Isolation and Characterization of *Saccharomyces cerevisiae* mRNA Transport-defective (*mtr*) Mutants

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Abstract. To understand the mechanisms of mRNA transport in eukaryotes, we have isolated *Saccharomyces cerevisiae* temperature-sensitive (ts) mutants which accumulate poly(A)$^+$ RNA in the nucleus at the restrictive temperature. A total of 21 recessive mutants were isolated and classified into 16 complementation groups. Backcrossed mRNA transport-defective strains from each complementation group have been analyzed. A strain which is ts for heat shock transcription factor was also analyzed since it also shows nuclear accumulation of poly(A)$^+$ RNA at 37°C. At 37°C the mRNA of each mutant is characterized by atypically long polyA tails. Unlike ts pre-mRNA splicing mutants, these strains do not interrupt splicing of pre-mRNA at 37°C; however four strains accumulate oversized RNA polymerase II transcripts. Some show inhibition of rRNA processing and a further subset of these strains is also characterized by inhibition of tRNA maturation. Several strains accumulate nuclear proteins in the cytoplasm when incubated at semipermissive temperature. Remarkably, many strains exhibit nucleolar fragmentation or enlargement at the restrictive temperature. Most strains show dramatic ultrastructural alterations of the nucleoplasm or nuclear membrane. Distinct mutants accumulate poly(A)$^+$ RNA in characteristic patterns in the nucleus.

THE export of mRNA from the nucleus involves components ranging from the sites of transcription to the sites of translation in the cytoplasm. It is therefore expected that many factors in both nucleus and cytoplasm are involved in this process, irrespective of whether mRNA export is carried out by an active or diffusional mechanism. In addition to mRNA, UsnRNAs, tRNA, and rRNA are exported form the nucleus to cytoplasm, the lattter as assembled ribosomal subunits. The export of these several varieties of RNA may depend upon common factors. The study of the mechanism of mRNA export is still in its infancy. Microinjection experiments with *Xenopus laevis* oocytes have provided information on RNA structural requirements and some *trans*-acting factors for the efficient export of tRNA, rRNA, UsnRNA, and mRNA (Tobian et al., 1985; Dworetzky and Feldherr, 1988; Featherstone et al., 1988; Bataillé et al., 1990; Guddat et al., 1990; Hamm and Mattaj, 1990; Dargemont and Kühn, 1992; Izaurralde et al., 1992), but this system has not yielded substantial insight into the transport machinery itself. Some information has also come from visualization of the distribution of transcripts in the nucleus (Carter et al., 1993; Xing et al., 1993; Zachar et al., 1993). Since none of the above approaches allows one to manipulate the interior of the nucleus or to define the machinery which accomplishes mRNA export, we and others have taken a genetic approach and isolated mRNA transport defective (*mtr*)[1] and ribonucleic acid trafficking mutants of the yeast, *Saccharomyces cerevisiae* (Amberg et al., 1992; Kadowaki et al., 1992). We chose *S. cerevisiae* as a model because the pre-mRNAs of this yeast undergo little processing (pre-mRNA splicing, nucleotide methylation) compared to animal cells (Sripati et al., 1976; Rymond and Rosbash, 1992). Thus, for example, inhibition of pre-mRNA splicing is not expected to block export of bulk mRNA. Our isolation of *mtr* mutants is dependent on the assumption that poly(A)$^+$ RNA will be sufficiently stable to accumulate in the nucleus at the restrictive temperature. We here provide a survey of the pro-

Table I. Summary of Mutant Isolation Using ^{3}H-Amino Acids

	YPH1	YPH2
Number of cells subjected to selection	1.0×10^5	1.2×10^5
Survivors after suicide selection	479	335
Temperature sensitive mutants	85	64
Nuclear positive mutants by FISH	5	4

In addition, three mutants (*mtr1-1*, *mtr1-2*, and *mtr1-3*) were recovered from an enrichment experiment utilizing toxic amino acid analogues (S-2-aminoethyl-L-cysteine and L-azetidine-2-carboxylate).

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1. *Abbreviations used in this paper*: cs, cold sensitive; FISH, fluorescent in situ hybridization; HSP, heat shock protein; *mtr*, mRNA transport defective; PABP, polyA-binding protein; *prp*, pre-mRNA processing; ts, temperature sensitive.

Table II. Summary of the Phenotype of mtr Mutants After Backcross

MTR group	Source	Strains used in experiment	ts/cs at °C	FISH phenotypes*	EM changes‡ (group #)	Nucleolar changes
1¶	Suicide selection + Hartwell collection	See Kadowaki et al., 1993	30–34**	Entire (3 h, 100%)	1	Fragmentation
2 (ts12α)	Suicide selection	See Kadowaki et al., 1994*a*	34	Spots (0.5, 100%)	4	Fragmentation
3 (ts20a)	"	See Kadowaki et al., 1994*b*	37	Crescent/entire (3, 90%)	3	Enlargement
4 (ts7a)	"	T188	37	Spot (3, 90%)	1	–
5 (ts37a)	"	T279	37	Circle/spots (3, 50%)	1	–
6 (ts54a)	"	T273	30, 16	Entire/spots (3, 30%)	1	–
7 (ts7α)	"	T229	34	Entire/spots (3, 20%)	5	–
8 (ts10α)	"	T301	34	Entire (3, 100%)	2	Fragmentation
10 (ts54α)	"	T255	34, 16	Spots (3, 50%)	6	–
11 (ts60α)	"	T330	34	Granular/entire (1.5, 20%)	None	–
12 (H157)	Hartwell collection	T205	34	Circle/spots (3.0, 80%)	1	–
13 (H194)	"	T212	34	Granular/entire (1.5, 80%)	2	–
14 (H209)	"	T263	34	Crescent/entire (4.5, 80%)	1	Fragmentation
15 (H397)	"	T292	34	Entire/spots (4.5, 80%)	2	Fragmentation
16 (H518)	"	T179	37	Entire/spots (4.5, 70%)	7	–
17 (H577)	"	T271	37	Crescent/entire (4.5, 80%)	3	Enlargement
mas3	M. Yaffe	MYY385	37	Spot (0.5, 100%)	2	Fragmentation

* Indicates the poly(A)$^+$ RNA accumulation pattern in the nucleus after 37°C incubation for time shown in parentheses. These times are optimal for obtaining a strong signal. The percentage of nuclear staining positive cells is also in parentheses. Some patterns depend on 37°C incubation time, as previously noted in *mtr1-1*.

‡ Ultrastructural changes detected by EM are the following: group 1, abundant osmiophilic material in the nucleoplasm; group 2, fine barley-like material in the nucleoplasm; group 3, tortuous substructure in the nucleoplasm; group 4, nucleolar fragmentation; group 5, gross alteration of the nuclear membrane; group 6, accumulation of electron-dense material resembling nuclear pores in the nucleoplasm; group 7, weaker version of group 5.

§ *N*, nuclear; *C*, cytoplasmic.

‖ Parentheses indicate the duration of 37°C preincubation before RNA labeling.

¶ Six independent *mtr1* mutants were isolated. *mtr1-1* (ts17a), *mtr1-2* (ts21a), and *mtr1-3* (ts16α) were from suicide selection. Three others (H249, H291, and H569) were from Hartwell collection.

** The restrictive temperature for *mtr1-1* is 34°C. The other *mtr1* mutants do not grow at 30°C.

cedures used and the phenotype of the 21 mutants isolated to date.

Materials and Methods

Isolation of mtr Mutants

YPH1/2 (Sikorski and Hieter, 1989) were treated with 3% EMS for 1.5 h at room temperature. The survival fraction was 10–15%. After quenching the reaction with 5% sodium thiosulfate, the cells were grown 20 h at 23°C in SD medium. Cells were then incubated 3 h at 37°C at 10^7 cells/ml in SD medium, washed twice with water (37°C) and labeled 30 min with 1 mCi/ml [^{3}H]lysine in lysine-free SD medium at 1.5×10^7 cells/ml at 37°C. The cells were then washed 3× in ice-cold water and stored in 20% glycerol-YAPD medium at −80°C. Samples were thawed weekly and when survival was less than 1%, replica plates were prepared to screen for ts growth at 37°C. The ts mutants were screened by FISH to localize intracellular poly(A)$^+$ RNA. The Hartwell collection of MNNG-mutagenized ts strains (provided by S. Butler, University of Rochester, Rochester, NY) was directly screened by FISH after a 3-h incubation at 37°C, Systematic backcrossing with parental strains yielded a set of *ura3-52* strains which were used for further analysis. These were T188 (*mtr4*), T279 (*mtr5*), T273 (*mtr6*), T229 (*mtr7*), T301 (*mtr8*), T255 (*mtr10*), T330 (*mtr11*), T205 (*mtr12*), T212 (*mtr13*), T263 (*mtr14*), T292 (*mtr15*), T179 (*mtr16*), and T271 (*mtr17*). Tetraploid strains were generated by expression of pGAL-HO (Herskowitz and Jensen, 1991) followed by flow cytometric analysis of single colonies maintained on YEPD.

Fluorescent In Situ Hybridization (FISH)

Each *mtr* mutant was incubated in YEPD medium at 23 or 37°C for various periods (see text) then fixed by 4% paraformaldehyde. FISH was carried out as described (Kadowaki et al., 1992).

Protein Synthesis

Each *mtr* mutant was continuously labeled at 23 or 37°C with SC medium supplemented with 0.03 mg/ml methionine and 2 μCi/ml [^{35}S]methionine. TCA-insoluble radioactivity was monitored during incubation.

RNA Processing Analysis

All *mtr* mutants were transformed with pLGSD5 (Guarente et al., 1982) to make them URA$^+$. Each *mtr* mutant was incubated in SC-URA medium at 23 or 37°C for appropriate time (see Table II) and then labeled with 0.1 mCi/ml [^{3}H]uridine for 10 min. The labeled RNA was isolated then analyzed by 1% Agarose-formaldehyde gel (for rRNA) or 10% polyacrylamide–8 M urea gel (for small RNAs).

Northern Hybridization

10 μg of total RNA prepared for polyA tail analysis was subjected to Northern hybridization using *CRY1* as a probe.

Table II. (continued)

NOP1 distribution at semipermissive temperature§	PolyA tail length increase	Oversized Polymerase II transcripts	Inhibition of rRNA processing‖	Inhibition of tRNA processing
N	+	+	+ (0.5 h)	+
N	+	−	+ (0.5)	−
N	+	+	+ (1)	+
N	+	+	− (2)	−
N	+	−	+ (2)	−
N	+	−	+ (2)	−
N	+	−	+ (2)	−
N	+	−	+ (2)	−
N	+	−	+ (2)	−
N	−	−	+ (1)	−
N	+	−	+ (2)	−
N+C	+	−	− (1)	−
N	+	−	+ (2)	−
N+C	+	−	+ (2)	−
N+C	+	−	− (2)	−
N	+	+	+ (2)	+
N	+	−	+ (0.5)	−

Indirect Immunofluorescence and Electron Microscopy

These were carried out as previously described in (Kadowaki et al., 1994*a*).

Results

Isolation of mtr Mutants

We predicted that conditional mutants deficient in mRNA transport (*mtr* mutants) should accumulate poly(A)$^+$ RNA in the nucleus at the restrictive temperature and gradually reduce protein synthesis. We therefore developed an enrichment procedure for ts mutants which had low protein synthetic activity using a ^{3}H-amino acid suicide selection. Strains of interest were then recovered following analysis of the distribution of poly(A)$^+$ RNA at the restrictive temperature: YPH1/2 (lys$^-$) (Sikorski and Hieter, 1989) mutagenized with EMS was preincubated at 37°C for 3 h, labeled with [^{3}H]lysine for 30 min and frozen in glycerol for several weeks. When survival was reduced 100–1,000-fold, temperature-sensitive strains were recovered. These candidate mRNA export mutants were incubated at 37°C and analyzed by fluorescent in situ hybridization (FISH) using biotinylated oligo-dT as a probe (Table I). The fluorescent signal detected by this procedure is sensitive to RNase treatment and specific to oligo-dT (Kadowaki et al., 1992). In other experiments, toxic amino acid analogues were used instead of [^{3}H]lysine. These experiments resulted in the isolation of twelve temperature-sensitive (ts) mutants which accumulated nuclear poly(A)$^+$ RNA at 37°C (*mtr* mutants). The screening procedure described above is powerful, but has restrictions in terms of the mutants that can be recovered. For example, the accumulated RNA must be polyadenylated and reasonably stable. Moreover, it may be difficult to recover mutants that rapidly lose viability at the restrictive temperature. To overcome this latter problem, a bank of yeast ts mutants mutagenized with MNNG (Hartwell, 1967) was directly screened by FISH. A total of nine *mtr* mutants were isolated from 447 ts mutants. All 21 *mtr* mutants were recessive and do not lose cell viability at 37°C for several hours, as judged by replating assays.

Complementation and Segregation Analysis

The 21 *mtr* mutants (Table II) were crossed in all combinations to assign them to complementation groups. The result of this analysis indicates that the *mtr* mutants are recessive and define 16 complementation groups (*mtr1-mtr17*; *mtr9* was subsequently lost); there are six *mtr1* mutants and one mutant in each of the remaining complementation groups. To confirm that a single ts mutation is responsible for the mRNA export defect, each mutant was repeatedly backcrossed with wild-type strains. Although some of the original strains had more than one ts mutation, a single ts mutation always cosegregated with the poly(A)$^+$ RNA accumulation phenotype after backcross. In two cases, the backcrossed strains were also cs and did not grow at 16°C. Thus, at least 16 genes are directly or indirectly involved in mRNA export in yeast. One backcrossed strain of each complementation group was used for the phenotypic analysis described below.

Analysis of Previously Isolated ts Mutants by FISH

We analyzed several previously isolated ts mutants by FISH to check possible effects of their nuclear mutations on accumulation of poly(A)$^+$ RNA (Table III). Strains which did not give an obvious nuclear signal after incubation at 37°C for 1–4 h include several pre-mRNA splicing (*prp*)-defective mutants, rRNA processing defective mutants, a polyA-binding protein mutant, NOP1 (fibrillarin homologue) mutants, a NSP1 (nuclear pore complex protein) mutant, RAP1 mutants, two actin mutants, a protein synthesis-defective mutant, and several heat shock protein deletion strains. Remarkably, the heat shock transcription factor ts mutant, *mas3*, accumulated poly(A)$^+$ RNA after 30 min incubation at 37°C (Fig. 1).

Distribution of Poly(A)$^+$ RNA in mtr1-mtr17 Mutants

The *mtr1-mtr17* mutants were incubated at 37°C for 0.5–4.5 h and then poly(A)$^+$ RNA distribution was analyzed by FISH (Fig. 1, Table II). As previously shown, *mtr1* exhibits uniform accumulation of poly(A)$^+$ RNA in the nuclei after a 3-h incubation at 37°C (Kadowaki et al., 1992), but *mtr1* and *mtr2-1* show focal accumulation (i.e., several spots) after 1.5 and 0.5–3 h, respectively, as does *mtr10*. Several mutants exhibit mixture of crescent and entire nuclear staining pattern by FISH (*mtr3-1*, *mtr14*, and *mtr17*) (Fig. 1 *A*). *mtr8* shows quite uniform nuclear accumulation of poly(A)$^+$

Table III. Summary of the FISH Analysis of Previously Isolated Mutants

	Strains	Source	References
Negative Mutants			
Pre-mRNA splicing defective mutants	*prp2-1* (368), *prp5-1* (108), *prp8-1* (219), and *prp11-1* (382)	Yeast Genetic Stock Center	Hartwell et al., 1970
	prp6-1 (Y45) and *prp9-1* (Y49)	M. Rosbash (Brandeis University, Waltham, MA)	Hartwell et al., 1970
	prp17-1 (Y106), *prp21-1* (Y108), and *prp22-1* (Y109)	M. Rosbash	Vijayraghavan et al., 1989
rRNA processing defective mutants	*rrp1* (G1/7-5-13C)	A. Hopper (Pennsylvania State University, University Park, PA)	Fabian and Hopper, 1987
	rrp2 (KS7-1D)	J. Warner (Albert Einstein College of Medicine, Bronx, NY)	Shauai and Warner, 1991
PolyA binding protein ts mutant	*pab1-F364L* (YAS120)	A. Sachs (University of California, Berkeley, CA)	Sachs and Davies, 1989
NOP1 ts mutants	*nop1-2,3,4,5*, and *7*	D. Tollervey (EMBL)	Tollervey et al., 1993
NSP1 ts mutant	*nsp1* (ts10A)	E. Hurt (EMBL)	Nehrbass et al., 1990
Actin ts mutants	*act1-1, act1-3*	R. Sternglanz (State University of New York, Stony Brook, NY) D. Drubin (University of California, Berkeley, CA)	DiNardo et al., 1984
RAP1 ts mutants	*rap1-1* (YDS486), *rap1-2* (YDS487), *rap1-4 (YDS488)*, and *rap1-5* (YDS489)	D. Shore (Columbia University, NY)	Kurtz and Shore, 1991
	rap1 (DG175)	K. Tatchell (North Carolina State University, Raleigh, NC)	Giesman et al., 1991
Protein synthesis defective mutant	*prt1-1* (187)	Yeast Genetic Stock Center	Hartwell and McLaughlin, 1968
HSP null mutants	Δ*HSP82* (A201 PLD82)*	S. Lindquist (University of Chicago, Chicago, IL)	Borkovich et al., 1989
	Δ*HSC82* (A193 CLD82)*		
	Δ*HSP26* (A606 LP57-1)	S. Lindquist	Petko and Lindquist, 1986
Positive mutants			
Heat shock transcription factor ts mutant	*mas3* (MYY385)	M. Yaffe (University of California, San Diego, CA)	Smith and Yaffe, 1991

* These genes are necessary for the optimal growth at 37.5°C.

RNA. *mtr4* and *mas3* accumulate poly(A)$^+$ RNA RNA as single dot in the nucleus. *mtr5* and *mtrl2* accumulate poly(A)$^+$ RNA as a peripheral circle and spots. *mtr6*, *mtr7*, *mtrl5*, and *mtrl6* show a mixture of entire nuclear and focal staining patterns. *mtrl1* and *mtrl3* exhibit a granular entire nuclear staining pattern. These distinct patterns are best distinguished in tetraploids, several of which are illustrated (Fig. 1 *B*). None of the *mtr* mutants showed a bright nucleus at 23°C.

Thiolutin Test

The bright nuclear signal and dark cytoplasm observed by FISH could be a result of rapid turnover of cytoplasmic poly(A)$^+$ RNA rather than an mRNA export defect. To test this possibility, we employed a thiolutin test using the nonspecific inhibitor of RNA polymerases, thiolutin (Tipper, 1973): Each mutant was incubated at 37°C to allow nuclear poly(A)$^+$ RNA accumulation. Thiolutin was then added for 2–4 h at the same temperature. Mutants defective only in the maintenance of cytoplasmic mRNA will lose their nuclear FISH signal during the reincubation while the nuclear signal will persist for mutants which have an mRNA transport defect if the nuclear pool of mRNA is sufficiently stable. Judging from the results of this test, all mutants are

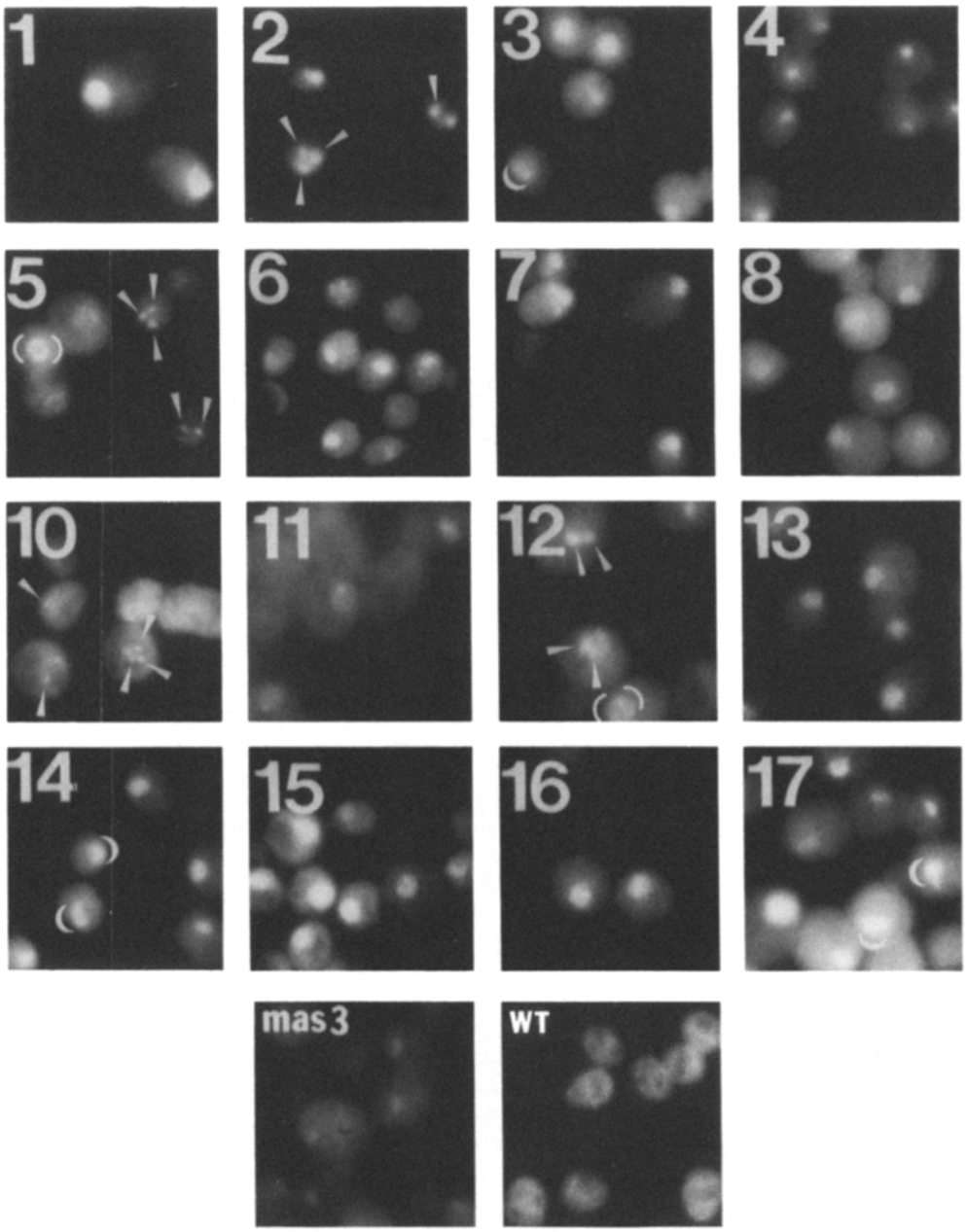
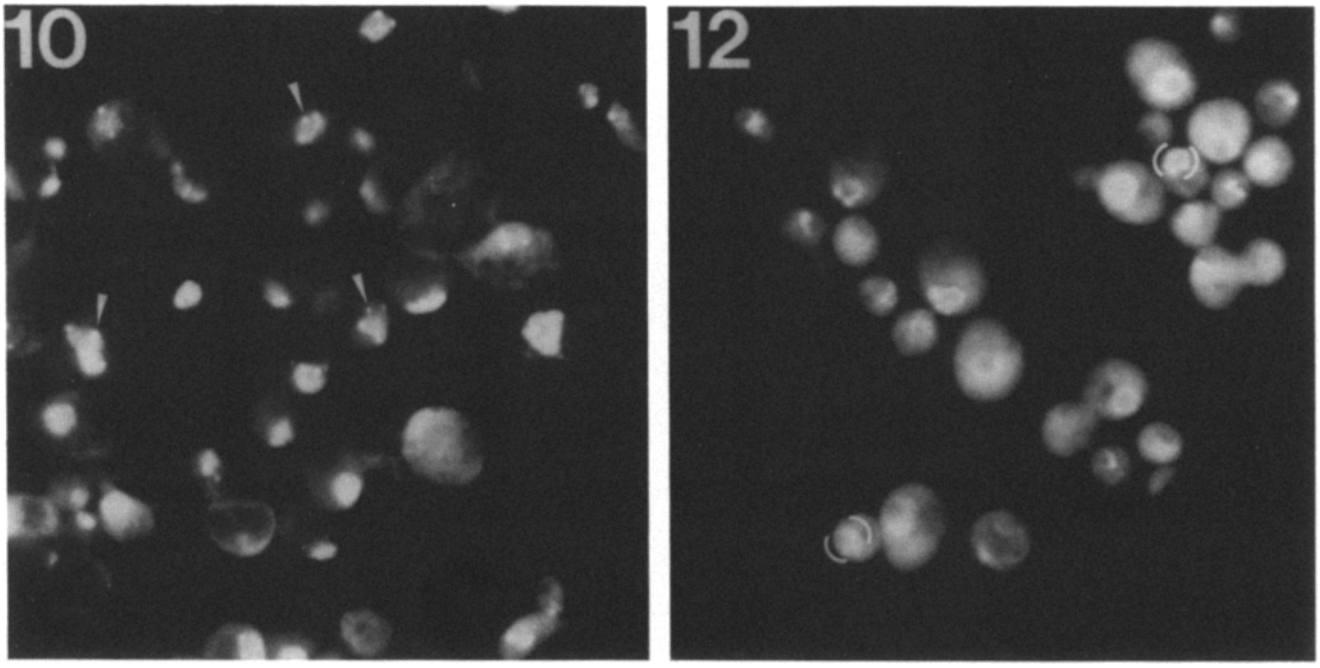


Figure 1. FISH analysis of *mtr* mutants. (*A*) Each *mtr* mutant was incubated at 37°C for the appropriate time (see Table II) and then poly(A)$^+$ RNA distribution was analyzed by FISH using biotinylated oligo-dT as a probe. Wild type and *mas3* are also shown. None of mutants showed nuclear poly(A)$^+$ RNA accumulation at 23°C. The position of the nucleus was confirmed by DAPI counter staining. Irregular staining patterns (*spots*, *arcs*, and *double arcs*, to designate circular patterns) are indicated. (*B*) *mtr10* and *mtr12* tetraploids were analyzed as in *A*. Note the distinctive accumulation pattern of poly(A)$^+$ RNA in the nucleus (spots for *mtr10*, many circles for *mtr12*).



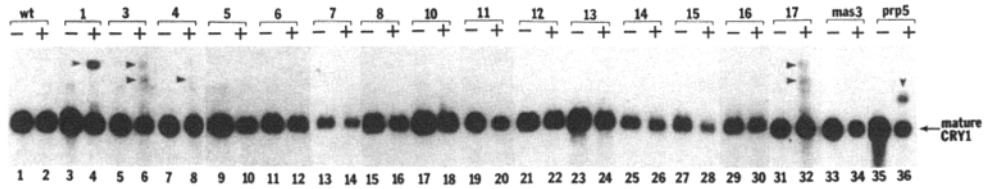


Figure 2. Northern hybridization analysis of *CRY1* mRNA in *mtr* mutants. 10 μg of total RNA isolated from cells treated as described in Fig. 2 was analyzed by Northern hybridization using *CRY1* as a probe. The position of mature *CRY1* mRNA is shown by the arrow. Pre-mRNA detected in *prp5* is indicated by the downward-pointing arrowhead. Oversized transcripts detected in *mtr1-1*, *mtr3-1*, *mtr4*, *mtr17* are shown by the arrowheads pointing to the right. No aberrant transcript was detected in *mtr2-1*.

defective in mRNA export rather than cytoplasmic mRNA stability (not shown).

PolyA Tail Length Analysis

RNA recovered from previously described mRNA export defective mutants has atypically long polyA tails at the restrictive temperature, consistent with the idea that trimming of mRNA polyA tail occurs in the cytoplasm (Forrester et al., 1992). We therefore analyzed polyA tail length in all *mtr* mutants at 23 and 37°C. Most *mtr* mutants exhibit elongation of polyA tails at 37°C. Certain strains already have somewhat longer than wild-type tails at the permissive temperature (*mtr4*, *mtr8*, *mtr10*, *mtr12*, and *mtr16*). The small increase of polyA tail length observed in *mtr11* corresponds to the fact that a relatively small number of the cells (~20%) shows nuclear accumulation of poly(A)$^+$ RNA in these mutants. Thus, the accumulation of nuclear poly(A)$^+$ RNA correlates well with the increase of polyA tail length (not shown).

Northern Blot Analysis of CRY1 mRNA

To analyze pre-mRNA splicing and mRNA processing (transcription initiation and 3′ end formation) in *mtr* mutants, equal amounts of total RNA isolated from cells incubated at 23 and 37°C were subjected to Northern blot analysis using *CRY1* (Larkin et al., 1987) as a probe, as shown in Fig. 2. The level of mature *CRY1* mRNA is decreased in most of the mutants. *mtr1*, *mtr3*, *mtr4*, and *mtr17* show the synthesis of oversized transcripts at 37°C. Since the sizes of these aberrant transcripts are different from those detected in *prp5*, these are not *CRY1* pre-mRNA. Two oversized transcripts detected in *mtr17* appear to be identical to those detected in *mtr3-1*. Thus, none of the *mtr* mutants accumulate pre-mRNA at the restrictive temperature, as do *prp* mutants. No aberrant *CRY1* transcripts were detected in *mtr2-1* (T. Kadowaki, unpublished observations). Judging from previous observations (Aebi et al., 1990; Forrester et al., 1992; Kadowaki et al., 1994*b*) and the present data, *mtr1/prp20/srm1*, *mtr3-1*, *mtr4*, and *mtr17* have defects in mRNA transcriptional precision (transcription initiation and 3′ end formation).

Protein Synthesis Analysis

As a result of poly(A)$^+$ RNA accumulation, we expected that protein synthesis would diminish in *mtr* mutants at 37°C. We thus monitored protein synthesis in each mutant by measuring [^{35}S]methionine incorporation into TCA insoluble material at 23 and 37°C. Protein synthesis is gradually reduced during 37°C incubation, by contrast to wild type (not shown). The rate of its reduction varied, *mtr4*, *mtr13*, and *mtr17* are the slowest to stop protein synthesis and continue to incorporate [^{35}S]methionine for 10 h, suggesting considerable leakiness of the mRNA transport defect in these three mutants.

Analysis of rRNA Processing

To analyze rRNA processing, *mtr* mutants were preincubated at either 23 or 37°C for 1–2 h and then pulse labeled with [^{3}H]uridine for 10 min at the same temperature. The [^{3}H]uridine labeling efficiency of RNA in most mutants is reduced at 37°C compared to 23°C, suggesting that either the synthesis of RNA, [^{3}H]uridine uptake or [^{3}H]UTP synthesis is decreased at 37°C. To normalize this effect, the labeled RNA samples analyzed on agarose gels each contained equal amounts of radioactivity. As shown in Fig. 3, rRNA processing appears to be little affected in *mtr4* and *mtr16*.

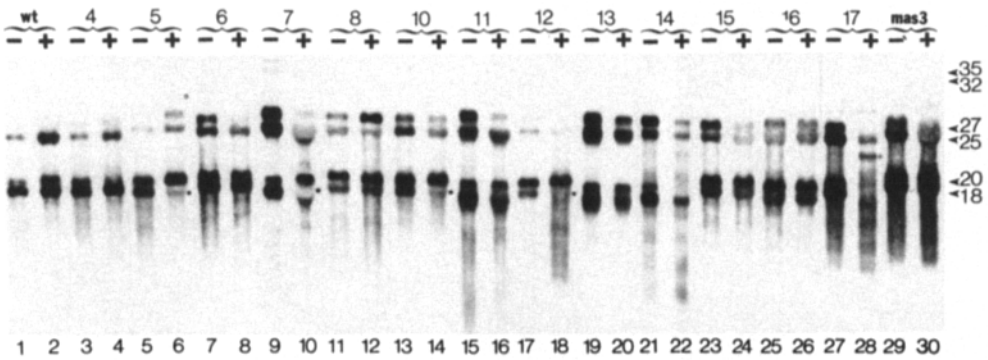


Figure 3. rRNA processing in *mtr* mutants. The *mtr* mutants were pre-incubated at 37°C (see Table II) and labeled for 10 min with [^{3}H]uridine at 23 (−) and 37°C (+). Each mutant is shown by the number at the top. The positions of 35, 32, 27, 25, 20, and 18S rRNA are shown by arrowheads. 18S rRNA decreased in *mtr5*, *mtr10*, and *mtr12* at 37°C (*asterisk*). The 29 and 23S RNA increased in *mtr5* and *mtr17* respectively are indicated by the circles.

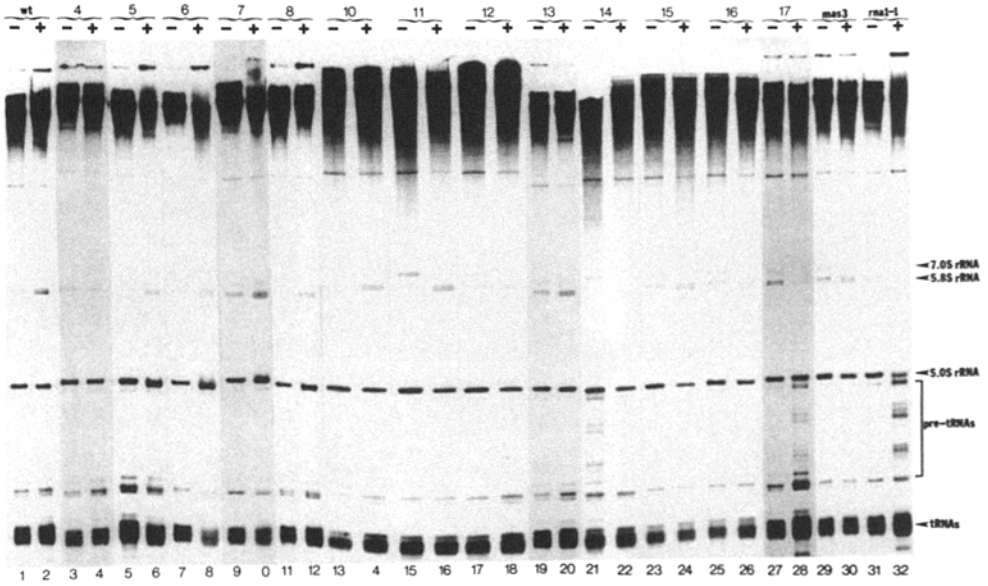


Figure 4. Small RNA processing in *mtr* mutants. The labeled RNA prepared in Fig. 3 was analyzed on 10% acrylamide–8 M urea gel. *rnal-1* is included for the identification of pre-tRNAs. The positions of 5.8, 5.0, and 7S rRNA, pre-tRNAs, and tRNAs are shown by the arrowheads and the bracket. Only *mtrl7* and *rnal-1* show significant increase of pre-tRNAs at 37°C. The level of 5.8S rRNA is almost undetectable in *mtrl4* at both temperatures. The level of pre-tRNAs is also high in *mtrl4* at 23°C.

The labeling of 18S rRNA is selectively reduced in *mtrl0* and *mtrl2*. Some increase of 35 and 29S RNA and reduction of 18S rRNA is observed in *mtr5*. An increase of 35 and 29S RNA and reduction of the rest of rRNA species is detected in *mtr3-1* (Kadowaki et al., 1994*b*). In *mtrl7* there is a major reduction in the level of 27, 20, and 18S rRNA. Moreover, a 23S RNA becomes conspicuous. The labeling of 27 and 25S rRNA appears to be reduced in *mtr6* and *mtrl5*. The labeling of all except 20S rRNA is reduced in *mtr7*. The processing of 27S to 25S rRNA seems to be slow in *mtr8* judging from the increase of the ratio of 27S to 25S rRNA. An increase of 35S and a decrease of the rest of rRNAs are detected in *mtrl1*, *mas3*, *mtrl-1* (Kadowaki et al., 1993), and *mtr2-1*. All rRNA species are reduced in *mtrl3* and *mtrl4*. Taken together, these observations show that although a mRNA export defect need not block rRNA processing, many backcrossed *mtr* mutants have defects at selected steps in rRNA processing.

Analysis of Small RNA Processing

The labeled RNA prepared above was also analyzed on 10% polyacrylamide–8 M urea gels to monitor the processing of small RNAs. For comparison, the *rna-1* strain was also studied. This mutant is also defective in mRNA export (Hopper et al., 1990). As shown in Fig.4, reduced labeling of the 5.8S doublet rRNA is found in *mtrl7* and *rnal-1*, at 37°C. We have made similar observations for *mtrl-1* (Kadowaki et al., 1993), *mtr2-1*, and *mtr3-1*. 5.8S rRNA is almost undetectable in *mtrl4* at both temperatures. The labeling of 5.0S rRNA is relatively stable in all mutants at 37°C. In general the relative labeling of 7S is greater at 37 than at 23°C, presumably because of the temperature dependence of 7S-to-5.8S processing. The accumulation of pre-tRNA is observed in *mtrl7*, *rnal-1*, *mtrl-1* (Kadowaki et al., 1993), and *mtr3-1* at 37°C. In *mtrl4* an inverse relation is seen. Taken along with previous data, these results indicate that only *rnal-1*, *mtrl/prp20/srml*, *mtr3-1*, and *mtrl7* have pre-tRNA splicing defects in addition to their mRNA export and rRNA processing defects at the restrictive temperature.

Analysis of NOP1 Distribution

To detect possible defects of nuclear protein import or retention in *mtr* mutants, *mtr* mutants were incubated at semipermissive temperatures (30–34°C) for 12 h and then the distribution of a nucleolar protein, NOP1 (Aris and Blobel, 1988), was analyzed by indirect immunofluorescence. Only *mtrl3*, *mtrl5*, and *mtrl6* exhibit an increase of cytoplasmic NOP1 levels in a fraction of the cells as shown in Fig. 5. Similar results were observed with histone H2B, indicating that this phenotype is not protein specific (not shown). Since the accumulation of NOP1 in the cytoplasm was not observed in these mutants when incubated at 37°C (~6 h), we conclude that newly synthesized nuclear proteins accumulate in the cytoplasm, possibly because their import is blocked.

The distribution of NOP1 in *mtr* mutants was also analyzed at 37°C. In addition to *mtrl-1* (Oakes et al., 1993; Kadowaki et al., 1994*a*), *mtr2-1*, and *rnal-1*, *mtr8*, *mtrl4*, *mtrl5*, and *mas3* exhibit nucleolar fragmentation (not shown). An enlarged somewhat diffuse staining pattern is observed in *mtrl7*, as in *mtr3-1*. Similar alterations of the distribution of nucleolar antigens were detected with the monoclonal antibody YN9C5 (gift of J. Broach, Princeton University, Princeton, NJ).

Ultrastructural Changes

All *mtr* mutants were analyzed by transmission electron microscopy before and after incubation at 37°C. The characteristic features described below after incubation at 37°C were not seen in 23°C controls. *mtrl1* does not show any significant changes at 37°C (not shown). The rest of the mutants are classified into seven different groups. Group 1 (*mtrl*, *mtr4*, *mtr5*, *mtr6*, *mtrl2*, and *mtrl4*) exhibits abundant osmiophilic material in much of the nucleoplasm. (*mtrl2* is illustrated in Fig. 6 *B*). Group 2 (*mtr8*, *mtrl3*, *mtrl5*, and *mas3*) shows fine "barley-like" material in the nucleoplasm (*mtrl5* is illustrated in Fig. 6 *C*). Group 3 (*mtr3-1* and *mtrl7*) shows small tortuous substructure in the nucleoplasm, possibly derived from the nucleolus (*mtrl7*, Fig. 6 *D*; *mtr3-1*. Group 4

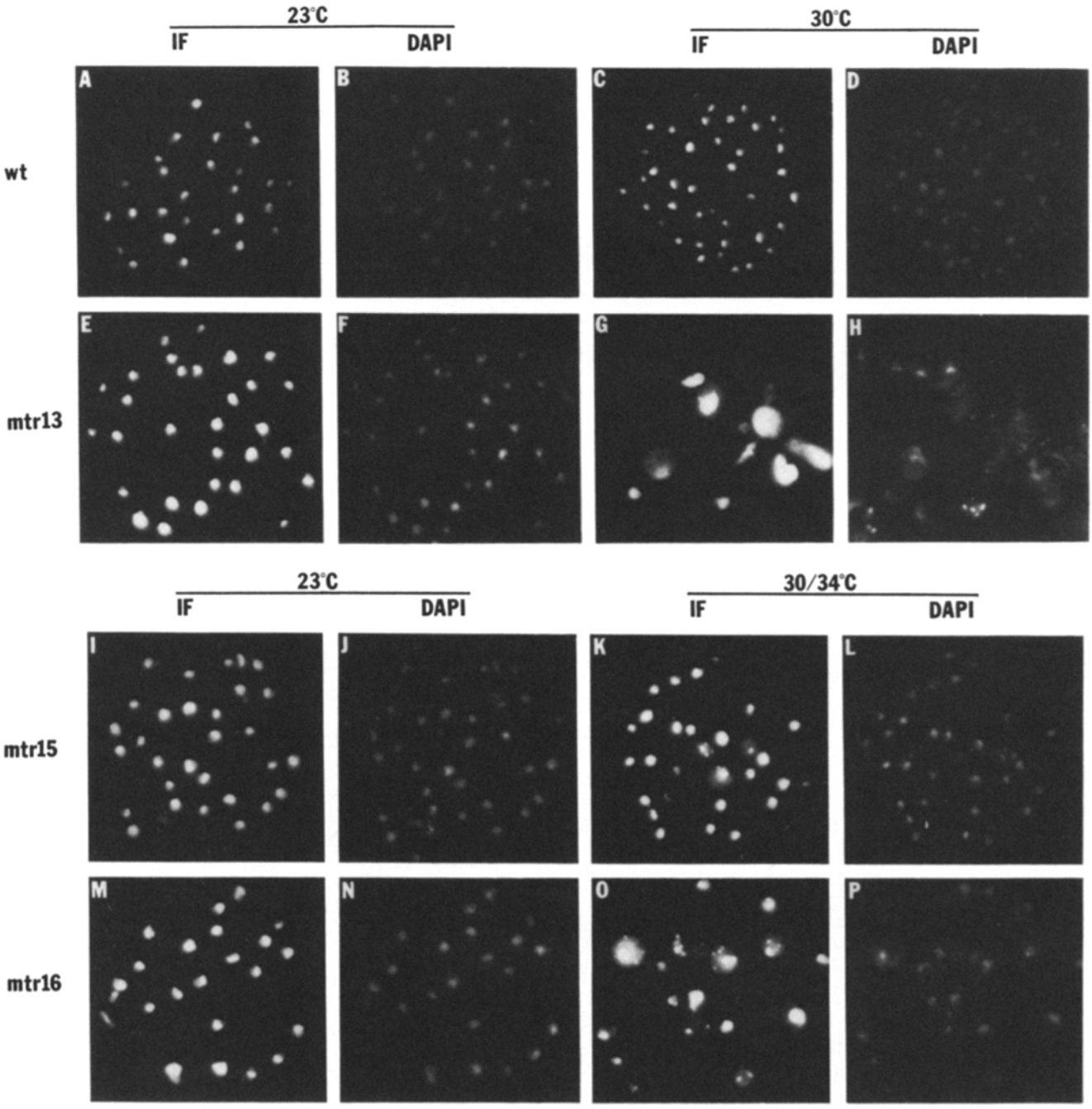


Figure 5. NOP1 distribution in *mtr13*, *mtr15*, and *mtr16* at semipermissive temperature. Wildtype (*A*, *B*, *C*, and *D*), *mtr13* (*E*, *F*, *G*, and *H*), *mtr15* (*I*, *J*, *K*, and *L*), and *mtr16* (*M*, *N*, *O*, and *P*) were incubated at 23°C (*A*, *B*, *E*, *F*, *I*, *J*, *M*, and *N*), 30°C (*C*, *D*, *G*, *H*, *K*, and *L*), or 34°C (*O* and *P*) for 12 h. NOP1 distribution was then analyzed by IF using anti-NOP1 monoclonal antibody, A66. The NOP1 staining is restricted in the nuclei of wt (at both 23 and 30/34°C, *mtr13*, *mtr15*, and *mtr16* (at 23°C) but is detected in the cytoplasm of *mtr13*, *mtr15*, and *mtr16* at 30/34°C. In addition, DAPI staining becomes ambiguous in cells which accumulate cytoplasmic NOP1.

(*mtr2-1*) shows nucleolar fragmentation. Group 5 (*mtr7*) exhibits gross dilation of the nuclear envelope, whose outer membrane appears to separate from some pore structures. There also is a characteristic elaboration of the ER (*mtr7*, Fig. 6 *E*). Group 6 (*mtr10*) accumulates electron-dense material resembling nuclear pores in the nucleoplasm and shows clustering of pores (Fig. 6 *F*). Group 7 (*mtr16*) looks like a weaker version of the Group 5 phenotype (Fig. 6 *G*).

Discussion

Isolation of mtr Mutants

Suicide selections using ^{3}H-labeled compounds and toxic analogs have been widely used to isolate animal cell and yeast mutants (Pouysségur and Franchi, 1987; Huffaker and Robbins, 1982). We combined such a strategy with FISH for

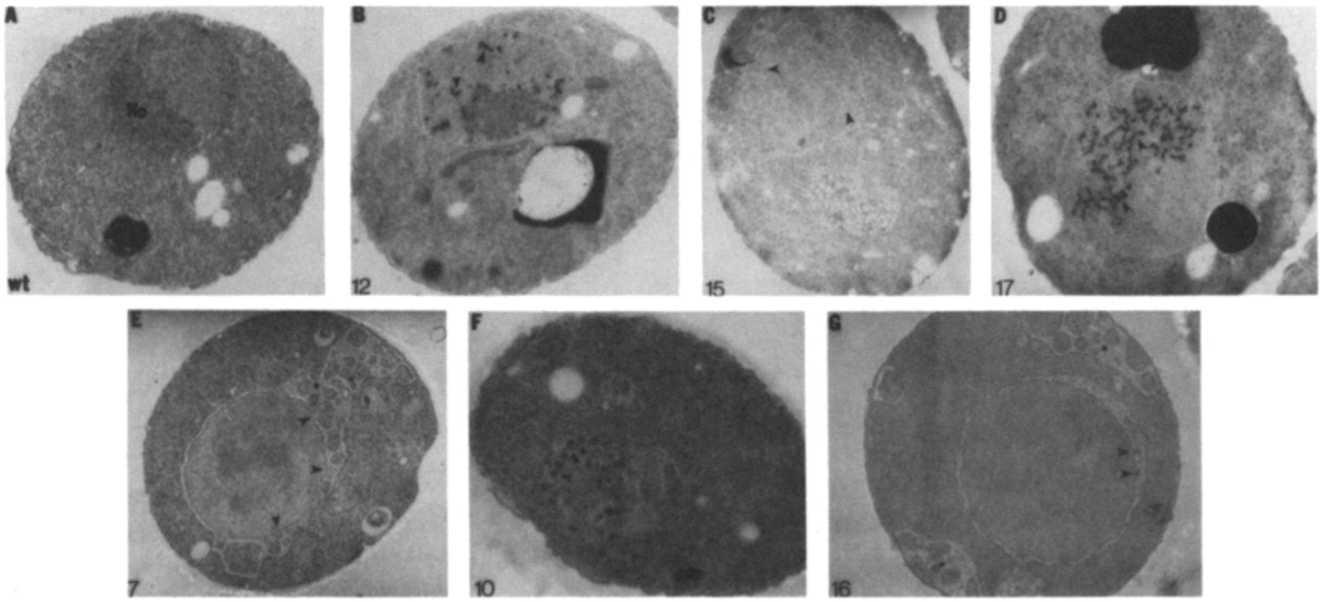


Figure 6. Ultrastructure of *mtr* mutants. The ultrastructure of *mtr* mutants incubated at 37°C was analyzed by electron microscopy. Wildtype (*A*) and one representative, *mtr12*, *mtr15*, *mtr17*, *mtr7*, *mtr10*, and *mtr16*, from group 1 ~ 7 (*B–G*) are shown. The uniform electron dense nucleolus and intact nuclear membrane are noted in wt. See text for description of the mutants. *mtr2-1* (*group 4*) shows fragmentation of the nucleolus. Arrowheads show the distinctive features observed in individual groups. Stars denote the putative expanded ER structure.

detection of poly(A)$^+$ RNA and have isolated 12 different *mtr* mutants. Furthermore, screening of a bank of yeast ts mutants yielded nine further *mtr* mutants. The value of the suicide procedure for enrichment of *mtr* mutants is not as good as expected. For example, nine *mtr* mutants were recovered from 149 ts mutants obtained by [^{3}H]lysine suicide selection (Table I) while nine *mtr* mutants were isolated from 447 random ts mutants. The difference in the enrichment factor in this case is only three, possibly because the 3 h preincubation at 37°C before selection is too short to stop protein synthesis by *mtr* mutants. Complementation analysis of a total of 21 *mtr* mutants indicated they are recessive and fall into 16 different complementation groups, *mtr1-mtr17*. Six mutants are in the first complementation group, including at least three different alleles (*mtr1-1*~*mtr1-3*) (Kadowaki et al., 1993). There is only one allele for the rest of the complementation groups, suggesting that the mutagenesis is not saturated. The recovery of a large number of alleles of *MTR1/PRP20/SRM1* may indicate that this locus is a hot spot for mutation or possibly that each domain of the seven approximate repeats in *MTR1/PRP20/SRM1* is essential for its function (Kadowaki et al., 1993).

The frequency of *mtr* mutants among all ts mutants generated by nitroguanidine mutagenesis (Hartwell, 1967) is about 2% (9/447), by comparison to the frequency of *prp* mutants (~5%) (Hartwell et al., 1970). The recent screening of EMS generated *prp* mutants based on the accumulation of pre-mRNA, lariat splicing intermediates and introns shows that *prp* mutants represent approximately 3.6% of all ts mutants (Vijayraghavan et al., 1989). Thus, the total number of *MTR* genes may be of the same order of magnitude as that of *PRP* genes. Those which have been identified to date are good candidates for being central to the mechanism of transport. *PRP* genes encode a succession of spliceosomal components, nonspliceosomal factors and proteins which indirectly affect the accumulation of pre-mRNA, lariat intermediates and introns (Vijayraghavan et al., 1989). We suspect that the *MTR* genes code for several subfamilies of components and complexes affecting multi-step reactions of mRNA transport.

To prove that *mtr* mutants have lesions in mRNA transport but not in cytoplasmic mRNA stability, a thiolutin test and polyA tail length analysis were performed. Judging from these experiments, all *mtr* mutants are likely to have defects in mRNA transport per se. Our previous observations on *mtr1* show that thiolutin itself does not block mRNA export (Kadowaki et al., 1992).

The analysis of *mtr* mutants by FISH reveals differences of the pattern of poly(A)$^+$ RNA accumulation in nuclei. Some mutants show a whole nuclear or crescent-like pattern while others exhibit foci, dot, or circular patterns. These observations suggest that mRNA is trapped at different steps along a transport pathway. Nevertheless, if *mtr* mutations induce nuclear disorganization, this disorganization could itself result in the varied patterns of accumulation of poly(A)$^+$ RNA. It will be interesting to analyze epistatic relations among *mtr* mutants that exhibit different accumulation patterns of nuclear poly(A)$^+$ RNA.

Processing of mRNA

Northern blot analysis of *CRY1* mRNA indicates that no *mtr* mutants grossly alter pre-mRNA splicing, as do *prp* mutants. Thus, there clearly is no coupling between mRNA splicing and export of the average poly(A)$^+$ RNA. Comparable observations were previously reported for *mtr2-1* and *mtr3-1*. It will be interesting to learn whether spliced transcripts are exported and whether other genes can be

identified which block both the export of the average poly(A)$^+$ RNA and splicing.

Interestingly, a group of mutants (*mtr1*, *mtr3-1*, *mtr4*, and *mtr17*) similar to those which interrupt tRNA processing (*mtr1*, *mtr3-1*, and *mtr17*) is characterized by the presence of over-sized *CRY1* transcripts at 37°C (Fig. 2). Some of the over-sized transcripts in *mtr17* are of the same size as those in *mtr3-1* and the smallest one is shared by *mtr4*. The larger over-sized transcript in *mtr3-1* and *mtr17* is of the same size as the one detected in *mtr1-1*. Since *mtr1/prp20/srm1* and *mtr3-1* affect transcription initiation and 3′ end formation (Forrester et al., 1992), the aberrant transcripts in *mtr4* and *mtr17* are likely to be produced in a similar manner. Those mutants which affect the length of transcripts may correspond to gene products which act early along the transport path.

Processing of rRNA and tRNA

The loss of cytoplasmic mRNA which slows protein synthesis in *mtr* mutants at 37°C correlates with the low RNA-labeling efficiency, probably due to inhibition of rRNA synthesis and to turnover of incompletely processed rRNA. This is very similar to observations made on the RNA polymerase II ts mutant, *rpb1-1* (Nonet et al., 1987), *prp* mutants, and wild-type cells treated with cycloheximide (Warner and Udem, 1972). Since several rRNA processing defective mutants do not exhibit a mRNA export defect (see above) and since some *mtr* mutants allow normal rRNA processing, rRNA processing and mRNA export are clearly separate processes. Nevertheless, the frequent observation of defects in rRNA processing among *mtr* mutants suggests a possible involvement of nucleolar components in mRNA export.

We particularly paid attention to the synthesis of 18S rRNA because the processing of 20 to 18S rRNA occurs in the cytoplasm (Udem and Warner, 1973). In fact, *mtr5*, *mtr10*, and *mtr12* appear to be defective in this step, suggesting that the export of 20S rRNA (as a 43S ribosomal subunit) is inhibited. These two export systems (mRNA, rRNA) thus may share common components represented by these three gene products.

The defects in *mtr17* are especially complex, including the production of an atypical 23S RNA, seemingly similar to that which has been characterized in other strains (Morrissey and Tollervey, 1993). The results of the analysis of tRNA processing show that only a limited subset of the rRNA processing defective strains (*mtr1*, *mtr3-1*, *mtr17*) is defective in pre-tRNA maturation at 37°C (Fig. 7).

Distribution of Nuclear Proteins

mtr13, *mtr15*, and *mtr16* accumulate nuclear proteins (NOP1 and histone H2B) in the cytoplasm when they are incubated at a semipermissive temperature (30–34°C). Since this phenotype was not observed at the fully restrictive temperature (37°C), it is likely that cytoplasmic accumulation of nuclear proteins requires ongoing protein synthesis. This phenotype can be explained by either: (*a*) a defect in retention of nuclear proteins or (*b*) a defect of import of nuclear proteins. We are not able to distinguish these possibilities at this point; however, under conditions of strong poly(A)$^+$ RNA accumulation (at 37°C) nuclear proteins remain in the nucleus in these mutants.

The analysis of the distribution of NOP1 and nucleolar antigens recognized by YN9C5 in *mtr* mutants at 37°C indicates that *mtr1*, *mtr2-1*, *mtr3-1*, *mtr8*, *mtr14*, *mtr15*, *mtr17*, and *mas3* induce clear nucleolar disorganization at the light microscopic level. This phenotype could be a secondary consequence of nuclear poly(A)$^+$ RNA accumulation as observed in *mtr1-1* and *mtr2-1* or these gene products may have a more direct role in the nucleolar organization, as suggested for MTR3. Several strains which do not show nucleolar fragmentation do exhibit reduced labeling of rRNA. We therefore do not believe that fragmentation is primarily a reflection of inhibition of RNA polymerase I.

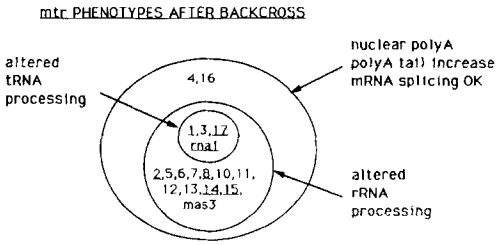


Figure 7. Venn diagram summarizing RNA processing in the *mtr* mutants. The underlined strains show fragmentation of the nucleolus.

Ultrastructural Analysis by EM

The analysis of most *mtr* mutants by EM reveals dramatic alterations of nuclear structures at 37°C. Several exhibit accumulation of a network of conspicuous electron-dense material in the nucleoplasm. Although we have not identified this material, it may derive from the nucleolus and/or include the poly(A)$^+$ RNA itself. Other mutants show structural changes in the nuclear pores reminiscent of but distinct from those reported when individual nuclear pore proteins are deleted (Wente and Blobel, 1993). The detailed analysis of these mutants should give insight into the roles of these components in the latest steps of mRNA export.

Involvement of Heat Shock Protein(s) in mRNA Export

Judging from the nuclear accumulation of poly(A)$^+$ RNA in *mas3* (at 37°C) which has a ts mutation in HSTF, one or more heat shock proteins (HSP) is important for mRNA export. We do not know if these HSPs are also required for mRNA export at 23°C. It will therefore be fruitful to see whether depletion of HSTF induces a mRNA export defect at 23°C. Hsp70/hsc70 are implicated in nuclear protein import (Imamoto et al., 1992; Shi and Thomas, 1992). Thus, HSPs are likely to be involved in macromolecular transport across the nuclear envelope in both directions. By analogy with their other roles they may be important for the normalization or unfolding of the export substrate: mRNA associated with proteins.

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