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 splitting 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 from the nucleus to cytoplasm, the latter 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; Bataille et al., 1990; Darge-mont and Kuhn, 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 (mtr1) 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 [3H]-Amino Acids

|        | YPH1   | YPH2   |
|--------|--------|--------|
| Number of cells subjected to selection | $1.0 \times 10^8$ | $1.2 \times 10^8$ |
| 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 cysteine and L-azetidine-2-carboxylate).

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.

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Materials and Methods

Isolation of mtr Mutants

YPHI/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. Cells were then washed 3× in ice-cold water and stored in 20% glycerol-YPAD 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 intracellularly the poly(A)^+ RNA. The Hartwell collection of MNNG-mutagenized ts mutants do not grow at 30°C.

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 [35S]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 [3H]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 poly(A) tail analysis was subjected to Northern hybridization using Cry1 as a probe.

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

| MTR group | Source | Strains used in experiment | ts/ea at °C | FISH phenotypes* | EM changes† (group #) | Nucleolar changes |
|-----------|--------|-----------------------------|-------------|-----------------|----------------------|------------------|
| 1*        |        |                             | 30-34**     | Entire (3 h, 100%) | 1                    | Fragmentation    |
| 2 (ts12a) | Suicide selection + Hartwell collection | See Kadowaki et al., 1993 | 34           | Spots (0.5, 100%)   | 4                    | Fragmentation    |
| 3 (ts20a) | Suicide selection | See Kadowaki et al., 1994a | 37           | Cresc/snt/entire   | 3                    | Enlargement      |
| 4 (ts7a)  | "      | T188                        | 37           | Spot (3, 90%)     | 1                    | -                |
| 5 (ts37a) | "      | T279                        | 37           | Circle/spots (3, 30%) | 1                    | -                |
| 6 (ts54a) | "      | T273                        | 30, 16       | Entire/spots (3, 30%) | 1                    | -                |
| 7 (ts7a)  | "      | T229                        | 34           | Entire/spots (2, 20%) | 5                    | -                |
| 8 (ts10a) | "      | T301                        | 34           | Entire (3, 100%)  | 2                    | Fragmentation    |
| 10 (ts54e) | "     | T255                        | 34, 16       | Spots (3, 50%)    | 6                    | -                |
| 11 (ts60e) | "     | T330                        | 34           | Granular/entire   | None                 | -                |
| 12 (H157) | Hartwell collection | T205          | 34           | Circle/spots (3, 0, 80%) | 1                    | -                |
| 13 (H194) | "      | T212                        | 34           | Granular/entire   | 2                    | -                |
| 14 (H209) | "      | T263                        | 34           | Cresc/snt/entire  | 1                    | Fragmentation    |
| 15 (H397) | "      | T292                        | 34           | Entire/spots     | 2                    | Fragmentation    |
| 16 (H518) | "      | T179                        | 37           | Entire/spots (4.5, 70%) | 7                    | -                |
| 17 (H577) | "      | T271                        | 37           | Cresc/snt/entire  | 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 mtr-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, long structure 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.
¶ Six independent mtr-1 mutants were isolated. mtr-1-1 (ts17a), mtr-1-2 (ts21a), and mtr-1-3 (ts16a) were from suicide selection. Three others (H249, H291, and H569) were from Hartwell collection.
** The restrictive temperature for mtr-1 is 34°C. The other mtr-1 mutants do not grow at 30°C.
Table II. (continued)

| NOPI distribution at semipermissive temperature | Polya- tail length increase | Oversized Polymerase II transcripts | Inhibition of rRNA processing | Inhibition of rRNA processing |
|------------------------------------------------|-----------------------------|------------------------------------|-----------------------------|-----------------------------|
| N                                              | +                           | +                                  | (0.5 h)                     | +                           |
| N                                              | +                           | -                                  | (1)                         | +                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (1)                         | -                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (1)                         | -                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (2)                         | -                           |
| N                                              | +                           | -                                  | (0.5)                       | -                           |

Indirect Immunofluorescence and Electron Microscopy

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

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 3H-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 [3H]lysine for 30 min and frozen in glycerol for several weeks. When survival was reduced 100-1000-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 [3H]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, NOPI (fibrillarin homologue) mutants, a NSPI (nuclear pore complex protein) mutant, RAPI 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-l 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-l, mtr4-l, 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                          | Vijayaraghavan et al., 1989 |
| rRNA processing defective mutants | rpl1 (G1/7-5-13C)                           | A. Hopper (Pennsylvania State University, University Park, PA) | Fabian and Hopper, 1987 |
|                                  | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
| rRNA processing defective mutants | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
|                                  | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
|                                  | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
|                                  | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
|                                  | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
|                                  | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
|                                  | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
|                                  | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
|                                  | rpl2 (KS7-1D)                              | J. Warner (Albert Einstein College of Medicine, Bronx, NY) | Shauai and Warner, 1991 |
| PolyA binding protein ts mutant  | pab1-F346L (YAS120)                        | A. Sachs (University of California, Berkeley, CA) | Sachs and Davies, 1989 |
| NOP1 ts mutants                  | npl-1-2,3,4,5, and 7                       | D. Tollervey (EMBL)                | Tollervey et al., 1993, 1999 |
| NSP1 ts mutant                   | nspl (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) | Dinardo et al., 1984 |
| RAP1 ts mutants                  | rap1-1 (YDS486), rap1-2 (YDS487), rap1-4 (YDS488), and rap1-5 (YDS489) | D. Drubin (University of California, Berkeley, CA) | Kurtz and Shore, 1991 |
|                                  | rap1 (DG175)                               | K. Tatchell (North Carolina State University, Raleigh, NC) | Giesman et al., 1991 |
| Protein synthesis defective mutant | prtl-1 (187)                              | Yeast Genetic Stock Center         | Hartwell and McLaughlin, 1968 |
| HSP null mutants                 | ΔHSP82 (A201 PLD82)*                       | S. Lindquist (University of Chicago, Chicago, IL) | Borkovich et al., 1989 |
|                                  | ΔHSP82 (A193 CLD82)*                       | S. Lindquist                       | Petko and Lindquist, 1986 |
|                                  | ΔHSP26 (A506 LP57-1)                       |                                  |                      |
| Positive mutants                 | 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 mtr12 accumulate poly(A)+ RNA as a peripheral circle and spots. mtr6, mtr7, mtr15, and mtr16 show a mixture of entire nuclear and focal staining patterns. mtr11 and mtr13 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).
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 prpS, 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., 1994b) and the present data, mtr1/prp20/srn1, 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 [35S]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 [35S]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 [3H]uridine for 10 min at the same temperature. The [3H]uridine labeling efficiency of RNA in most mutants is reduced at 37°C compared to 23°C, suggesting that either the synthesis of RNA, [3H]uridine uptake or [3H]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.
The labeling of 18S rRNA is selectively reduced in mtr10 and mtr12. 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., 1994b). In mtr17 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 mtr15. The labeling of all except 20S rRNA is reduced in mtr7. The processing of 27S to 25S rRNA seems to be slow in mtrS 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 mtr11, mas3, mtrl-1 (Kadowaki et al., 1993), and mtr2-1. All rRNA species are reduced in mtr13 and mtr14. 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 NOP1 Distribution**

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 mtr17 and rnal-1, at 37°C. We have made similar observations for mtr1-1 (Kadowaki et al., 1993), mtr2-1, and mtr3-1. 5.8S rRNA is almost undetectable in mtr14 at both temperatures. The labeling of 5.0S rRNA is almost undetectable in mtr14 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 mtr17, rnal-1, mtr1-1 (Kadowaki et al., 1993), and mtr3-1 at 37°C. In mtr14 an inverse relation is seen. Taken along with previous data, these results indicate that only rnal-1, mtr1/prp20/ srml, mtr3-1, and mtr17 have pre-tRNA splicing defects in addition to their mRNA export and rRNA processing defects at the restrictive temperature.

**Analysis of Small RNA Processing**

To detect possible defects of nuclear protein import or retention in mtr mutants, mtr mutants were incubated at semi-permissive 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 mtr3, mtr5, and mtr6 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 mtr1-1 (Oakes et al., 1993; Kadowaki et al., 1994a), mtr2-1, and rnal-1, mtr8, mtr14, mtr15, and mas3 exhibit nucleolar fragmentation (not shown). An enlarged somewhat diffuse staining pattern is observed in mtr17, 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. mtr11 does not show any significant changes at 37°C (not shown). The rest of the mutants are classified into seven different groups. Group 1 (mtr1, mtr4, mtr5, mtr6, mtr12, and mtr14) exhibits abundant osmophilic material in much of the nucleoplasm. (mtr12 is illustrated in Fig. 6 B). Group 2 (mtr8, mtr13, mtr15, and mas3) shows fine "barley-like" material in the nucleoplasm (mtr15 is illustrated in Fig. 6 C). Group 3 (mtr3-1 and mtr17) shows small tortuous substructure in the nucleoplasm, possibly derived from the nucleolus (mtr17, Fig. 6 D; mtr3-1. Group 4
Figure 5. NOP1 distribution in mtr13, mtr15, and mtr16 at semipermisive 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.

Discussion

Isolation of mtr Mutants

Suicide selections using ³H-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 [3H]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 MTRI/PRP20/SRM1 may indicate that this locus is a hot spot for mutation or possibly that each domain of the seven approximate repeats in MTRI/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 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 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. Nevertheless, the frequent observation of defects in poly(A)$^+$ RNA and splicing.

Interestingly, a group of mutants (mtrl, mtr3-1, mtr4, and mtr17) similar to those which interrupt tRNA processing (mtrl, mtr3-1, and mtr17) is characterized by the presence of over-sized 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 mtrl/prp20/srmr and mtr1-3 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, rpbl-1 (Nonet et al., 1987), prp1 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 rRNA processing show that only a limited subset of the rRNA processing defective strains (mtrl, mtr3-1, mtr17) is defective in pre-rRNA 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 anti-
References

Aebi, M., M. W. Clark, U. Vijayraghavan, and J. Abelson. 1990. A yeast mutant, PRP20, altered in RNA metabolism and maintenance of the nuclear structure, is defective in a gene homologous to the human gene RCP1 which is involved in the control of chromosome condensation. Mol. & Gen. Genet. 224:72–80.

Amberg, D. C., A. L. Goldstein, and C. N. Cole. 1992. Isolation and characterization of a yeast RNA processing-defective mutant. Mol. Cell Biol. 12:2186–2192.

Amberg, D. C., A. L. Goldstein, and C. N. Cole. 1992. Isolation and characterization of a yeast RNA processing-defective mutant. Mol. Cell Biol. 12:2186–2192.

Amberg, D. C., A. L. Goldstein, and C. N. Cole. 1992. Isolation and characterization of a yeast RNA processing-defective mutant. Mol. Cell Biol. 12:2186–2192.

Amberg, D. C., A. L. Goldstein, and C. N. Cole. 1992. Isolation and characterization of a yeast RNA processing-defective mutant. Mol. Cell Biol. 12:2186–2192.

Amberg, D. C., A. L. Goldstein, and C. N. Cole. 1992. Isolation and characterization of a yeast RNA processing-defective mutant. Mol. Cell Biol. 12:2186–2192.

Amberg, D. C., A. L. Goldstein, and C. N. Cole. 1992. Isolation and characterization of a yeast RNA processing-defective mutant. Mol. Cell Biol. 12:2186–2192.