indicated that these effects did not apply to all classes of mRNA (Fig. 8 and Table 2). To better understand the effect of expressing Nmd3pΔ100, additional mRNAs were also analyzed, with the results of these analysis found in Table 2. To test whether the increased abundance of the *CYH2* pre-mRNA was an effect on the nonsense-mediated mRNA decay pathway, or a general stabilization of a subset of normal mRNAs, we measured the abundance of the *can1–100* mRNA in cells expressing either pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ100*. The *can1–100* mRNA contains a premature UAA codon and is normally a substrate for the nonsense-mediated mRNA decay pathway; therefore, it should be stabilized if the dominant-negative effect was specifically targeted to nonsense-containing substrates (Maderazo A, He F, Mangus D, Jacobson A, in prep.). However, steady-state levels of *can1–100*

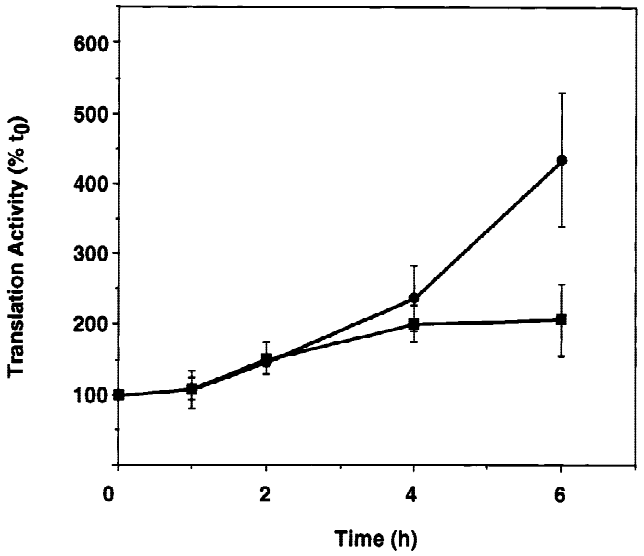

FIGURE 6. Amino acid incorporation in cells harboring pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ100*. Cells were subjected to galactose induction for different lengths of time, and incorporation of ^{35}S-labeled amino acids was measured as described in Materials and Methods. Data are expressed as the percentage of incorporation at t_0, and are averages of triplicate samples. The error bars denote the standard deviations of three separate experiments. Circles depict cells containing pRS316-*GAL-NMD3FL* and squares depict cells with pRS316-*GAL-nmd3Δ100*.

mRNA were not increased in cells expressing the truncated form of *NMD3*, indicating that the increased abundance detected for *CYH2* pre-mRNA was not due to inhibition of nonsense-mediated mRNA decay (Table 2). Given that overexpression of Nmd3pΔ100 resulted in impaired translation, increased abundance of *CYH2* pre-mRNA and mRNA, and the fact Nmd3p cosedimented with the 60S fractions on sucrose gradients, we reasoned that Nmd3pΔ100 might be affecting the stability of other ribosomal protein mRNAs. To test this possibility, we analyzed the steady-state levels of the *TCM1* and *RP51a* ribosomal protein mRNAs. In cells expressing Nmd3pΔ100, *RP51a* mRNA decreased steadily as a function of time after galactose induction such that, by 6 h post-galactose induction, cells contained only 48% of their t_0 amount of the mRNA (Table 2). In contrast, cells expressing Nmd3pΔ100 maintained almost identical levels of *RP51a* mRNA at all time points after galactose induction (Table 2). Unlike the *RP51a* mRNA, the levels of the *TCM1* mRNA were similar in cells expressing either Nmd3p or Nmd3pΔ100 (Table 2), indicating that the increased abundance conferred by expression of Nmd3pΔ100 is specific only to a subset of ribosomal protein mRNAs.

The differences in *CYH2* and *RP51a* mRNA levels brought on by expression of Nmd3pΔ100 could reflect increases in the synthesis or stability of these RNAs. To distinguish between these possibilities, half-lives of the *CYH2* mRNA and pre-mRNA were determined in cells expressing either pRS316-*GAL-nmd3Δ100* or pRS316-*GAL-NMD3FL*. These plasmids were transformed into yRP582, a yeast strain harboring a temperature-sensitive allele of RNA polymerase II. Control experiments indicated that it was necessary to induce these cells with galactose for 10 h to obtain changes in mRNA abundance comparable to those obtained in the experiments shown in Figure 8 (data not shown). Cells treated in this way were then shifted to 37 °C and mRNA decay rates determined by northern blot analysis of RNA samples isolated at different times after the temperature shift. These experiments demonstrated that expression of pRS316-*GAL-nmd3Δ100* reduced the half-lives of the *CYH2* pre-mRNA and mRNA approximately twofold (Fig. 9), but had no effect on the half-life of the *STE2* mRNA (data not shown). These results indicate that the elevated levels of *CYH2* transcripts were caused by changes in RNA synthesis and suggest that such increased synthesis may trigger feed-back mechanisms that regulate stability of the respective RNAs (see Discussion).

Overexpression of Nmd3pΔ100 disrupts normal rRNA processing

Because expression of pRS316-*GAL-nmd3Δ100* inhibited protein synthesis and altered the abundance of some ribosomal protein mRNAs, we hypothesized that these effects might be indirect consequences of an Nmd3pΔ100 effect on rRNA processing. To determine whether overexpression of Nmd3pΔ100 affected rRNA processing, total RNA was isolated from cells harboring pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ100* at different times after galactose induction and analyzed by northern blotting.

The yeast 35S pre-rRNA is shown in Figure 10A, together with its principal processing sites and the location of sequences complementary to the oligonucleotide probes used in our analysis. Figure 10B illustrates the effects of pRS316-*GAL-nmd3Δ100* expression on the accumulation of mature 25S and 18S rRNA. At 6 h post-galactose induction, a significant decrease in the abundance of mature 18S rRNA was detected in the *nmd3Δ100* strain, indicating that overexpression of Nmd3pΔ100 was indeed inhibiting normal rRNA processing. To better determine the point in the processing pathway at which Nmd3pΔ100 was acting, the membrane used in Figure 10B was stripped and then rehybridized with a probe complementary to ITS1 (internal transcribed spacer region 1). Hybridization with the ITS1 oligonucleotide revealed that 35S pre-rRNA and an aberrant 24S processing intermediate accumulate in cells expressing Nmd3pΔ100 (Fig. 10C). The 24S rRNA intermediate is known to accumulate when initial cleavages at 35S rRNA sites A_0, A_1, and D are inhibited, and cleavage then occurs within ITS2, followed by $3' \rightarrow 5'$ exonucleolytic digestion up to site E (Venema & Tol-

TABLE 1. Plasmids.

Plasmid	Relevant yeast sequences	Source
pRS316 3XHA-*NMD3*	0.5-kb *Not*I-*Nco*I PCR fragment containing the *NMD3* promoter region, *Nco*I-*Eco*RI fragment containing a triple hemagglutinin epitope tag, ~2.0-kb *Eco*RI-*Sal*I fragment containing the *NMD3* coding region, 3′ UTR and a small portion of the pBSKS+ polylinker.	This study
pRS316 *GAL1-NMD3FL*	Contains a 0.6-kb *Xba*I-*Hin*dIII *GAL1* promoter fragment and an ~3.0-kb *Hin*dIII-*Hin*dIII fragment of *NMD3* containing the complete coding region (5′ *Hin*dIII site introduced by PCR at −7 position).	This study
pRS316 *GAL1-nmd3Δ100*	pRS316 *GAL-NMD3FL* containing a 0.4-kb *Bam*HI-*Mlu*I fragment containing a stop codon at the 3′ end, replaces the ~0.80-kb *NMD3* coding sequence, resulting in the removal of the C-terminal 300 bp of the *NMD3* coding sequence (unique *Mlu*I site introduced immediately downstream of the stop codon of *NMD3*).	This study
pRS316 *GAL1-nmd3Δ200*	pRS316 *GAL-NMD3FL* containing a unique *Mlu*I site engineered immediately downstream of the stop codon of *NMD3*. A 0.2-kb *Bam*HI-*Mlu*I fragment containing a stop codon at the 3′ end replaces the ~0.8-kb *NMD3* coding sequence, resulting in the removal of the C-terminal 600 bp of the *NMD3* coding sequence.	This study
pBS-*nmd3::HIS3*	1.2-kb *Xba*I-*Eco*RI fragment containing the 5′ promoter region to the second codon of the *NMD3* region. 1.8-kb *Eco*RI-*Sac*I fragment of *HIS3* and the 0.36-kb *Sac*I-*Hin*dIII fragment containing the 3′ UTR of *NMD3*.	This study
pGAD2-*NMD3*	0.8-kb *Cla*I-*Hin*dIII fragment containing the C-terminal coding region of *NMD3*.	He & Jacobson, 1995
pMA424-*UPF1*	3.6-kb *Eco*RI-*Bam*HI fragment containing the entire *UPF1*-coding region and the ~1.0-kb downstream sequences cloned into the *Eco*RI-*Bam*HI sites of pMA424.	He & Jacobson, 1995

lervey, 1996; Fig. 10A). To confirm that the processing intermediate detected in Figure 10C was indeed the 24S pre-rRNA, the blot was stripped and rehybridized with a 5′ ETS (external transcribed sequence) probe that is complementary to sequences 5′ to the A_0/A_1 region of the pre-rRNA (Fig. 10A). This blot showed that hybridization with either the 5′ ETS or the ITS1 probe detected intermediates of the same size (Figs. 10C and 10D), leading us to conclude that rRNA processing is altered in cells overexpressing Nmd3pΔ100.

DISCUSSION

Nonsense-mediated mRNA decay, the rapid turnover of mRNAs with premature translational termination codons, requires the activity of the *UPF1* gene in yeast, roundworms, and humans (Leeds et al., 1991, 1992; Peltz et al., 1993a; Weng et al., 1996; Sun et al., 1998; P. Anderson, pers. comm.). To identify additional factors involved in yeast nonsense-mediated mRNA decay, we conducted a two-hybrid screen for Upf1p-interacting proteins (He & Jacobson, 1995). The screen identified six potential interactors, two of which, Nmd2p and Dbp2p, have been analyzed previously and shown to have important roles in the degradation of nonsense-containing mRNAs (He & Jacobson, 1995; He et al., 1996, 1997; Bond A, He F, Mangus D, Jacobson A, in prep.). In this article, we have characterized *NMD3*, another gene identified in the screen for *UPF1*-interactors. *NMD3* is essential for viability (Fig. 3), has sequence motifs characteristic of zinc-finger proteins and ribosomal protein S13, and has well conserved homologs in *C. elegans, D. melanogaster*, and *H. sapiens* (Fig. 1). The high degree of sequence conservation and the essential nature of the yeast *NMD3* gene indicate that Nmd3p may have a vital function in numerous organisms.

To gain insight into the role of Nmd3p, we characterized a conditional *nmd3* allele. Overexpression of a truncated form of Nmd3p, lacking its C-terminal 100 amino acids and the majority of its Upf1p-interacting domain, was shown to have a dominant-negative effect on cell growth. High-level expression of the *nmd3Δ100* allele also had consequences for the protein synthesis apparatus and its function, causing an accumulation of half-mer polyribosomes (Fig. 5), up to a twofold reduction in rates of amino acid incorporation (Fig. 6), and alterations in the normal course of ribosomal RNA processing (Fig. 10). These effects were not observed with

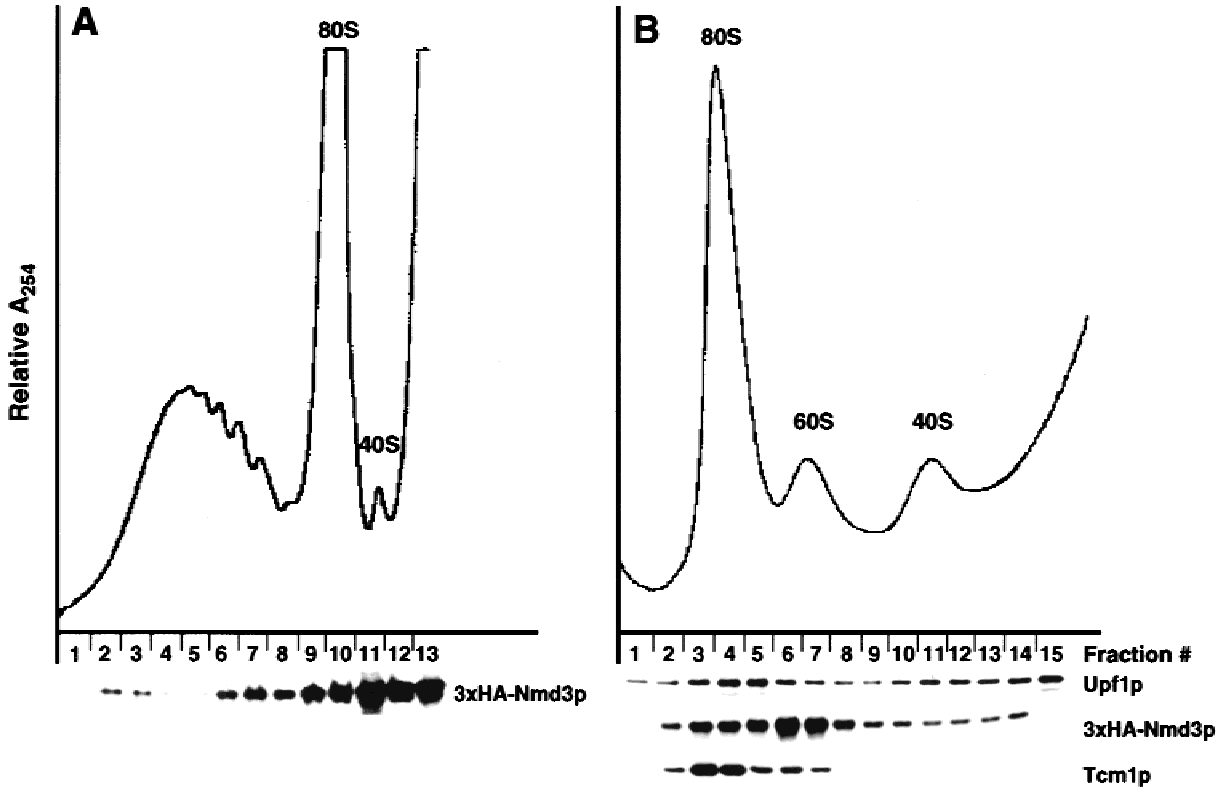

FIGURE 7. Nmd3p cofractionates with the 60S ribosomal subunit. Extracts from strain JBY001, a haploid nmd3::*HIS3* disruption strain containing a plasmid-borne 3xHA-*NMD3* allele, were fractionated on sucrose gradients that were subsequently analyzed by western blotting. In each panel, the top depicts the OD profile, with sedimentation proceeding from right to left and the 80S, 60S, and 40S peaks indicated. The bottom of each panel presents the results of western blotting analyses of the gradient fractions. Panels were serially stripped and rebound with the antibodies indicated to the right of the blot. **A**: 15–50% sucrose gradient; **B**: 7–47% sucrose gradient.

overexpression of full-length Nmd3p or Nmd3pΔ200, suggesting that removal of amino acids 419–518 from Nmd3p creates novel interaction capabilities for the protein that are abrogated by further removal of amino acids 319–418.

The detection of polyribosome half-mers, which are generally caused by the binding to mRNA of 40S subunits without concurrent binding of 60S subunits (van Venrooij et al., 1977; Hesler et al., 1981; Nelson & Winkler, 1987; Moritz et al., 1991; Ohtake & Wickner, 1995; Eisinger et al., 1997), as well as the 60S subunit association of epitope-tagged Nmd3p (Fig. 7), suggests that the novel interactions of truncated Nmd3p may occur with components of the 60S ribosomal subunit. Because the translation phenotypes caused by overexpression of Nmd3pΔ100 require the cell to pass through approximately two doublings before they are apparent, it is likely that newly synthesized ribosomes are preferentially affected. If truncated Nmd3p had an effect on existing ribosomal subunits, an earlier onset of the dominant-negative phenotypes would have been expected.

Further evidence for the association of Nmd3p with the 60S subunit comes from studies of the *GRC5/QSR1* gene. Eisinger et al. (1997) have shown that Grc5p/Qsr1p is an integral 60S ribosomal protein necessary for the proper joining of the 40S and 60S ribosomal subunits and that cells with a temperature-sensitive mutation in *GRC5/QSR1* display an aberrant polyribosome sedimentation profile comparable to that detected in cells overexpressing the *nmd3Δ100* allele. In another study, we identified a *grc5/qsr1* allele in a screen of 4,000 temperature-sensitive mutants for strains that stabilized the *CYH2* pre-mRNA (Zuk & Jacobson, 1998; Zuk et al., 1999). The mutant strain stabilized inherently unstable mRNAs two- to threefold and both the temperature-sensitive and mRNA decay phenotypes of this strain were suppressed by expression of a triple HA-tagged version of *NMD3* expressed on a high copy plasmid.

The detection of *NMD3* in a two-hybrid screen in which the *UPF1* gene was used as bait raised the possibility that Nmd3p, like two other Upf1p-interactors, Nmd2p and Dbp2p, would have a role in nonsense-mediated mRNA decay. Although the level of the *CYH2* pre-mRNA increased in cells overexpressing truncated Nmd3p (Fig. 8), the half-life of this transcript actually decreased in these cells (Fig. 9). Moreover, the abun-

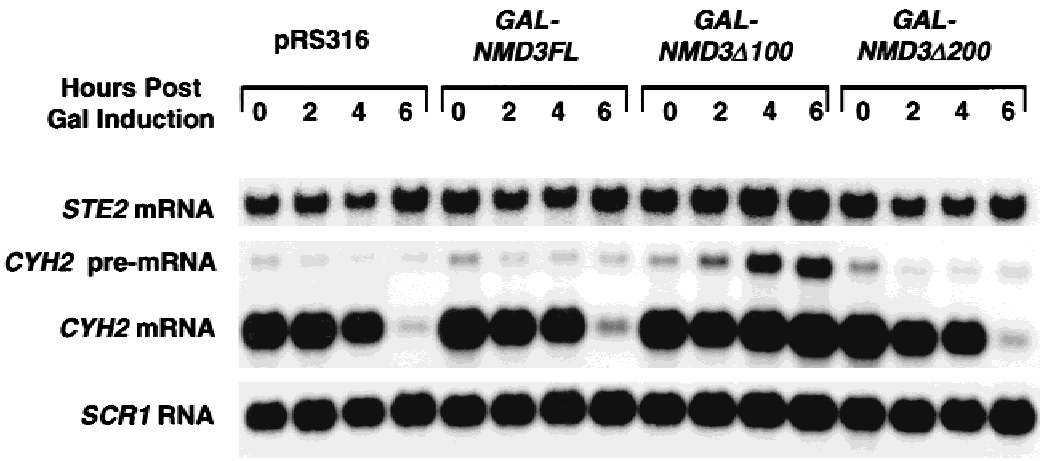

FIGURE 8. Overexpression of pRS316-*GAL-nmd3Δ100* increases the abundance of *CYH2* transcripts. Cells harboring pRS316 with no insert or the indicated inserts were grown in SC/raffinose media lacking uracil to an OD_{600} of 0.4 and then shifted to media containing 2% galactose. Samples were taken at 2-h intervals for RNA isolation and northern blot analysis (using 20 μg of RNA per lane). The blots were hybridized to a radiolabeled *CYH2* probe, exposed, and then sequentially stripped and reprobed with *STE2* and *SCR1* DNAs, the latter as a loading control.

dance of the *can1–100* nonsense-containing mRNA decreased in response to overexpression of truncated Nmd3p. Because the *CYH2* gene encodes ribosomal protein L28, these results suggested that the increased abundance of the *CYH2* pre-mRNA might be attributable to stimulation of the synthesis of components of the ribosome, rather than to an effect on a specific decay pathway.

The possibility that ribosome biogenesis might be altered in cells expressing Nmd3pΔ100 is consistent with the observed increases in the levels of two ribosomal protein mRNAs (*CYH2* mRNA and *RP51a* mRNA [encoding protein S17a]; Table 2), the increased accumulation of 35S pre-rRNA (Fig. 10), and the alterations in amounts of rRNA processing intermediates (Fig. 10). The increased abundance of the ribosomal protein mRNAs, as well as the decreased stability of the *CYH2* transcripts (Fig. 9), suggests that the translation defect(s) brought on by overexpression of truncated Nmd3p leads to destabilization of at least some ribosomal protein mRNAs. Work by Nam and Fried (1986) has shown that defects in 60S ribosomal subunit assembly can cause such destabilization. The enhanced abundance of some ribosomal protein mRNAs and the 35S pre-rRNA may indicate the existence of a feedback loop that compensates for loss of ribosomes by increasing the transcription of the respective genes. The failure of the *TCM1* mRNA, encoding ribosomal

TABLE 2. Relative levels of mRNAs after galactose induction.

Plasmid/induction (h)	*CYH2* mRNA	*CYH2* pre-mRNA	*STE2* mRNA	*can1–100* mRNA	*TCM1* mRNA	*RP51a* mRNA
pRS316						
0	100	100	100	ND[a]	ND	ND
2	77	54	77	ND	ND	ND
4	56	43	68	ND	ND	ND
6	6	34	93	ND	ND	ND
pRS316-*GAL-NMD3FL*						
0	100	100	100	100	100	100
2	66	57	70	94	111	92
4	38	51	66	77	62	59
6	8	49	77	76	51	48
pRS316-*GAL-nmd3Δ100*						
0	100	100	100	100	100	100
2	64	140	99	47	55	77
4	100	274	117	53	52	104
6	99	287	128	52	52	103

Levels of all transcripts were determined by northern blotting, corrected for loading using *SCR1*, and normalized to the respective t_0 values.

[a]ND: Value not determined.

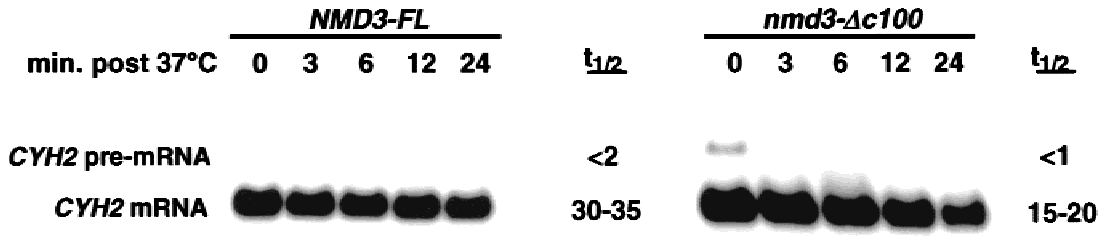

FIGURE 9. Overexpression of pRS316-*GAL-nmd3Δ100* decreases *CYH2* transcript stability. YRP582 cells harboring pRS316-*GAL-NMD3FL* or pRS316-*GAL-nmd3Δ100* were grown in SC/galactose media lacking uracil to an OD_{600} of 0.6, shifted to 37 °C, and aliquots of the cultures were removed at the indicated times. RNA was isolated from each sample and analyzed by Northern blotting using a radiolabeled *CYH2* probe.

protein L3, to also increase under these circumstances may be attributable to differences in the factors responsible for the respective transcriptional regulatory events. In this regard, it is interesting to note that transcription of the *CYH2, RP51a,* and pre-rRNA genes is regulated by the positive-acting factor Rap1p, whereas that of *TCM1* is regulated by Abf1p (Moehle & Hinnebusch, 1991; Mizuta et al., 1998; Hodges et al., 1999).

Collectively, the data presented here and elsewhere (Eisinger et al., 1997; Dick et al., 1997; Dick & Trumpower, 1998; Ho & Johnson, 1999; Zuk et al., 1999) are consistent with a role for Nmd3p in the formation, function, or maintenance of the 60S ribosomal subunit and suggest that overexpression of the *nmd3Δ100* allele may disrupt the formation of these subunits. Because Upf1p appears to be a regulator of translational termination (Czaplinski et al., 1998; Maderazo A, He F, Mangus D, Jacobson A, in prep.), this interpretation also suggests that Nmd3p may provide a link for Upf1p to the ribosome.

MATERIALS AND METHODS

Strains, plasmids, and general methods

Yeast strains and plasmids used in this study are listed in Tables 3 and 1, respectively. Preparation of standard yeast media and methods of cell culture were as described previously (Rose et al., 1990). Transformation of yeast was done by the high-efficiency method of Schiestl and Gietz (1989). DNA manipulations were performed according to standard techniques (Sambrook et al., 1989). All PCR amplifications were performed with Taq DNA polymerase (White et al., 1989) and confirmed, where appropriate, with DNA sequencing. DNA sequences were determined by the method of Sanger et al. (1977). Overlapping fragments of the *NMD3* gene were subcloned in Bluescript and sequenced by annealing of oligonucleotide primers specific to the T3 or T7 promoter regions of the plasmid or by use of oligonucleotide primers that annealed within the subcloned inserts. Plasmid DNAs were propagated in *Escherichia coli* strain DH5α. All designations of ribosomal protein names followed the recently revised nomenclature of Mager et al. (1997). Computer searches for protein:protein homologies utilized the NCBI BLAST program (Altschul et al., 1997). Sequence alignment was generated using the pileup feature of the Wisconsin Package Version 9.1 of the GCG sequence analysis program.

Isolation of the *NMD3* gene and disruption of the chromosomal *NMD3* locus

A *Saccharomyces cerevisiae* genomic DNA library of Sau3A partial fragments constructed in YCp50 was provided by M. Rose (Rose et al., 1987). Colony hybridization was performed as described in Sambrook et al. (1989) under the same conditions described for genomic DNA Southern hybridizations (see below). Approximately three genomic equivalents were screened using a 0.8-kb *Cla*I-*Hin*dIII fragment of *NMD3* as probe. Disruption of the *NMD3* gene (Rothstein, 1991) was performed by transformation of the diploid strain HFY103 with a *Not*I-*Sal*I fragment from Bs-*nmd3::HIS3* (the *Not*I and *Sal*I sites are in the Bluescript polylinker) and selecting His+ transformants. The disruption event was confirmed by Southern analysis.

Transcript mapping

Primer extension analysis was performed according to the procedure described by Boorstein and Craig (1989). Total RNAs from HFY104 and HFY121 were hybridized to an oligonucleotide primer (NMD3-3; 5′-CCCATCGATTGGGGTACCACA-3′) complementary to nt 63–83 of the *NMD3* sense strand.

Southern blot analysis

Total yeast genomic DNA was extracted according to the method of Rose et al. (1990). After restriction digestion with *Xba*I, the DNA was electrophoresed on 0.8% agarose gels and then transferred and cross-linked to Zetaprobe membranes as described in Sambrook et al. (1989). Filters were prehybridized for 2–3 h at 42 °C in 5× SSPE, 40% formamide, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 4 mg/mL of salmon sperm DNA (Sambrook et al., 1989). Filters were hybridized overnight at 42 °C to a radiolabeled *NMD3* probe (1.2-kb *Xba*I-*Cla*I fragment of the 5′ region of *NMD3*), generated by random priming (Feinberg & Vogelstein, 1983) with a kit from Boehringer Mannheim Biochemicals. After hybridization, filters were washed twice in 1× SSC, 0.1% SDS, at room temperature, and once in 0.1× SSC, 0.1% SDS, at 58 °C, before analysis on a Betagen Blot Analyzer.

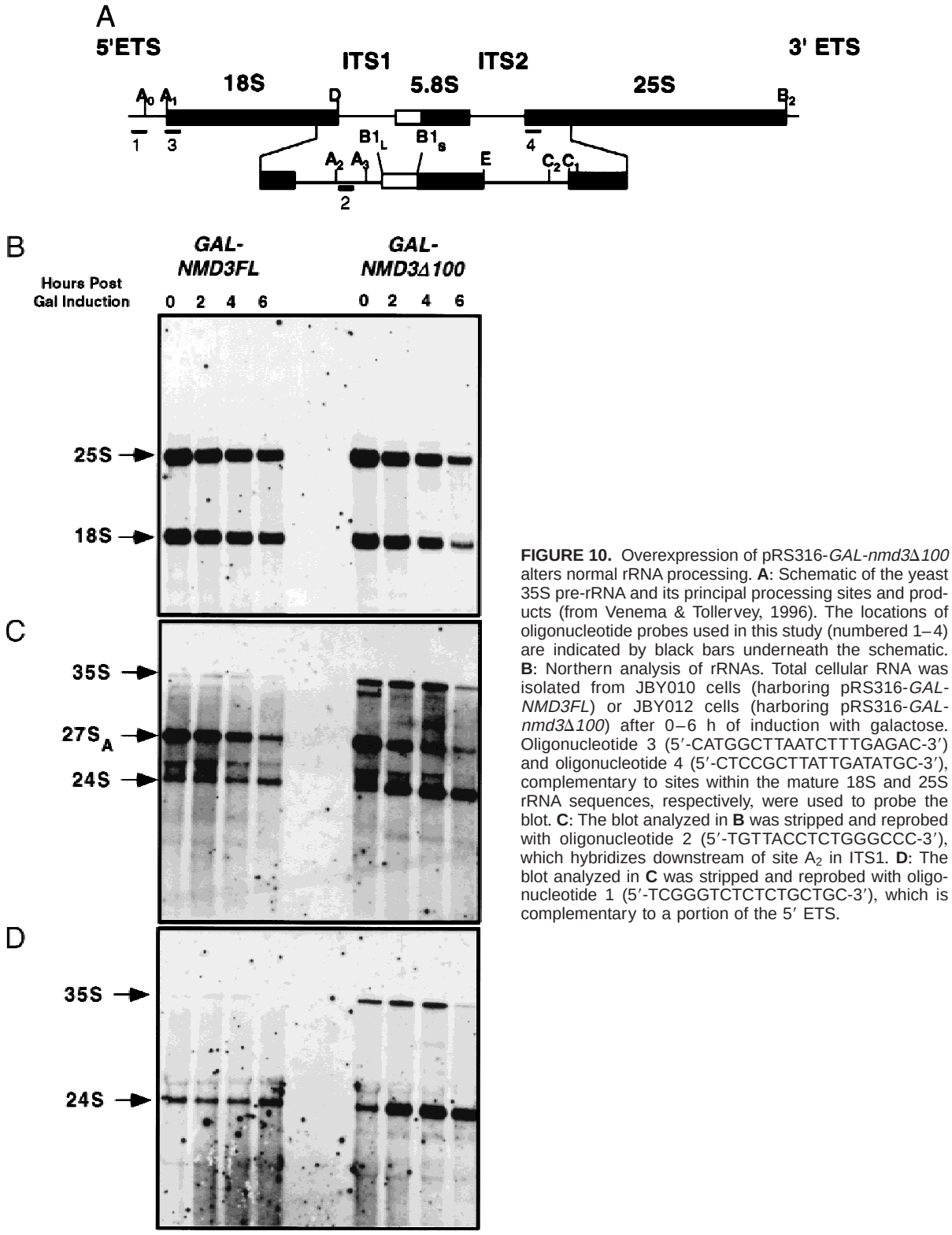

FIGURE 10. Overexpression of pRS316-*GAL-nmd3Δ100* alters normal rRNA processing. **A**: Schematic of the yeast 35S pre-rRNA and its principal processing sites and products (from Venema & Tollervey, 1996). The locations of oligonucleotide probes used in this study (numbered 1–4) are indicated by black bars underneath the schematic. **B**: Northern analysis of rRNAs. Total cellular RNA was isolated from JBY010 cells (harboring pRS316-*GAL-NMD3FL*) or JBY012 cells (harboring pRS316-*GAL-nmd3Δ100*) after 0–6 h of induction with galactose. Oligonucleotide 3 (5′-CATGGCTTAATCTTTGAGAC-3′) and oligonucleotide 4 (5′-CTCCGCTTATTGATATGC-3′), complementary to sites within the mature 18S and 25S rRNA sequences, respectively, were used to probe the blot. **C**: The blot analyzed in **B** was stripped and reprobed with oligonucleotide 2 (5′-TGTTACCTCTGGGCCC-3′), which hybridizes downstream of site A_2 in ITS1. **D**: The blot analyzed in **C** was stripped and reprobed with oligonucleotide 1 (5′-TCGGGTCTCTCTGCTGC-3′), which is complementary to a portion of the 5′ ETS.

RNA extraction and northern blot analysis

RNA used for analysis of cytoplasmic mRNAs was isolated by the hot phenol method as described previously (Herrick et al., 1990). Aliquots (20 μg) of each RNA sample were analyzed by northern blotting, using radiolabeled probes prepared by random priming as described above. mRNA decay rates, expressed as half-lives ($t_{1/2}$), were determined by counting the blots with a BioRad Molecular Imager, normalizing the data such that t_0 after a 10-h shift to galactose equaled 100%,

TABLE 3. Yeast strains.

Strains	Genotype	Source
GGY1:171	*gal4Δ gal80Δ URA3::GAL1-lac Z his3 leu2*	Fields & Song, 1989
HFY91	*MAT **a**/Mat α NMD3/nmd3 ade2-1/ade2-1 his3-11,15/his3-11,16 leu2-3,112/leu2-3,112 trp1-1/trp1-1*	He & Jacobson, 1995
HFY103 (W303)	*MAT **a**/Mat α ade2-1/ade2-1 his3-11,15/his3-11,16 leu2-3,112/leu2-3,112 trp1-1/trp1-1*	He & Jacobson, 1995
HFY121	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1*	This study
HFY122	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1*	This study
JBY010	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1* [pRS316 *GAL-NMD3FL*]	This study
JBY012	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1* [pRS316 *GAL-nmd3Δ100*]	This study
JBY014	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1* [pRS316 *GAL-nmd3Δ200*]	This study
YRP582	*MAT**a** rpb1-1 ura3-52 leu2-2,112*	Decker & Parker, 1993
YRP582-FL	*MAT**a** rpb1-1 ura3-52 leu2-2,112* [pRS316 *GAL-NMD3FL*]	This study
YRP582-Δ*100*	*MAT**a** rpb1-1 ura3-52 leu2-2,112* [pRS316 *GAL-nmd3Δ100*]	This study

and plotting the data with respect to time on semi-log axes. Probes for the CYH2, RP51a, TCM1, and STE2 transcripts have been described previously (Herrick et al., 1990; He et al., 1993). *CAN1* mRNA was detected with a probe made from a 1.0-kbp *Eco*RI-*Sal*1 fragment of YEplac195-CAN1 (He F, Jacobson A, in prep.; Maderazo A, He F, Mangus D, Jacobson A, in prep.). Total RNA used for analysis of nuclear pre-rRNAs was isolated by the glass bead/phenol method (Ulery et al., 1991) and then analyzed by northern blotting, using the oligonucleotide hybridization conditions of Peltz et al. (1993a).

Polyribosome analysis

Cytoplasmic extracts were prepared as described previously (Mangus & Jacobson, 1999). The extracts were fractionated at 4 °C on 15–50% or 7–47% sucrose gradients buffered with 50 mM Tris-acetate, pH 7.4, 50 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). A Beckman SW41 rotor, centrifuged at 45,000 rpm for 150 min, was used for the 15–50% gradients and an SW27 rotor, centrifuged at 27,000 rpm for 300 min, was used for the 7–47% gradients. All gradients were harvested from the bottom and the distribution of RNA was analyzed by continuous monitoring of A_{254}. Polyribosome fractions were analyzed as described previously (Mangus & Jacobson, 1999). Relative stoichiometry of ribosomal subunits was determined by comparisons of the areas under the respective peaks.

Measurement of amino acid incorporation

Cells were grown in 200 mL of SC −ura,−met,+raffinose medium at 30 °C to an OD_{600} of 0.1, harvested by centrifugation, resuspended in 20 mL of fresh medium, and shaken for 10 min at 30 °C. Galactose (4 mL of 20% solution) was then added to a final concentration of 2% galactose per culture. Triplicate 1 mL aliquots were removed at the indicated times and incubated with a mixture of 5 μCi of ^{35}S-translabel (ICN; 70% methionine and ~15% cysteine) and 5 μL of 50 mM unlabeled methionine for 10 min at 30 °C. Incorporation of the radiolabeled amino acids was monitored by trichloroacetic acid (TCA) precipitation. TCA (5 mL of a 5% solution) was added to each aliquot, followed by heating at 90 °C for 20 min and subsequent incubation on ice. The precipitates were collected on GF/C filters, which were washed with 2 × 25 mL of 5% TCA and 25 mL of ethanol, dried under a heat lamp, and counted by scintillation spectrometry. Each experiment was repeated at least three times.

ACKNOWLEDGMENTS

This work was supported by a grant to A.J. from the National Institutes of Health (GM27757) and by a postdoctoral fellowship to F.H. from the Charles A. King Trust, Fleet Bank, Boston, Massachusetts. We thank the members of the Jacobson lab for plasmids, methods, and helpful comments on the manuscript, Jonathan Warner for the generous gift of Tcm1p monoclonal antibody, and Arlen Johnson for graciously communicating results prior to their publication.

Received January 4, 1999; returned for revision March 1, 1999; revised manuscript received May 19, 1999

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