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Mutations in Nucleolar Proteins Lead to Nucleolar Accumulation of PolyA$^+$ RNA in *Saccharomyces cerevisiae*

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Synthesis of mRNA and rRNA occur in the chromatin-rich nucleoplasm and the nucleolus, respectively. Nevertheless, we here report that a *Saccharomyces cerevisiae* gene, *MTR3*, previously implicated in mRNA transport, codes for a novel essential 28-kDa nucleolar protein. Moreover, in *mtr3–1* the accumulated polyA$^+$ RNA actually colocalizes with nucleolar antigens, the nucleolus becomes somewhat disorganized, and rRNA synthesis and processing are inhibited. A strain with a ts conditional mutation in RNA polymerase I also shows nucleolar accumulation of polyA$^+$ RNA, whereas strains with mutations in the nucleolar protein Nop1p do not. Thus, in several mutant backgrounds, when mRNA cannot be exported it concentrates in the nucleolus. mRNA may normally encounter nucleolar components before export and proteins such as Mtr3p may be critical for export of both mRNA and ribosomal subunits.

INTRODUCTION

The mechanism and path of export of mRNA are largely unknown. Microinjection studies of *Xenopus* oocytes indicate that mRNA export is saturable. It is not readily competed by other varieties of RNA and, although a m^{7}G 5′ cap may facilitate export, neither the cap nor a polyA tail is essential (Jarmolowski *et al.*, 1994). To understand the succession of events that accomplishes transport, we and others have isolated conditional recessive mutants of *Saccharomyces cerevisiae*. As visualized by fluorescent in situ hybridization, these mutants accumulate polyA$^+$ RNA in the nucleus at 37°C (Hopper *et al.*, 1990; Amberg *et al.*, 1992; Kenna *et al.*, 1993; Kadowaki *et al.*, 1994a; Rout and Wente, 1994; Tartakoff and Schneiter, 1995).

The haploid mRNA transport mutant, *mtr3–1*, was recovered from a ^{3}H-amino acid suicide selection and shows strong nuclear accumulation of polyA$^+$ RNA after 1–3 h of incubation at 37°C (Kadowaki *et al.*, 1994a). Our earlier studies have also shown as follows, that in *mtr3–1* at 37°C: 1) the average polyA$^+$ RNA tail length increases, 2) pre-mRNA splicing continues, although some oversized polymerase II transcripts are seen, 3) protein synthesis progressively slows, and 4) once polyA$^+$ RNA accumulates it persists upon reincubation in the absence of RNA synthesis (Kadowaki *et al.*, 1994a). In the present study we describe the structure of the *MTR3* gene, and show that the *Mtr3p* product is a nucleolar protein and that the site of accumulation of polyA$^+$ RNA in *mtr3–1* is actually within the nucleolus. Several earlier studies have also suggested that the nucleolus is important for mRNA processing (Schneiter *et al.*, 1995).

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MATERIALS AND METHODS

Yeast Strains

The original *mtr3–1* (ts 20) mutant was backcrossed with YTK100 three times. At this point temperature sensitivity and accumulation of polyA$^+$ RNA cosegregated. The mutation is recessive. YTK304 was constructed by transformation of YTK303 with pTK304 followed by tetrad dissection. A LEU$^+$ URA$^+$ spore was picked and called YTK304. YTK305 and YTK306 were constructed by transformation of YTK304 with pTK305 or pTK306, followed by isolation of 5-FOA resistant colonies, respectively (Table 1).

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Table 1. List of yeast strains

Strain	Genotype		Source
SH1	*MATα*	*ade5*	S. Henry
YPH501	*MATa/α*	*ade2-101/ade2-101 ura3-52/ura3-52 lys2-801/ lys2-801 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 trp1-Δ63/trp1-Δ63*	P. Hieter (Sikorski and Hieter, 1989)
S395D-1	*a/a*		Berkeley Yeast Genetics Stock Center
S394B-1	*α/α*		Berkeley Yeast Genetics Stock Center
RY262	*MATα*	*rpb1-1 ura3-52 his4-539*	R. Young (Nonet *et al.*, 1987)
YTK100	*MATa*	*ura3-52*	Kadowaki *et al.*, 1993
YTK300	*MATa*	*mtr3-1 ura3-52*	This study
YTK301	*MATα*	*mtr3-1 ura3-52*	This study
YTK302	*MATa*	*mtr3-1 rpb1-1 ura3-52 his4-539*	This study
YTK303	*MATa/α*	*ade2-101/ade2-101 ura3-52/ura3-52 lys2-801/lys2-801 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 trp1-Δ63/trp1-Δ63 MTR3/ΔMTR3::LEU2*	This study
YTK304	*MATα*	*ade2-101 ura3-52 lys2-801 his3-Δ200 leu2-Δ1 trp1-Δ63 ΔMTR3::LEU2 pTK304*	This study
YTK305	*MATα*	*ade2-101 ura3-52 lys2-801 his3-Δ200 leu2-Δ1 trp1-Δ63 ΔMTR3::LEU2 pTK305*	This study
YTK306	*MATα*	*ade2-101 ura3-52 lys2-801 his3-Δ200 leu2-Δ1 trp1-Δ63 ΔMTR3::LEU2 pTK306*	This study

Cloning of the MTR3 Gene

YTK300 was transformed with a yeast genomic DNA library on YCp50 (Sikorski and Hieter, 1989; Rose and Broach, 1991). A plasmid was isolated from one TS^+ transformant and the complementing DNA fragments were determined by subcloning. pTK300 was digested with *Hpa*I and used for transformation of YTK300 (*mtr3-1*). A TS^+ URA^+ transformant was crossed with SH1 and YTK301, followed by tetrad analysis. All spores in 20 tetrads from the cross with SH1 (*MTR3 URA3*) were TS^+ and the segregation pattern of *URA3* (PD:T:NPD) was 4:14:2. The analysis of 20 tetrads from a cross with YTK301 (*mtr3-1 ura3-52*) showed all spores were PD (2 TS^+:2 ts spores). These results indicate that the cloned DNA fragment corresponds to *MTR3* gene. A series of nested deletions was prepared from pTK303 for sequencing (Sambrook *et al.*, 1989) (Table 2).

Gene Disruption

For disruption of *MTR3*, pTK302 was constructed by replacing the *Bam*HI–*Nhe*I fragment in pTK301 with the *LEU2 Bgl*II–*Bgl*II fragment isolated from PS118 (Silver *et al.*, 1988) after converting cohesive to blunt ends by treatment with Klenow enzyme. YPH501 was transformed with *Bss*HII-digested pTK302 (which releases the *ΔMTR3::LEU2* DNA fragment) and Leu^+ colonies were recovered. The resulting strain is YTK303. The gene replacement was confirmed by genomic Southern hybridization. Upon sporulation and tetrad dissection of YTK303, only leu^- colonies were obtained.

Gene Tagging

The HA-epitope tagging of Mtr3p was performed by polymerase chain reaction (PCR) using pTK303 and the following primers: *primer 1*, 5′-TCCCCCGGGAATGAATGTTCAAGACAGAAGG-3′; *primer 2*, 5′-TCCCCCGGGCTAAGTTTCCTGGTTCATTAA-3′; and *primer 3*, 5′ -TCCCCCGGGCTAAGTAGCGTAGTCTGG-GACGTCGTATG GGTATTCCTGGTTCATTAAACAGCTTAT-3′. The combination of primers 1/3 and primers 1/2 yields tagged and untagged PCR versions of *MTR3*, respectively. The HA-epitope (YPYDVPDYA) was inserted between glu and thr at the C-terminus of Mtr3p. PCR products were digested with *Sma*I and cloned into the *Bam*HI site of pGAP316 after blunting with Klenow enzyme. The *GAL1* promoters of the resulting plasmids were replaced by endogenous *MTR3* promoters by exchanging the *Bam*HI–*Eco*RI fragment with the *Bam*HI–*Xba*I fragment containing the 5′ sequences from *MTR3*. *MTR3* gene cassettes were then cloned into pRS313 (Sikorski *et al.*, 1989) to yield pTK306 (which carries tagged *MTR3*) and pTK305 (which carries untagged *MTR3*).

Table 2. List of plasmids

pTK300	*Xba*I–*Kpn*I DNA fragment including *MTR3* cloned in pRS306 (Sikorski and Hieter, 1989)
pTK301	The same DNA fragment as pTK300 cloned in $pBTSKII^+$
pTK302	Identical to pTK301 but the internal *BAMHI–NheI* fragment of *MTR3* has been replaced by a *Bgl*II–*Bgl*II fragment including *LEU2* from PS118 (Silver *et al.*, 1988)
pTK303	*Xba*I–*Hpa*I fragment including *MTR3* cloned in $pBTSKII^+$
pTK304	The same DNA fragment as pTK303 cloned in pRS316 (Sikorski and Hieter, 1989)
pTK305	Untagged Mtr3p driven by the *MTR3* promoter and terminated by a PGK terminator in pRS313 (Sikorski and Hieter, 1989)
pTK306	Same as pTK305 except that the *MTR3* ORF includes the carboxy-terminal HA-epitope tag

Immunofluorescence and In Situ Hybridization

These procedures were carried out as in Kadowaki *et al.*, 1993 and 1994a.

RESULTS

To localize the site of polyA$^+$ RNA accumulation in the nucleus, in situ hybridization and indirect immunofluorescence were combined. In wild-type cells the chromatin-rich nucleoplasm and nucleolar region–where the Nop1p antigen is concentrated (Aris and Blobel, 1988)–appear poor in polyA$^+$ RNA, by comparison with the cytoplasm (Figure 1A). After incubation of *mtr3* for 3 h at 37°C, polyA$^+$ RNA (green) largely coincides with Nop1p (red) and is strikingly separated from the histone H2B-rich part (red) of the nucleus (Figure 1B). Comparable observations were made for *mtr17* (Figure 1B) and *mtr14* (our unpublished observations). A ts mutation in a second nucleolar protein, the large subunit of RNA polymerase I, also causes accumulation of polyA$^+$ RNA in association with the nucleolus (Schneiter *et al.*, 1995). In this case, as for *mtr1–1* and *mtr2–1*, the nucleolus fragments (Kadowaki *et al.*, 1994b). These observations on *pol*I ts strains appear to reflect a structural role of *pol*I (Oakes *et al.*, 1993; Kadowaki *et al.*, 1994b) rather than

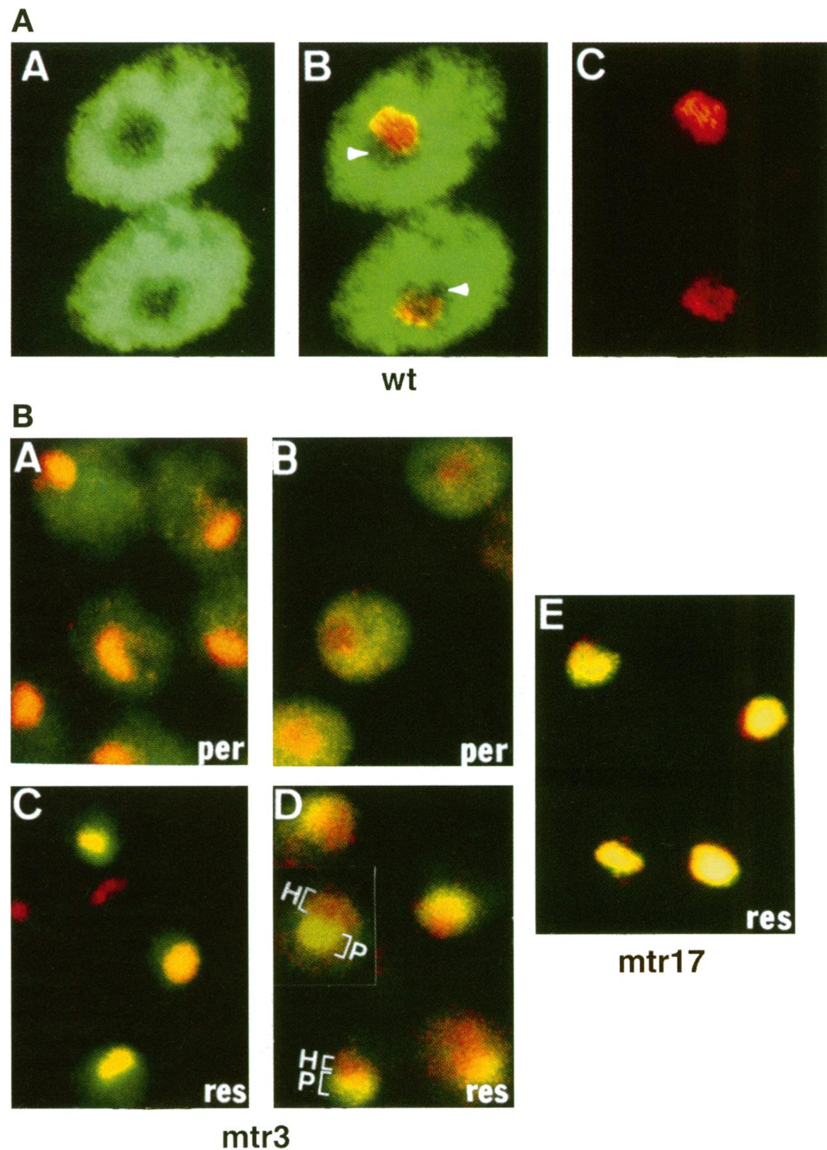

Figure 1. Localization of polyA$^+$ RNA in wild-type and mutant cells. (A) Wild-type tetraploid cells were grown at 30°C, fixed, and processed for simultaneous detection of polyA$^+$ RNA (fluorescein channel, panel A) and Nop1p (rhodamine channel, panel C). In wild-type cells, there is no uniform overlap between polyA$^+$ RNA and the nucleolar antigen, as illustrated by the merging of the two channels (panel B). The yellow regions are inevitable due to some superposition of fluorochromes. The dark area (arrowheads) adjacent to the nucleolus coincides with the DNA-rich chromatin when stained with DAPI (our unpublished observations). Controls employing oligo-dA in place of oligo-dT or pretreatment of cells with the RNA polymerase inhibitor thiolutin (Tipper, 1973), document the specificity of the in situ hybridization signal. The tetraploid was produced by crossing the diploid strains S395D-1 and S394B-1 (Berkeley Yeast Genetic Stock Center). (B) PolyA$^+$ RNA accumulated in haploid *mtr3* (panels A–D) and *mtr17* (panel E) mutant cells colocalizes with the nucleolar Ag Nop1p and is excluded from the histone-rich part of the nucleoplasm. Cells were incubated at the permissive (per) temperature of 23°C (panels A and B), or shifted to the restrictive (res) temperature of 37°C for 3 h (panels C, D, and E) before fixation. PolyA$^+$ RNA and nuclear antigens were simultaneously detected by a combination of in situ hybridization and indirect immunofluorescence. In *mtr3–1*, at 23°C, no uniform colocalization of polyA$^+$ RNA (fluorescein channel) with the rhodamine-labeled nuclear antigens Nop1p (panel A), or histone H2B (panel B), was observed. The orange color in panels A and B is the inevitable result of some superposition of fluorochromes because of the small size of the haploid nucleus. It is not comparable to the true yellow seen in panels C and E. After incubation of *mtr3–1* at the restrictive temperature, accumulated fluorescein-labeled polyA$^+$ RNA (P) largely colocalizes with Nop1p to give a yellow signal (panel C) and is excluded from the chromatin (histone)-rich (H; rhodamine-labeled) part of the nucleus (panel D). Panel E shows the yellow overlap between polyA$^+$ RNA and Nop1p in a *mtr17* mutant strain after incubation at 37°C for 3 h. A polyA$^+$ RNA-free chromatin-rich region comparable to that seen in Figure 1A cannot be consistently resolved because of the small size of haploids. Histone 2B was detected with a rabbit antiserum obtained from M. Grunstein. Nop1p was detected with the monoclonal antibody A66.2 (Aris and Blobel, 1988). Samples were observed in a Bio-Rad (Richmond, CA) laser confocal microscope.

the absence of rRNA synthesis, i.e., accumulation of polyA$^+$ RNA is not seen upon inhibition of rRNA synthesis in strains in which rDNA transcription is driven by an RNA polymerase II galactose promoter (our unpublished observations). Accumulation is also not seen in several mutants bearing mutations in the nucleolar protein Nop1p, nor in the rRNA processing *rrp1* or *rrp2* mutants (Kadowaki *et al.*, 1994a).

rRNA processing in *mtr3–1* was analyzed in ^{3}H-[methyl]methionine pulse-chase experiments after pre-incubation at 37°C (Figure 2). Cells were pulse labeled for 3 min and chased for 5 min or 10 min. Possibly because of the nucleolar localization of Mtr3p (see below), the synthesis and processing (lane 8) of 35S pre-rRNA are inhibited at 37°C. Unlike most *mtr* mutants (Kadowaki *et al.*, 1994a), ^{3}H-18S rRNA becomes almost undetectable (lanes 8–10), possibly reflecting inhibition of ribosomal subunit export, because the 20s to 18s conversion occurs in the cytoplasm (Udem and Warner, 1973). Comparable defects are seen for [^{3}H]uridine-labeled RNA (our unpublished observations). Additionally, after a 1-h pre-incubation of *mtr3–1* at 37°C, a modest accumulation of pre-tRNA species is observed in [^{3}H]uridine labeling experiments (our unpublished observations).

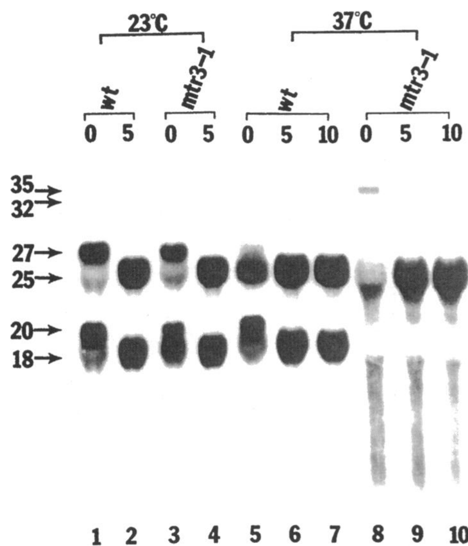

Figure 2. Processing of rRNA in wild-type and *mtr3–1* mutant cells. YTK100 (wt; lanes 1, 2, 5, 6, and 7) and YTK300 (*mtr3–1*; lanes 3, 4, 8, 9, and 10) were preincubated at 23°C (lanes 1, 2, 3, and 4) or 37°C (lanes 5, 6, 7, 8, 9, and 10) for 1 h followed by a 3-min ^{3}H-[methyl]methionine pulse (lanes 1, 3, 5, and 8), or a 3-min pulse followed by 5 min (lanes 2, 4, 6, and 9) and 10 min (lanes 7 and 10) chase at the same temperature. Labeled RNA (equal amounts of radioactivity) was analyzed on a 1% agarose-formaldehyde gel. Note the overabundance of labeled 35S, reduction of 20S, and especially 18S species in *mtr3–1* at 37°C.

The nucleolus forms a homogeneous electron-dense crescent at the edge of the nucleoplasm in wild type and *mtr3–1* at 23°C (Figure 3). By contrast, after incubation of *mtr3–1* at 37°C, it occupies a larger area revealing an unprecedented tortuous substructure, resembling the "nucleonema" of animal cells (Goessens, 1984). To learn whether nucleolar disorganization in *mtr3–1* is a secondary consequence of polyA$^+$ RNA accumulation, as is nucleolar fragmentation for *mtr1–1* and *mtr2–1* (Kadowaki *et al.*, 1994b), cells were incubated at 37°C with the RNA polymerase inhibitor thiolutin (Tipper, 1973). Also, the *mtr3–1* mutation was combined in a double mutant with a ts RNA polymerase II mutation *rpb1–1*, in which mRNA transcription stops within 10 min (Nonet *et al.*, 1987). In both cases, some nucleolar disorganization is still seen. Thus, Mtr3p may directly affect nucleolar organization.

The *MTR3* gene was cloned. The coding sequence predicts that Mtr3p is a novel 250-amino acid hydrophilic protein without recognizable motifs or significant homology to known proteins by BLAST and FASTA search (Pearson and Lipman, 1988; Altschul *et al.*, 1990) (Figure 4). The codon adaptation index (Sharp and Li, 1987) value of *MTR3* is 0.141, suggesting low expression of the Mtr3p protein. When one *MTR3* gene is disrupted in a diploid wild-type strain and the diploid is then sporulated, only two leu$^-$ spores are recovered from a dozen tetrads at 23°, 30°, or 37°C. When the *MTR3* ORF is expressed from a *GAL1* promoter in *mtr3–1*, the transformant grows in galactose medium at 37°C but not in glucose. In addition, when YTK304 (which has a disrupted chromosomal *MTR3* gene and carries a *URA3 MTR3* plasmid) was streaked on a 5-FOA plate, no 5-FOA–resistant colonies appeared (our unpublished observations). Thus, the *MTR3* gene is essential for vegetative growth.

A gene encoding Mtr3p with the HA epitope at its carboxy terminus was constructed. A centromeric plasmid carrying this construct restored viability to a *MTR3* null mutant. Furthermore, the growth rate of YTK305 (a strain bearing untagged Mtr3p) and YTK306 (with tagged Mtr3p) were identical. The intracellular localization of HA-epitope–tagged Mtr3p in YTK306 was analyzed by subcellular fractionation (Figure 5A) and by indirect immunofluorescence (Figure 5B). In crude fractions, tagged Mtr3p co-fractionates with the nucleolar protein Nop1p (Aris and Blobel, 1988), and judging by immunofluorescence, it co-localizes with Rpa190p, the largest subunit of RNA polymerase I (Mémet *et al.*, 1988) at the edge of the 4,6-diamidino-2-phenylindole (DAPI)-stained and histone-containing region. Thus, Mtr3p is concentrated in the nucleolus.

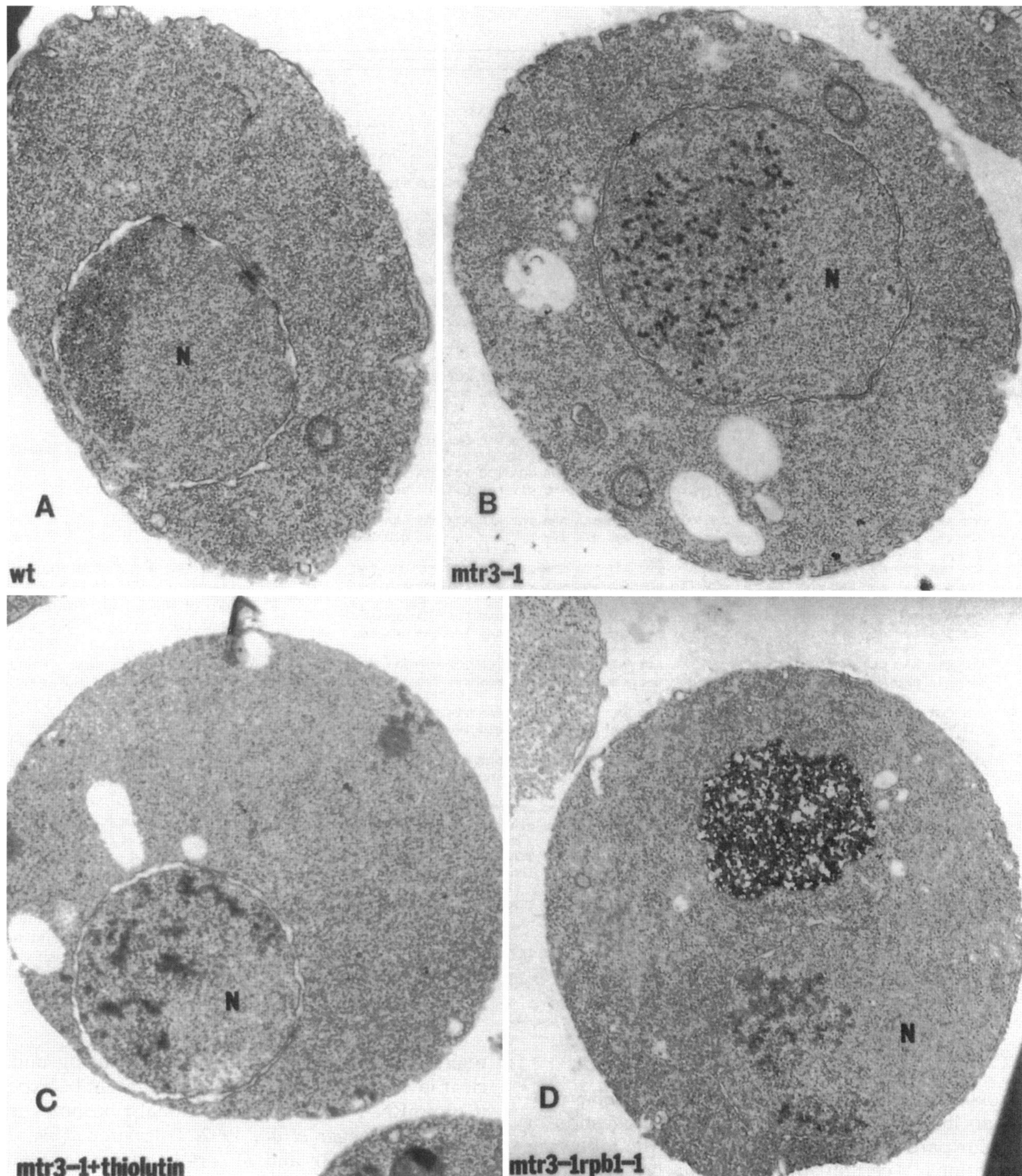

Figure 3. Nucleolar structure in wild type and *mtr3–1*. YTK100 (wt; panel A), YTK300 (*mtr3–1*; panels B and C), and YTK302 (*mtr3–1 rpb1–1*; panel D) were incubated 3 h at 37°C and then processed for transmission electron microscopy. One of the YTK300 cultures was treated with 3 μg/ml thiolutin for 3 h to inhibit RNA synthesis (panel C). Somewhat dispersed electron-dense material with a labyrinthine substructure is routinely detected in *mtr3–1* at 37°C (panel B) and a conventional condensed nucleolar crescent is absent. Immunogold staining shows that the labyrinthine region is positive for Nop1p and negative for osmium-amine staining of DNA (our unpublished observations). The fine structure of the electron-dense region is also altered in YTK300 treated with thiolutin (panel C) and in the double mutant (panel D). Staining of thin sections was with 2% uranylacetate in EtOH followed by Reynold's lead citrate.

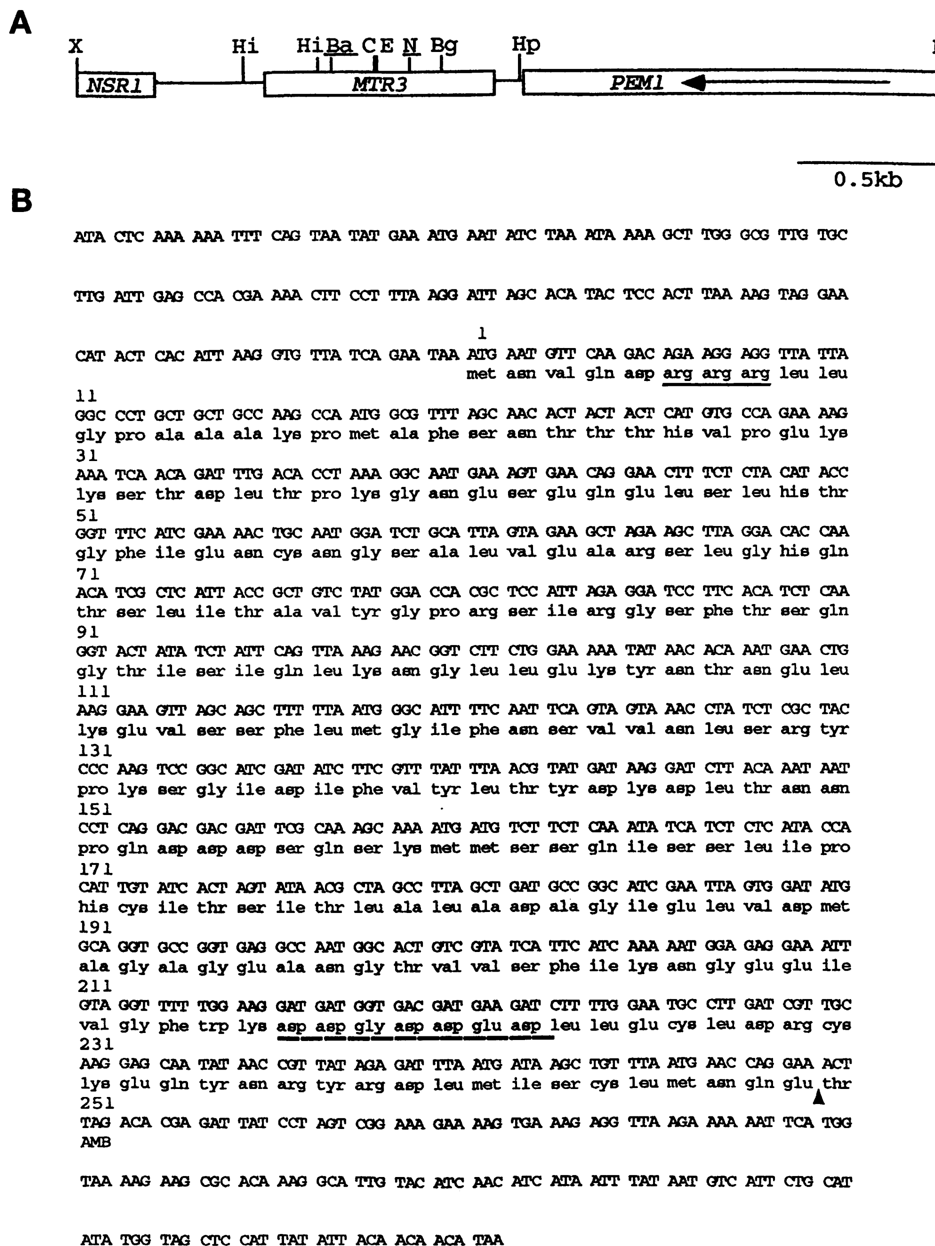

Figure 4. Restriction map and sequence of MTR3. (A) The restriction map of the 3.1-kb *Xba*I–*Kpn*I fragment containing *MTR3*. ORFs of *NSR1* (Lee *et al.*, 1991), *MTR3*, and *PEM1* (Kodaki and Yamashita, 1987) are indicated by open boxes and the direction of transcription is left to right, except for *PEM1* (shown by arrow). The *Bam*HI and *Nhe*I sites used for gene disruption are underlined. The symbols are as follows: X, *Xba*I; Hi, *Hin*dIII; Ba, *Bam*HI; C, *Cla*I; E, *Eco*RV; N, *Nhe*I; Bg, *Bgl*II; Hp, *Hpa*I; and K, *Kpn*I. (B) The nucleotide and predicted amino acid sequence of *MTR3*. A putative nuclear localization signal and a cluster of acidic amino acids are indicated by solid and dashed lines, respectively. The site of HA-epitope insertion is shown by the arrowhead.

DISCUSSION

Our observations indicate that yeast nucleolar proteins do influence mRNA export. This suggestion of nucleolar participation is supported by the following observations: 1) certain animal cell transcripts are concentrated in the nucleolus (Bond and Wold, 1993); 2) nucleolar ablation in animal cells interrupts gene expression (Deák *et al.*, 1972); 3) the HTLV and HIV proteins Rev and Rex, which promote the export of unspliced pre-mRNA, are normally concentrated in the nucleolus (Nosaka *et al.*, 1989; Meyer and Malim, 1994); 4) stop codons near the 5′ end of the coding region of transcripts regulate both splicing and nuclear levels of the same mRNAs (as might be expected if pre-mRNA encounters functional ribosomes in the nucleus) (Naeger *et al.*, 1992; Belgrader *et al.*, 1993; Dietz *et al.*, 1993); and 5) certain ribosomal proteins inhibit splicing of their mRNAs (Eng and Warner, 1991; Li and Woolford, 1994). Observations 4 and 5 are most readily explained by postulating a nuclear encounter between pre-mRNA and ribosomal proteins and subunits, which are concentrated in the yeast nucleolus (Sillevis-Smitt *et al.*, 1973). It may also be significant that one hnRNP protein is concentrated

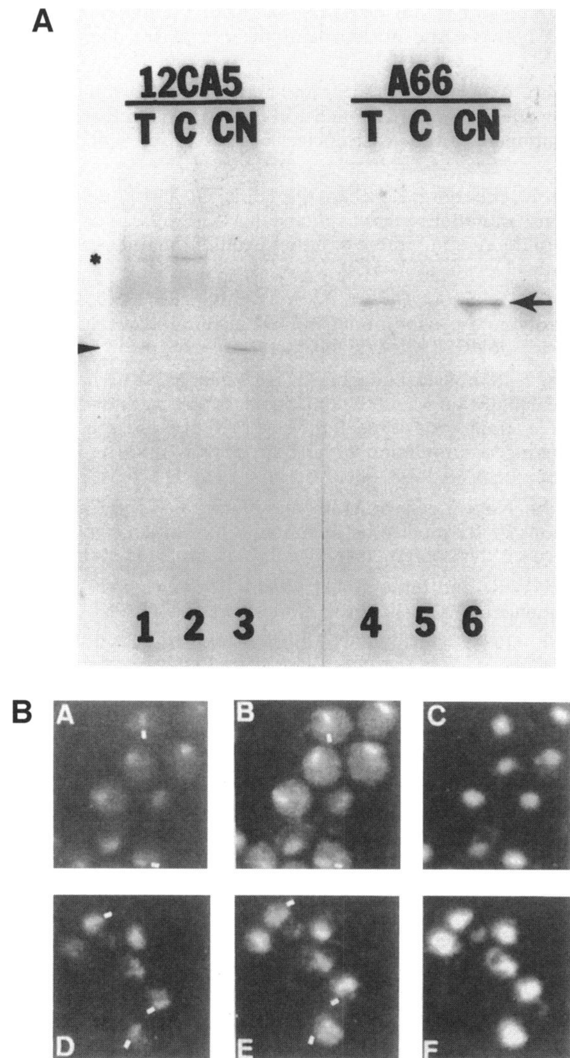

Figure 5. Intracellular localization of Mtr3p. (A) Epitope-tagged Mtr3p was localized by subcellular fractionation of YTK306. Fifty micrograms of protein from total (lanes 1 and 4), cytosolic (lanes 2 and 5), and crude nuclear (lanes 3 and 6) fractions were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot analysis using 12CA5 (recognizing HA epitope) (lanes 1, 2, and 3) and A66 (recognizing Nop1p) (lanes 4, 5, and 6). The epitope-tagged Mtr3p (arrowhead) is concentrated in the nuclear fraction, as is Nop1p (arrow). It is not sufficiently abundant to be detected in the cell lysate at this level of load. The asterisk indicates a cytosolic protein cross-reacting with 12CA5. It is also detected in YTK305, which carries untagged Mtr3p. Cell fractionation was as reported by Mirzayan *et al.*, 1992. (B) Epitope-tagged Mtr3p was localized by double immunofluorescent labeling of YTK306 (carrying HA-tagged Mtr3p) with the mouse monoclonal 12CA5 (panels A and D) and rabbit anti-Rpa190p (panel B) or rabbit anti-histone H2B (panel E) antibody. The tagged Mtr3p colocalizes with Rpa190p, the largest subunit of RNA polymerase I, but not with histones or DNA. The white lines designate nucleoli. The Rpa190p antigen was studied in this experiment, instead of Nop1p, because it allowed simultaneous localization of the epitope tag with a mouse antibody. Panels C and F are the corresponding DAPI images. Samples were observed in a Nikon epifluorescence microscope.

adjacent to the nucleolus (Ghetti *et al.*, 1992) and that mRNAs transcribed from the rDNA repeat can be exported (Petes, 1980).

We therefore consider it likely that RNA polymerase II transcripts encounter components of the nucleolus before export. Such encounters might occur either in the chromatin-rich nucleoplasm or in the nucleolus and may involve the cyclic exit and reimport of nucleolar proteins, as is known to occur for B23, nucleolin, and Rev (Yung *et al.*, 1985; Borer *et al.*, 1989; Nosaka *et al.*, 1989; Meyer and Malim, 1994; Kalland *et al.*, 1994). Possibly, nucleolar proteins such as Mtr3p package, pilot, and/or motor both rRNA and mRNA to the cytoplasm. Because, at steady-state, only minimal polyA$^+$ RNA localizes to the nucleolus (Carter *et al.*, 1991; Visa *et al.*, 1993; Huang *et al.*, 1994), any transit of polyA$^+$ RNA through the nucleolus itself must be rapid. Thus, it might appear more reasonable to propose that the nucleolus is not on the normal transport path, i.e., that any encounters normally occur outside the nucleolus, while in the *mtr3* or *rpa190* mutant background, transcripts that cannot be efficiently exported are redirected to the nucleolus. This might result from the "loosening" of nucleolar structure that is seen in *mtr3*; however, we do not see a nucleolar accumulation of polyA$^+$ RNA in *nop1* mutants (Kadowaki *et al.*, 1994a). It is nevertheless striking that ongoing transcription by RNA polymerase II causes nucleolar fragmentation in certain mutant backgrounds (Kadowaki *et al.*, 1994b) as might be expected if transcripts actually pass through the nucleolus.

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