INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Nucleocytoplasmic transport of mRNA in *Saccharomyces cerevisiae*

Kadowaki, Tatsuhiko, Ph.D.
Case Western Reserve University, 1994
Nucleocytoplasmic transport of mRNA in
Saccharomyces cerevisiae

by

TATSUMIKO KADOWAKI

Submitted in partial fulfillment of the requirement
for the degree of Doctor of Philosophy

Thesis advisor: Dr. Alan M. Tartakoff

Department of Pathology
CASE WESTERN RESERVE UNIVERSITY
January, 1994
CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

We hereby approve the thesis of

TATSUHIKO KADOURAKI

candidate for the Ph.D. degree.*

(signed)  [signature]
(chair)

[signature]

[signature]

[signature]

date  9/13/93

*We also certify that written approval has been obtained for any proprietary material contained therein.
I grant to Case Western Reserve University the right to use this
work, irrespective of any copyright, for the University's own
purposes without cost to the University or to its students, agents
and employees. I further agree that the University may
reproduce and provide single copies of the work, in any format
other than in or from microforms, to the public for the cost of
reproduction.

[Signature]
Nucleocytoplasmic transport of mRNA in *Saccharomyces cerevisiae*

Abstract

by

TATSUHIKO KADOWAKI

Nucleocytoplasmic transport of macromolecules across nuclear membrane was the essential invention during evolution of eukaryotes. This bidirectional transport is carried out by a machine, the nuclear pore complex, that is anchored in both layers of the nuclear envelope. Although significant progress has been made in understanding of the mechanisms of nuclear protein import, very little is known about the mechanisms of RNA export from nucleus to cytoplasm.

To learn molecular and cellular mechanisms of mRNA transport, a genetic approach using *Saccharomyces cerevisiae*, i.e., the isolation of mRNA transport defective (mtr) mutants followed by their characterization was undertaken. Twenty one temperature sensitive mutants that accumulate polyA+RNA in the nucleus at the restrictive temperature were isolated by a suicide selection enrichment procedure and by a screen of random temperature sensitive
mutants. These mtr mutants were classified into sixteen different complementation groups, mtrl-mtrl6. The mutants also exhibit different accumulation patterns of polyA\textsuperscript{+}RNA in the nucleus, suggesting they might have lesions in the different steps of mRNA transport pathway.

The first of mtr mutants, mtrl-1 has been characterized in detail and it has multiple defects on RNA processing and transport. Since the structure of mRNA synthesized in mtrl-1 is substantially normal, the mtrl-1 mutation affects the mRNA transport machinery. MTR1 and its homologs are nuclear proteins consisting of seven repeat units and appear to encode guanine nucleotide release proteins on small nuclear GTPases. It is therefore proposed MTR1 and its homologs act as regulators for RNA processing and transport by activating nuclear GTPases. This function is universal in various species and not related to the regulation of cell cycle progression or the state of chromatin organization.

The second mtr mutant, mtr2-1 has been characterized, and mRNA export is blocked at the intermediate path of mRNA export in this mutant. PolyA\textsuperscript{+}RNA accumulated in the nucleus closely associates with nucleolar proteins, suggesting the possible interaction between the nucleolus and the mRNA transport machinery. MTR2 is a 21kD protein
which is poorly expressed and concentrated in the nucleus.
ACKNOWLEDGMENTS

I would like to acknowledge all people involved in this research project since 1989. Starting this project was a gamble for me and I respect Alan's long-term interest in macromolecular transport of eukaryotes. I would also be grateful to Dr. Sandra K. Lemmon for her advice on yeast genetics.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xi</td>
</tr>
<tr>
<td><strong>Chapter 1: Introduction</strong></td>
<td></td>
</tr>
<tr>
<td>1. Nucleocytoplasmic transport of RNA</td>
<td>1</td>
</tr>
<tr>
<td>2. The role of nuclear pore complex (NPC) on nucleocytoplasmic transport of RNA</td>
<td>2</td>
</tr>
<tr>
<td>3. Transport of tRNA</td>
<td>4</td>
</tr>
<tr>
<td>4. Transport of rRNA</td>
<td>5</td>
</tr>
<tr>
<td>5. Transport of U snRNA</td>
<td>6</td>
</tr>
<tr>
<td>6. Transport of mRNA</td>
<td>7</td>
</tr>
<tr>
<td>7. Import of nuclear proteins and U snRNPs</td>
<td>14</td>
</tr>
<tr>
<td>8. Specific aim</td>
<td>17</td>
</tr>
<tr>
<td><strong>Chapter 2: Isolation of <em>Saccharomyces cerevisiae</em> mRNA transport defective mutants</strong></td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>19</td>
</tr>
<tr>
<td>Introduction</td>
<td>20</td>
</tr>
<tr>
<td>Results</td>
<td>22</td>
</tr>
<tr>
<td>Discussion</td>
<td>30</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>35</td>
</tr>
</tbody>
</table>
Chapter 3: Regulation of RNA transport and processing by a nuclear guanine nucleotide release protein and its homologs

Summary 39
Introduction 41
Results 42
Discussion 93
Materials and Methods 100

Chapter 4: MTR2, An essential nuclear protein which governs mRNA transport within the nucleus and the distribution of nucleolar components in Saccharomyces cerevisiae

Summary 109
Introduction 110
Results 111
Discussion 135
Materials and Methods 140

Chapter 5: Concluding remarks 148
Literature cited 151
LIST OF FIGURES

Figure 1  FISH analysis of mtrl-mtr16 mutants.  27
Figure 2  Protein synthesis and polyA-RNA localization in mtrl-l.  43
Figure 3  The "thiolutin block test" of mtrl-l.  47
Figure 4  PolyA-RNA levels and polyA-RNA turnover in wild type, mtrl-l, rpbl-l and mtrl-lrpbl-l.  49
Figure 5  Reversibility of polyA-RNA accumulation and its requirement for ATP in mtrl-l.  53
Figure 6  Transcripts which accumulate in the nucleus of mtrl-l at 37°C exit and are translated when temperature is subsequently returned to 23°C.  56
Figure 7  Analysis of 5' cap structure.  59
Figure 8  Analysis of polyA tail length.  61
Figure 9  Detection of CRY1 transcripts in wild type, mtrl-l and prp5-l strains.  64
Figure 10 Analysis of rRNA and tRNA processing.  66
Figure 11 Distribution of polyA-RNA in five different mtr1 mutants.  72
Figure 12 The effects of MTR1 depletion on cell growth and polyA-RNA distribution.  75
Figure 13 Identification of mutations in the MTR1 gene.  78
Figure 14 PolyA-RNA distribution in BHK-21 and tsBN2 cells.  82
Figure 15 PolyA-RNA distribution and chromosome condensation in S. pombe piml-46 mutant.  85
Figure 16 Distribution of polyA-RNA in S. cerevisiae single and double mutants.  89
Figure 17  Distribution of polyA·RNA in *S. pombe* single and double mutants.  91
Figure 18  Distribution of polyA·RNA in wt, *mtr2-1* and *mtr2-1rpbl-1*.  112
Figure 19  PolyA tail length distribution in *mtr2-1*.  115
Figure 20  Pre-mRNA splicing in *mtr2-1*.  118
Figure 21  tRNA and rRNA processing in *mtr2-1*.  123
Figure 22  Distribution of nucleolar antigens in *mtr2-1*.  127
Figure 23  Co-localization of polyA·RNA and nucleolar proteins.  129
Figure 24  Sequence of MTR2 gene.  132
LIST OF TABLES

Table 1  Summary of mutant isolation.  
Table 2  Summary of the phenotypes of mtr mutants  
Table 3  β-galactosidase activity in SL7 and YTK203 transformants.
LIST OF ABBREVIATIONS

cAMP  cyclic adenosine monophosphate
ATP  adenosine triphosphate
SHK  baby hamster kidney
bp  base pair
BSA  bovine serum albumin
cpm  count per minute
D  dalton
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
DNP  dinitrophenyl
DTT  dithiothreitol
EDTA  ethylenediaminetetraacetic acid
EM  electron microscopy
FITC  fluorescein isothiocyanate
g  gram
GDP  guanosine diphosphate
GTP  guanosine triphosphate
h  hour
Hepes  N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HPLC  high performance liquid chromatography
Ig  immunoglobulin
kb  kilobase(s)
kbp  kilobase pair(s)
kD  kilodalton(s)
liter(s)  liter(s)
μl  microliter(s)
ml  milliliter(s)
m  meter
μm  micrometer(s)
M  molar
mAb  monoclonal antibody
mol  mole(s)
MW  molecular weight
N  normal (concentration of ionizable groups)
NP-40  Nodinet P-40
OD  optical density
PBS  phosphate buffered saline
RNA  ribonucleic acid
mRNA  messenger RNA
rRNA  ribosomal RNA
snRNA  small nuclear RNA
tRNA  transfer RNA
RNase  ribonuclease
RNP  ribonucleoprotein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxyribonucleic acid transferase</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1. Nucleocytoplasmic transport of RNA

The consequences of the compartmentalization in eukaryotes are extremely dramatic in terms of the complexity of macromolecular transport across the nuclear membrane. The transport through the nuclear membrane is bidirectional. Since most RNA is synthesized in the nucleus and exerts its functions in the cytoplasm, it has to be exported from the nucleus to the cytoplasm. Conversely, many nuclear proteins are initially synthesized in the cytoplasm followed by selective import into the nucleus.

The eukaryotic RNAs synthesized in the nucleus are divided into three different classes depending on whether they are transcribed by RNA polymerases I, II or III. rRNA is transcribed by RNA polymerase I (pol I), mRNA and some snRNAs are transcribed by pol II and 5S rRNA and other small nuclear and cytoplasmic RNAs are transcribed by pol III. Immediately after transcribed in the nucleus, the RNAs associate with various proteins to form different sets of ribonucleoproteins (RNPs). This implies that the transport machinery for specific classes of RNA recognizes
RNA sequences, proteins or both.

2. The role of nuclear pore complex (NPC) on nucleocytoplasmic transport of RNA

The macromolecular transport across nuclear membrane is solely mediated by the NPC. It has a molecular mass estimated at 10^6 daltons and is five times the mass of a SV40 virus particle or about 30 times the mass of a ribosome (Reichelt et al., 1990). It is organized with eightfold symmetry around a central channel and spans both the inner and outer layers of the nuclear envelope. The recent report about three dimensional analysis of the NPC has revealed its symmetric framework, anchoring in the nuclear membrane and distinct interconnected subunits (Hinshaw et al., 1992). Interestingly, eight small peripheral channels were also identified in addition to a central channel and they could be probable routes for passive diffusion of ions and small molecules. The fibrils extending from NPC to both cytoplasmic and nuclear faces are also detected in sectioned material (Dingwall and Laskey, 1992). The components of these fibrils are not yet identified. A recent study of the nuclear envelope by high resolution scanning electron microscopy has shown that the nucleoplasmic face of the NPC forms baskets which attach to a regular fibrous lattice (Goldberg and Allen, 1992).

There is currently no evidence to suggest that some
pores are specialized for particular substrates. Indeed there is evidence to the contrary. Gold particles coated with a nucleophilic protein and introduced into the cytoplasm of Xenopus oocytes have been shown to migrate into the nucleus, while those coated with RNA and introduced into the nucleus migrate to the cytoplasm. Direct microscopic observation of oocytes which had been consecutively injected with both substrates showed that both types of gold particles could be found in association with the same pore, presumably on the way into or out of the nucleus (Dworetzky and Feldherr, 1988). Similarly, a monoclonal antibody against the NPC and the lectin, wheat germ agglutinin (WGA), have been shown to inhibit both RNA and protein transport (Featherstone et al., 1988).

Several nuclear pore proteins have been isolated from rat liver and yeast, and amino acid sequences have also been determined for some of them (Snow et al., 1987; Starr et al., 1990). Yeast nuclear pore proteins (ex. NUP1 and NSP1) contain a heptad repeat sequence that appears to be unique in this family of proteins (Davis and Fink, 1990; Nehrbass et al., 1990). A temperature sensitive nspl mutant was shown to be defective in nuclear protein import. RNA transport has not been characterized in this mutant. Several other yeast nuclear pore proteins and genes have been recently identified (Wimmer et al., 1992; Wente et
al., 1992; Loeb et al., 1993). It is expected that the study of yeast strains which have mutations in these genes will facilitate our understanding of the roles of the NPC in nucleocytoplasmic transport of RNA and protein.

3. Transport of tRNA

Transport of tRNA was studied in Xenopus oocytes by microinjection. It was shown that tRNA transport was carrier mediated translocation process and highly specific (Zasloff et al., 1982; Zasloff, 1983; Tobian et al., 1985). A single base change in human tRNA$^{Met}$ reduces its export 20 fold. The mutations which affect transport efficiency are clustered in the highly conserved D stem-loop and T stem-loop regions. It suggests that the tRNA transport machinery specifically recognizes secondary and tertiary structures of these regions. The fact that all tRNA mutants resulting in inefficient transport also show processing defects may indicate tRNA transport and the processing machinery share common components.

To identify protein(s) involved in tRNA transport, a HeLa nuclear protein that bound to wild-type tRNA$^{Met}$ but not to the G57U mutant was purified (Singh and Green, 1993). The single base substitution G57U mutant tRNA was shown to be defective in efficient transport. Surprisingly, the purified protein was identical to the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The
effects of three additional single nucleotide substitution tRNA\textsuperscript{Met} mutants on GAPDH binding was also analyzed. The C56U mutant, which like G57U is export-defective, bound to GAPDH with lower affinity than wild-type tRNA. In contrast, the C62U mutant, which has a small effect on the rate of nuclear export, and C61U mutant, whose rate of export has not been determined, both bound to GAPDH with the same affinity as wild-type tRNA. It is not yet determined whether GAPDH participates in tRNA transport.

4. Transport of rRNA

The export of rRNA is carried out as ribosome particles. Synthesis and processing of rRNA takes place in nucleolus, where ribosomes are assembled. The export of ribosomal subunits (40S and 60S) was studied in Xenopus oocytes by microinjection (Bataillé et al., 1990). Their transport displayed properties characteristic of a facilitated, energy-dependent process; the rate of export was saturable and transport was completely inhibited either by lowering the temperature or by depleting nuclei of ATP. Ribosomal subunit transport was inhibited when WGA was injected into the nucleus. Since ribosomal subunits from yeast and Escherichia coli were also efficiently exported from Xenopus oocyte nuclei, it was suggested that the export of some RNP complexes were directed by a collective biochemical property rather than by specific macromolecular
primary sequences or structures.

The export of 5S rRNA is probably performed by the same mechanism as other rRNA species if 5S rRNA is incorporated in 60S subunit. But intra-nuclear transport of 5S rRNA should be different from other rRNAs since it is transcribed by RNA polymerase III in different sites from nucleolus.

The endogenous 5S rRNA in stage VI (fully grown) Xenopus oocytes exists either incorporated into ribosomes or as a 7S complex. The transport of this 7S RNP complex was studied by microinjection of 5S rDNA and \( \lambda^{32}P\)-GTP (Guddat et al., 1990). The newly transcribed RNA transiently interacted with La antigen, and La was then replaced with either ribosomal protein L5 or the 5S genespecific transcription factor TFIIB. Each of these RNP complexes was transported out of the nucleus and accumulated in the cytoplasm. Since RNA molecules impaired in their ability to interact with either L5 or TFIIB can be exported, there appear to be two equivalent but independent pathways for nuclear export of 5S rRNA.

5. Transport of U snRNA

The data on U snRNA export was obtained using U1 snRNA in Xenopus oocytes. Pulse-chase experiments indicated that transcripts of microinjected U1 snRNA genes were transported with a half-time in the nucleus of less
than 10 min. The export of U1 snRNA was inhibited by WGA, and this inhibitory effect was relieved by coinjection of N-acetyl-glucosamine (Neuman de Vegvar and Dahlberg, 1990). It suggests that U1 transport is mediated by NPC.

Efficient export of U1 snRNA is dependent on its 5' monomethylguanosine cap structure. If U1 snRNAs were transcribed by RNA polymerase III instead of II, and therefore carried 5' triphosphate ends, they were retained in the nucleus (Hamm and Mattaj, 1990). Furthermore, U1 snRNA export was specifically inhibited by the microinjection of cap analog m7GpppG or EtGpppG (Izaurralde et al., 1992). These results indicated the presence of a nuclear cap binding protein whose binding specificity was different from the cytoplasmic cap binding protein (eIF-4E). A 80 kD nuclear cap binding protein was purified from HeLa cells and it bound to specific cap analogs that were inhibitory to U1 snRNA export (Izaurralde et al., 1992). The roles of this 80 kD protein on U1 snRNA export remain to be answered.

6. Transport of mRNA

Cellular mRNA is transcribed by RNA polymerase II, which is followed by extensive processing in the nucleus and cytoplasm. Unlike other RNA species (rRNA, tRNA and U snRNA), mRNA is extremely diverse in primary sequence except the 3' end portion. It is therefore unlikely that
the primary or higher structures derived from mRNA sequence act as signals for mRNA export. Common structural elements among different mRNAs are 5' monomethylguanosine cap, polyA tail and 3' stem-loop structure in animal histone mRNAs.

The role of 5' monomethylguanosine cap on mRNA transport was examined in Xenopus oocytes. Efficient export of adenovirus mRNA was dependent on the mono-methylated guanosine cap and di-, or tri-methylated guanosine caps were ineffective for transport (Hamm and Mattaj, 1990). In addition, the export of 5' monomethylguanosine capped mRNA was inhibited by co-injection of cap dinucleotide m7GpppG (Dargemont and Kühn, 1992). These results suggest that nuclear cap binding protein is actively involved in mRNA export in addition to U snRNAs transcribed by RNA polymerase II.

The direct role of 3' polyA tail on mRNA export has not been studied. Since animal histone mRNAs lack polyA tail, polyA tail is certainly not necessary for export of all mRNAs. The polyA binding proteins (PABP) were identified from several species including yeast (Baer and Kornberg, 1983; Sachs et al., 1986; Sachs and Kornberg, 1985; Grange et al., 1987). PABP was suggested to be involved in polyA tail shortening and translation initiation in yeast (Sachs and Davis, 1989). Its role on polyA-RNA export has not been examined in any species.
Animal histone mRNAs contain a unique stem-loop structure at the 3' end instead of a polyA tail. The importance of this structure on the export process was examined by introducing mutations. The results indicated that both primary sequence and secondary structure of the stem-loop were important for the efficient export (Eckner et al., 1991; Sun et al., 1992).

The interaction of processing and export machineries of mRNA has been studied in several different systems. The effects of 3' end processing on the export of intron-less neomycin resistance (neo) gene has been reported (Eckner et al., 1991). The neo gene was engineered to contain either the β-globin poly(A) signal or histone processing signal. The export of neo mRNA was stimulated by these signals and not efficient if neo gene had only plasmid vector sequence at the 3' end. Furthermore, the export of two neo mRNAs which could acquire a histone 3' end through cleavage by either the normal cellular histone pre-mRNA processing machinery or by the action of a cis-acting ribozyme were analyzed. Efficient export was only observed when the authentic 3' end formation pathway was utilized. Ribozyme-cleaved transcripts were transport deficient and accumulated in the nucleus. These results provided evidence for a mechanistic linkage between 3' end formation and mRNA export.
The interaction of mRNA splicing and transport machineries has been shown to be competitive. The introns act as nuclear retention signals for pre-mRNA. In yeast, a pre-mRNA with an intron was not exported unless the intron was excised. Unspliced pre-mRNA was only exported if splicing complex formation was disturbed by mutating either highly conserved splicing signals or some trans-acting factors, including U1 snRNA, PRP6 and PRP9, whose mutations were presumed to affect early steps in the formation of spliceosomes (Legrain and Rosbash, 1989). Interference with other splicing factors resulted in the retention of pre-mRNA in the nucleus, probably due to the formation of dead-end complexes. Thus, intron-containing transcripts were incorporated in spliceosomal complexes and released from them only after the removal of the intron.

A similar conclusion was made by studying the effect of intron mutations on pre-mRNA export in animal cells and Xenopus oocytes (Chang and Sharp, 1989; Hamm and Mattaj, 1990). Mutated pre-mRNAs that could not be spliced out but could form defective splicing complexes were retained in the nucleus. Conversely, pre-mRNAs unable to form detectable splicing complexes were rapidly exported into the cytoplasm. These results suggest that the factors which are involved in the commitment of pre-mRNA into splicing pathway and subsequent spliceosome formation may
prevent the transport of mRNA to the cytoplasm. According to this model, the function of HIV Rev protein (Emerman et al., 1989; Felber et al., 1989; Malim et al., 1989) is to prevent the formation of spliceosomal complexes, and therefore viral late transcripts with multiple introns are exported to the cytoplasm upon Rev binding.

It is, however, important to mention that the presence of one or more introns does not always result in mRNA retention in the nucleus. A large number of pre-mRNAs are subjected to alternative splicing pathways. Some transcripts are exported to the cytoplasm with more than one introns. Whether export of these RNAs is due to inefficient recognition of splice sites by the splicing machinery or to activities which disrupt the association between splice sites and splicing factors is not known.

Although previous descriptions predict that the deletion of introns from particular genes will result in the rapid accumulation of mRNA in the cytoplasm, this is, however, not always the case. Several eukaryotic and viral genes (ex. rabbit β-globin and SV40 late transcripts) require the presence of one or more excisable introns in order to achieve normal levels of cytoplasmic mRNA (Buchman and Berg, 1988; Neuberger and Williams, 1988; Ryu and Mertz, 1989). Furthermore, the presence of introns in genes described above is also necessary for stabilization
of primary transcripts in the nucleus. The reason why an intron is only required in some transcripts remains unknown. Some transcripts may require the commitment to the splicing pathway for their efficient intra-nuclear transport.

Immediately after being transcribed, pre-mRNAs associate with the abundant family of hnRNP proteins (Dreyfuss, 1986). It was thought hnRNP proteins dissociated from RNA before or during transport. But a recent report has shown that some hnRNPs (ex. A1, A2 and E) shuttle between the nucleus and cytoplasm during interphase (Piñol-Roma and Dreyfuss, 1992). These proteins do indeed accumulate in the cytoplasm upon extended incubation with transcriptional inhibitors. UV cross-linking experiments demonstrated that A1 protein which was accumulated by this treatment is associated with polyA-RNA. These results suggest that mRNA may be exported from the nucleus in association with subsets of hnRNP proteins. The hnRNP-mRNA complexes could be the direct substrate for the mRNA transport machinery.

A kinetic study of mRNA transport was conducted with Xenopus oocytes by microinjecting in vitro synthesized mRNA substrates (Dargemont and Kühn, 1992). The transport was saturable with an apparent Km of 3.6 X 10⁸ molecules/oocyte nucleus and its half-time was ~2 min under non-saturating
conditions. mRNA export showed properties of an energy-
dependent mechanism, since it was inhibited at 4°C or by
ATP depletion. The export was inhibited by co-injection of
WGA and tRNA. Thus, transport machinery for mRNA (at least
translocation through NPC) is similar to that for other
types of RNA.

Several specific mRNAs have been visualized in animal
cell nuclei by fluorescent in situ hybridization (FISH).
Nuclear transcripts were detected as a focus, track, or
highly elongated track (which represents accumulated RNA)
depending on the genes examined (Lawrence et al., 1989;
Xing et al., 1993). They were closely positioned at their
genomic DNA which was localized in the nuclear interior,
and therefore there was no evidence supporting the idea
(Blobel, 1985) that actively transcribed chromatin DNA was
preferentially localized near the nuclear periphery to
facilitate mRNA transport. The absence of intron sequences
from a portion of the focus or track defined by the cDNA
probe indicates that intron splicing occurs within this
accumulation of RNA. In addition, hybridization with
intron probes also produced a less intense, slightly
punctate fluorescence throughout the nucleoplasm, excluding
the nucleolus. It suggests that the excised introns are
free to diffuse in contrast to unprocessed RNA molecules
which accumulate at the site of transcription. Although
mRNA transport and processing are likely to occur along this track, RNA tracks do not extend to the nuclear envelope. Therefore, the export of mature mRNA through nuclear membrane might occur by more than one route and it is not a rate limiting step of the entire mRNA transport process. The highly organized nature of nuclear transcripts is consistent with evidence indicating that RNA processing and transport occur in association with the nuclear matrix (King and Lawrence, 1991).

7. Import of nuclear proteins and U snRNPs

Nuclear protein import is an active, ATP-requiring process. In the absence of ATP, or at 0°C, transport substrates binds to sites on the cytoplasmic face of the NPC, suggesting that there are at least two steps in the import pathway: ATP-independent binding to the NPC, followed by ATP-dependent translocation events through the NPC into the nucleus. The lectin, WGA, blocks translocation but not binding; this suggests that the O-glycosylated NPC proteins are involved in the translocation step (Newmeyer, 1993).

Using cell-free or permeabilized-cell transport assays, cytosolic transport factors have been identified by several groups (Newmeyer and Forbes, 1990; Adam et al., 1990; Adam and Gerace, 1991; Moore and Blobel, 1992; Sterne-Marr et al., 1992). For example, one factor is necessary
for the nuclear pore-binding step and the other is required
for the efficient translocation through the NPC. The
precise relationship between factors identified by
different groups is still unclear.

The existence of nuclear localization signal (NLS) in
nuclear proteins suggested that NLS binding proteins must
be present in the cell. The identification of such factors
was made by cross-linking experiments. The purified NLS-
binding proteins have been shown to be necessary for
nuclear import in permeabilized cells (Adam and Gerace,
1991; Stochaj and Silver, 1992).

In addition to factors described above, heat shock
proteins, hsc70/hsp70, appear to bind to NLS peptides and
are required for nuclear import of karyophilic proteins and
even of NLS peptide-conjugates (Shi and Thomas, 1992;
Imamoto et al., 1992). Hsc70/hsp70 proteins might
potentiate the interaction of the NLS with other NLS
recognition proteins; alternatively, they might facilitate
the release of the NLS from its receptor at a later stage
of nuclear import, perhaps, for example, after the
karyophilic protein has entered the nuclear interior.

The transport of U snRNAs through the nuclear membrane
is bidirectional. U snRNAs transcribed in the nucleus are
exported to cytoplasm where they assemble with Sm proteins
and one or more polypeptides associated specifically with
the individual U snRNA into snRNP particles. During this phase the U snRNAs are hypermethylated at their guanosine cap, processed at their 3' ends and possibly modified at some of their internal nucleotides. Hypermethylation results in the conversion of the 7 methyl-guanosine cap structure into a 2,2,7 trimethyl-guanosine cap. The snRNP particles are then able to move back to the nucleus.

The recent work has demonstrated that, in the case of U1 and U2 snRNAs, migration back to the nucleus is dependent on both the binding of the common U snRNP proteins and the presence of the trimethylated cap structure (Hamm et al., 1990; Fischer and Lührmann, 1990; Fischer et al., 1991). Artificial U1 and U2 snRNAs with ApppG cap structures can bind the common proteins but remain in the cytoplasm, as do RNAs with trimethyl cap structure but which lack the Sm binding site and thus do not associate with the common proteins. The karyophilic signals that allow import of U1 and U2 snRNPs into the nucleus of Xenopus oocytes is therefore bipartite, and both components are required for nuclear migration. In addition, injection of a free m^2,2,7GpppG dinucleotide into the cytoplasm inhibits nuclear uptake of U1 and U2 snRNPs. The inhibition is specific since similar concentrations of m^7GpppG dinucleotide have no effect. This result not only provides additional evidence for the involvement of the
trimethylated cap structure on U1 and U2 nuclear import but also suggests the existence of a saturable trans-acting factor that recognizes the trimethylated cap structure. In contrast to U1 and U2, the U4 and U5 snRNPs do not have a stringent requirement for the trimethylated cap structure for transport. It is therefore suggested that proteins specifically associated with U4 and U5 snRNAs might have different roles on nuclear import compared to those associated with U1 and U2 snRNAs.

The competition experiments has indicated that the import of U2 snRNP is not competed by bovine serum albumin conjugated with SV40 NLS (Michaud and Goldfarb, 1991). Similarly, it was shown that injection of the m$^{2,2',7'}$GpppG dinucleotide had no effect on karyophilic protein import. In contrast, the uptake of U6 snRNA to nucleus was not affected by cap dinucleotide but was inhibited by NLS peptide conjugate. U6 snRNA is a pol III transcript and thus does not have an inverted m$^7$G cap structure. The U6 snRNP protein whose binding is required for transport is therefore presumed to have an NLS of the same class as that of SV40 large T antigen, and U6 transport seems to depend only on this NLS.

8. Specific aim

The study of nucleocytoplastic transport of RNA is still in its infancy. This is because of the lack of
knowledge about the composition of the RNPs which are substrates for transport and the components of the transport machinery with which RNPs interact. Another big problem is that our incomplete understanding of the effects of nuclear structure and compartmentalization on RNA metabolism. Furthermore, it will be difficult to establish a RNA export system that allows biochemical manipulation of nuclear components in vitro. For these reasons, we attempted a genetic approach by the use of the yeast, *Saccharomyces cerevisiae* as a system to shed light on our understanding of mRNA transport in eukaryotes.
CHAPTER 2

Isolation of *Saccharomyces cerevisiae* mRNA transport defective mutants

SUMMARY

To isolate *Saccharomyces cerevisiae* mRNA transport defective (mtr) mutants, amino acid analog and $^{3}$H·amino acid suicide selections were developed. Following the selection of temperature sensitive mutants, FISH was used to isolate mutants which accumulated polyA⁺RNA in the nucleus at the restrictive temperature. Two selections yielded twelve mutants which were classified into ten different complementation groups. In addition, screening of a bank of ts mutants yielded six more complementation groups. These mutants exhibit different accumulation patterns of polyA⁺RNA in the nucleus, suggesting the possibility that different steps of the transport pathway might be blocked.
INTRODUCTION

To understand the molecular and cellular mechanisms of mRNA transport in eukaryotes, the genetic approach using yeast, *Saccharomyces cerevisiae*, seems to be the best for the following reasons. The mRNAs in yeast have a relatively simple structure compared to those in higher eukaryotes. They lack internal m^6^A-methylation so that 5' monomethylguanosine cap is the only methylated residue (Sripati et al., 1976). Most yeast mRNAs do not have introns (Woolford, 1989) and they are competent for transport irrespective of the splicing pathway. This is very important because the inhibition of splicing could result in the retention of pre-mRNA in splicesome. It is expected that general inhibition of pre-mRNA splicing does not lead to an export block of bulk mRNA in yeast.

Our approach is to isolate yeast temperature sensitive (ts) mutants which are defective in mRNA transport (*mtr* mutants). Amino acid analog and ^3^H-amino acid suicide selections have been used to facilitate the isolation of such mutants. These selections are expected to concentrate protein synthesis defective mutants at the restrictive temperature. Among ts mutants obtained by the above methods, FISH using oligo-dT as a probe is applied to identify the mutants which accumulate polyA^-RNA in the nucleus at the restrictive temperature. This is based on
the assumption that polyA'RNA can be stably maintained in the nucleus if its export is blocked. The similar phenotype could be observed in mutants which fail to maintain the stability of cytoplasmic mRNA. The "thiolutin block test" is used to distinguish these possibilities.

By two selections, twelve different mutants have been isolated and they are classified into ten complementation groups. In addition, a bank of yeast ts mutants has been directly screened by FISH and six additional complementation groups are obtained. In this chapter, the selection scheme and basic characterization of mtr mutants are described.
RESULTS

Selection of mtr mutants

To enrich for mutants that can not export mRNA at 37°C (restrictive temperature), suicide selection procedures have been used based on the assumption that cells that have not exported mRNA for 3 hr will tolerate incubation in the presence of high concentration of toxic amino acid analogs or 3H-labeled amino acid. Both methods were successfully used to isolate animal cell mutants which were defective in protein synthesis or amino acid transport (Pouysségur and Franchi, 1987). The EMS-mutagenized YPH258/259 and YPH1/2 (Sikorski and Hieter, 1989) were subjected to toxic amino acids (lysine analog S-2-aminoethyl-L-cysteine and proline analog L-azetidine-2-carboxylate) and 3H-amino acid (3H-lysine) suicide selection, respectively. After isolating ts mutants from survivors, they were screened by FISH to identify mutants which accumulate polyA+RNA in the nucleus at 37°C. Each ts mutant was incubated at 37°C for 3hr then fixed for the analysis. The mutants which gave a strong nuclear signal compared to the cytoplasm were picked as mtr mutants. A total of twelve different mtr mutants were isolated and the fraction of nuclear positive cells in different mutants varied from 20 to 100%. The results of two selections are summarized in Table 1.
Table 1
Summary of mutant isolation

<table>
<thead>
<tr>
<th></th>
<th>Selection A</th>
<th>Selection B</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3H-amino acid)</td>
<td>YPH1: (1.0 \times 10^5)</td>
<td>YPH258: (2 \times 10^7)</td>
</tr>
<tr>
<td>(toxic amino acid analogs)</td>
<td>YPH2: (1.2 \times 10^5)</td>
<td>YPH259: (2 \times 10^7)</td>
</tr>
<tr>
<td>Number of cells subjected to selection</td>
<td>(1.0 \times 10^5)</td>
<td>(2 \times 10^7)</td>
</tr>
<tr>
<td>Survivors after suicide selection</td>
<td>479</td>
<td>335</td>
</tr>
<tr>
<td>Colonies picked</td>
<td>479</td>
<td>335</td>
</tr>
<tr>
<td>Temperature sensitive mutants</td>
<td>85</td>
<td>64</td>
</tr>
<tr>
<td>Nuclear positive mutants by FISH</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

* Relatively small colonies were picked up from survivors.
Direct screening of a bank of temperature sensitive mutants by FISH

The selection described above has restrictions in terms of the mutants that can be recovered. It may be difficult to recover the mutants that rapidly lose viability at the restrictive temperature because cells are pre-incubated at 37°C for 3 hr prior to the addition of analogs or radioisotope. To overcome this problem, a bank of yeast temperature sensitive mutants was directly screened by FISH. This screening also gives an idea about the efficiency of the enrichment for mtr mutants by suicide selection. A total of 9 mtr mutants were isolated from 447 ts mutants.

Complementation analysis of mtr mutants

To classify mtr mutants into different complementation groups, the original mutants were backcrossed with parent strains to obtain both MAT a and ᵁ strains. All heterozygous diploids were TS⁺ (that can grow at 37°C), indicating mutations are recessive. All mutants were crossed to each other to check for temperature sensitivity. Two mutants which gave rise to TS⁺ diploids were classified into different complementation groups. Twenty one mutants were classified into sixteen, mtrl-16, complementation groups. There were six mutants that belonged to mtrl and the allele difference among three mtrl
mutants was confirmed by the appearance of TS* diploids from heterozygous ts diploids upon mitotic recombination induced by UV irradiation.

**Segregation analysis of mtr mutants**

Each mtr mutant was backcrossed several times with wild type yeast strains to look for co-segregation of temperature sensitivity and nuclear polyA•RNA accumulation. Backcrossing has been recently finished and all mutants show co-segregation of these two phenotypes. It suggests that mRNA export is blocked in these mutants due to the mutation(s) in single gene.

**The pattern of nuclear polyA•RNA accumulation in mtr mutants**

The individual mtr mutants were carefully analyzed by FISH to see the detail of polyA•RNA accumulated in the nucleus. As shown in Figure 1, the pattern of polyA•RNA accumulation in the nucleus is different among mutants. Most of them exhibit uniform signal throughout the entire nucleus but some show foci, dot or a peripheral signal.

**Thiolutin block test**

The phenotype of mtr mutants observed by FISH (bright nucleus and dim cytoplasm) could be due to a defect in cytoplasmic mRNA maintenance instead of an mRNA export defect. To distinguish between these possibilities, a thiolutin block test was applied to each mutant. Thiolutin
is an inhibitor that stops transcription by all three RNA polymerases (Tipper, 1973). The mtr mutants were incubated at 37°C for the appropriate time followed by the addition of thiolutin. After 1-2 hr incubation at 37°C, cells were analyzed by FISH. If the nuclear signal remained, cells are defective in mRNA export, and if not, cells are defective in cytoplasmic mRNA maintenance. All mutants were tested by above methods and they have an mRNA export defect judging from the results of this test (data not shown).

Analysis of pre-mRNA splicing defective mutants by FISH

To confirm that a pre-mRNA splicing defect does not result in the simultaneous mRNA export block, several pre-mRNA splicing defective mutants (prp2, 5, 8, and 11) were subjected to FISH analysis. After 1-3 hr incubation at 37°C, none of them showed significant accumulation of polyA+RNA in the nucleus (data not shown). This indicates that the inhibition of pre-mRNA splicing does not affect the export of bulk mRNA in yeast.
Figure 1  FISH analysis of mtr1-mtr16 mutants

The mtr mutants were incubated at 37°C for various periods (0.5-4.5 hr) followed by identical fixation. Each mutant was analyzed by FISH using biotinylated oligo-dT as a probe. The arrowheads show irregular staining patterns observed in several mtr mutants. The uniform signal throughout entire cell was detected in wild type strain.
Table 2
Summary of the phenotypes of mtr mutants

<table>
<thead>
<tr>
<th>mtr mutant</th>
<th>number of alleles</th>
<th>FISH phenotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>entire</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>spots</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>half</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>one spot</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>circle/spots</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>spots</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>entire</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>entire</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>spots</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>granular/entire</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>circle/spots</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>entire</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>one spot</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>granular/entire</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>entire</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>entire</td>
</tr>
</tbody>
</table>

* This indicates the pattern of polyA^-RNA accumulation in the nucleus.
DISCUSSION

Isolation of mtr mutants

The suicide selection using toxic analogs and $^3$H-labeled compounds has been widely used to isolate different types of animal cell mutants (Pouysségur and Franchi, 1987). We adopted this technique to enrich for mtr mutants. In addition, the development of FISH for yeast cells has been critical for the screening procedures. By combining these techniques, 12 different mutants were isolated. Furthermore, a bank of yeast ts mutants was directly screened by FISH and it yielded 9 different mutants. These results show that the enrichment of mtr mutants by suicide selections is not as good as expected. For example, 9 mtr mutants were recovered from 149 ts mutants obtained by $^3$H-lysine suicide selection (Table 1) and 9 mtr mutants were isolated from 447 random ts mutants. The enrichment factor in this case is only 3. The reason for this is that 3 hr pre-incubation at 37°C prior to selection is probably too short to stop protein synthesis of mtr mutants. In fact, most mtr mutants do not completely stop protein synthesis after 3 hr incubation at 37°C (C.Kumagai, T.Kadowaki, and A.Tartakoff, unpublished). This is also consistent with the fact some mtr mutants are weak alleles judging from the number of nuclear positive cells determined by FISH.

Complementation analysis of a total of 21 mtr mutants
indicated they are classified into 16 different complementation groups, mtr1-16. Six mutants are mtr1 and at least 3 different alleles are identified among them. There is only one allele for the rest of the complementation groups, suggesting the number of mutants is not saturated. It also indicates that the number of genes directly or indirectly involved in mRNA transport is more than 16 in yeast. We do not completely understand why so many alleles of mtr1 have been recovered. The MTR1 locus might be a hot spot for mutation in the yeast genome or each domain of the seven repeats in MTR1 is important for its function (see Chapter 3 for details).

To prove mtr mutants have lesions in mRNA export but not in cytoplasmic mRNA maintenance, the "thiolutin block test" was used. Judging from the results of the test, all mtr mutants are indeed defective in mRNA transport. It also suggests the normal pool of nuclear polyA+RNA is not sufficient enough to exhibit a nuclear signal by FISH even with a low level of cytoplasmic polyA+RNA.

The pattern of polyA+RNA accumulation in the nuclei of mtr mutants

The analysis of mtr mutants by FISH reveals the differences in the polyA+RNA accumulation pattern in the nuclei. As illustrated in Figure 1, some show a whole nuclear staining pattern and the others exhibit foci, dot,
or peripheral staining patterns. Since all mutants are identically fixed and processed for FISH, the different staining patterns demonstrate polyA-RNA is accumulated in the nucleus in a different manner. It suggests that mRNA might be trapped at the different steps in a transport pathway but this interpretation should be made cautiously. If mtr mutations induce nuclear disorganization, this could simply result in the aberrant accumulation of polyA-RNA in the nucleus. This will make it difficult to dissect intranuclear mRNA transport pathway by, for example, epistatic analysis of mtr mutants. The phenotypes of mtr mutants are characterized in Table 2.

What kinds of mutants can be recovered by this screen?

The most critical step in this screen is the detection of mutants which give a strong nuclear signal by FISH. It is expected that mutations which affect mRNA transcription, mRNA structure (ex. 5' monomethylguanosine cap), mRNA binding proteins, NPC, nuclear organization as well as cytoplasmic proteins could result in nuclear mRNA accumulation. It will be difficult to determine if the mutations are directly involved in mRNA export since mRNA export is dependent on many aspects (mRNA structure, mRNA binding proteins, mRNA transport machinery and nuclear organization) and lack of our knowledge about how they
influence mRNA export.

The results of FISH analysis of pre-mRNA splicing defective mutants indicate the export of bulk mRNA is independent of splicing in yeast as expected. Protein synthesis inhibition (either by ts mutation or cycloheximide) does not induce nuclear polyA+RNA accumulation (Chapter 3), suggesting mRNA translation by ribosomes is not essential for mRNA export. Since pre-mRNA splicing and protein synthesis defects simultaneously result in synthesis and processing defects of rRNA (Shulman et al., 1977; Hartwell et al., 1970), it is likely that mRNA export is independent of ribosome biogenesis. Therefore, mutants that have specific lesions in pre-mRNA splicing, protein synthesis and ribosome biogenesis should not be recovered by this screen.

So far, similar screening procedures by other labs identified the RNA1 and RAT1 genes, but their functions on mRNA export are still unknown (Amberg et al., 1992). RNA1 is a cytoplasmic protein and affects multiple RNA processing events (Hopper et al., 1990). It has recently been suggested that RNA1 modulates cAMP levels which in turn may affect RNA processing in nucleus. RAT1 is a nuclear protein and has exo-ribonuclease activity in vitro (Kenna et al., 1993). The same gene was also identified as a gene whose mutation activates the expression of a tRNA
gene with a defective internal promoter (Di Segni et al., 1993). It is difficult to link the in vitro activity and possible in vivo functions of this gene product.

Ongoing study of mtr mutants reveals that MTR gene products include a heat shock transcription factor (Sorger and Pellham, 1988; Wiederrecht et al., 1988), a putative RNA binding protein and an RNA helicase in addition to MTR1, 2 and 3. The study by transmission electron microscope shows some mtr mutants might be defective in the organization of the nuclear interior and nuclear envelope. These results indicate MTR gene products must include proteins closely associating with RNA and maintaining the nuclear structure.
MATERIALS AND METHODS

Selection of mtr mutants

The YPH1/2 (Selection A) and YPH258/259 (Selection B) were treated with 3% EMS for 1.5 hr at room temperature. The survival fraction was 10-15%. After quenching the reaction with 5% sodium thiosulfate, the cells were grown 20 hr at 23°C in SD medium. Two selection procedures have been used. Selection A: Cells were then incubated 3 hr at 37°C at 10⁷ cell/ml in SD medium, washed twice with water (37°C) and labeled 30 min at 37°C with 1mCi/ml ³H-lysine in lysine-free SD medium at 1.5 X 10⁷ cell/ml. The cells were then washed 3X 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. Selection B: Cells were incubated 3 hr at 37°C at 7.4 X 10⁶ cell/ml in SD medium, washed twice with water (37°C) and resuspended in lysine-free SD medium supplemented with the lysine analog S-2-aminoethyl-L-cysteine and the proline analog L-azetidine-2-carboxylate added to final concentration of 3mM and 2 mM, respectively. The incubation was continued at 37°C for 24 hr then cells were inoculated to YEFPD plates. The survival fraction of the cells after this treatment was 0.1-0.2%. Replica plates were made to screen for ts growth.

To evaluate the temperature dependence of protein
synthesis, individual colonies were grown overnight at 23°C and labeled in SD medium supplemented with 80μCi/ml ³H-amino acid mixture for 0-3 hr at 23°C and 37°C. After incubation, aliquots of the cells were spotted onto 10% TCA presoaked 3MM paper and processed by successive washes in TCA, 90% ethanol and ether to determine total incorporation.

**Fluorescent in situ hybridization (FISH)**

For detection of polyA⁺RNA, cells grown for 0-3 hr at 23°C or 37°C in YEPD (1-2 OD₆₀₀nm) were fixed in suspension by addition of formaldehyde to a final concentration of 4%, incubated 15 min with shaking, sedimented and resuspended in 4% paraformaldehyde in 5mM MgCl₂, 0.1M KPO₄ buffer, pH 7.5 and held without shaking for 2 hr at room temperature. They were then washed 3X in 1.2 M sorbitol, 0.1 M KPO₄ buffer, pH7.5 (solution A). 2.5 X 10⁶ cells in 250μl of solution A were then incubated with 0.5μl β-mercaptoethanol and Zymolyase 100T (final concentration 80μg/ml) with gentle shaking for 1-2 hr at 37°C. The cells were washed 2X in solution A, resuspended in 20μl of solution A and spotted for 0.5 hr onto a poly-L-lysine coated glass slide at 4°C. After a wash with 70% ethanol, cells were dehydrated through 70, 90, and 100% ethanol and air dried. 10μl of hybridization solution consisting of 0.25μM
biotinylated oligo-dT, 1mg/ml yeast tRNA, 1mg/ml salmon sperm DNA, 2X SSC, 10mM vanadyl complex, 0.2% BSA, 10% dextran sulfate was then added for 3 hr at 37°C in a humid chamber. Each preparation was then washed 3X with 2X SSC at 37°C (10 min each), 3X with 1X SSC at room temperature (10 min each) and once with 4X SSC, 0.1% Triton X-100 for 10 seconds. A fresh solution of 100µl of 2µg/ml FITC-avidin, 1% BSA, 4X SSC was added and incubated for 30 min in the dark at the room temperature. The solution was then aspirated and the preparation was washed 2X with 4X SSC (10 min each), 2X with 4X SSC, 0.1% Triton X-100 (10 min each), 2X with 4X SSC at room temperature, mounted in 90% glycerol, 1mg/ml p-phenylenediamine, 45ng/ml DAPI, PBS and examined.

The biotinylated oligo-dT<sub>25-30</sub> probe was prepared using biotin-16-dUTP and terminal deoxynucleotidyl transferase to end-label oligo-dT.

In the "thiolutin block test", cells were preincubated at 37°C in YEPD medium then thiolutin (dissolved in DMSO) was added to a final concentration of 3µg/ml. After continued incubation at 37°C, cells were fixed and processed as described above.

**Genetic methods**

The standard yeast genetics was used as described (Guthrie and Fink, 1991).
Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPH1</td>
<td>MATa</td>
<td>ade2-101 ura3-52 lys2-801</td>
</tr>
<tr>
<td>YPH2</td>
<td>MATα</td>
<td>ade2-101 ura3-52 lys2-801</td>
</tr>
<tr>
<td>YPH258</td>
<td>MATa</td>
<td>ade2-101 ura3-52 lys2-801 his3-Δ200 leu2-Δ1</td>
</tr>
<tr>
<td>YPH259</td>
<td>MATα</td>
<td>ade2-101 ura3-52 lys2-801 his3-Δ200 leu2-Δ1</td>
</tr>
<tr>
<td>368</td>
<td>MATa</td>
<td>ade1 ade2 ura1 his7 lys2 tyr1 gall prp2-1</td>
</tr>
<tr>
<td>108</td>
<td>MATa</td>
<td>ade1 ade2 ura1 his7 lys2 tyr1 gall prp5-1</td>
</tr>
<tr>
<td>219</td>
<td>MATa</td>
<td>ade1 ade2 ura1 his7 lys2 tyr1 prp8-1</td>
</tr>
<tr>
<td>382</td>
<td>MATa</td>
<td>ade1 ade2 ura1 his7 lys2 tyr1 gall prp11-1</td>
</tr>
</tbody>
</table>
CHAPTER 3

Regulation of RNA transport and processing by a nuclear guanine nucleotide release protein and its homologs

SUMMARY

One mtr mutant, mtrl has been characterized in detail. The export of polyA'-RNA is rapidly inhibited and the intranuclear polyA'-RNA is remarkably stable in this mutant. The polyA'-RNA pool accumulated in the nucleus at 37°C can be exported and translated when the temperature is reduced to 23°C. Consistent with this, the structural analysis of mRNA synthesized at 37°C shows mRNA has a normal 5' cap and elongated polyA tail, suggesting the mtrl mutation affects the mRNA export machinery. The effects of the mtrl mutation are pleiotropic in terms of RNA processing, i.e., the processing of rRNA and tRNA is also inhibited in mtrl at 37°C.

The MTRI gene is shown to be identical to the previously isolated SRM1/PRP20 genes. Furthermore, Schizosaccharomyces pombe and mammalian homologs have also been identified and the biochemical analysis indicates

39
proteins encoded by these genes have guanine nucleotide releasing activities on recently identified nuclear GTPases. The role of MTR1 and its homologs on RNA transport is universal in all eukaryotes. It is likely that they stimulate nuclear GTPases by exchanging GDP to GTP. Then activated GTPases influence the activities of effectors which control multiple events in the nucleus.
INTRODUCTION

The mtr1 was the first mutant isolated during the mutant screening described in the previous chapter. It is the best mutant to conduct the primary characterization of mtr mutants because of the availability of multiple mutant alleles. The phenotypes of mtr1-1 are extensively characterized. The MTR1 gene is cloned and the point mutations in three alleles are also identified. It is found that this gene and its homologs have previously been implicated in other aspects of mRNA processing and in cell cycle control. Moreover, previous studies have shown that MTR1 homologs bind nucleosomes and, indirectly, control DNA synthesis. RNA processing and cell cycle progression has been further characterized in mtr1 alleles and the consequences of ts mutations of MTR1 homologs in a BHK-21 cell mutant and a mutant of Schizosaccharomyces pombe have been investigated. These investigations lead to the first identification of a higher eukaryotic cell with a ts mutation causing intranuclear accumulation of polyA+RNA.
RESULTS

Basic phenotypes of \textit{mtrl-1}

Figure 2A illustrates the characteristic kinetics of protein synthesis of \textit{mtrl-1} by comparison to wild type (wt) at 23°C and 37°C. Incorporation of labeled amino acids is quasi-linear except for \textit{mtrl-1}, which stops after 1-2 hr at 37°C. Comparable kinetics of protein synthesis are seen for a mutant which is ts for mRNA synthesis, \textit{rpbl-1} (Nonet et al., 1987) and for \textit{rnl-1}. The FISH to detect polyA+RNA revealed a uniform distribution of fluorescence in wt cells grown at 23°C or 37°C and in \textit{mtrl-1} grown at 23°C (Figure 2B). The fluorescent signal was eliminated by pretreatment with non-specific RNase. Moreover, in a temperature sensitive RNA polymerase II mutant, \textit{rpbl-1}, incubation at 37°C leads to a progressive disappearance of fluorescence from the nucleus and cytoplasm. When \textit{mtrl-1} cells are incubated for increasing periods at 37°C, the cytoplasmic signal faded and the nuclear signal increased for at least 3 hr. During this period, the intranuclear signal grows from a focal spot (40 min) to a more complex foci (80 min). As judged by simultaneous staining with DAPI, the fluorescent signal ultimately fills the entire nucleus. The nuclear signal was not seen when comparable experiments were performed with a \textit{mtrl-1rpbl-1} double mutant, suggesting the accumulation of nuclear polyA+RNA requires
Figure 2  Protein synthesis and polyA⁺RNA localization in mtr1-1

Panel A  Protein synthesis in wild type and mtr1-1. Each cell type was incubated for increasing periods of time at 23°C or 37°C in the presence of mixed ³H-labeled amino acids. After the indicated periods of time, samples were withdrawn for TCA precipitation. As described in the text, incorporation is roughly linear with time except for mtr1-1, which reaches a plateau after 1-2 hr at 37°C.

Panel B  FISH analysis of wild type, rpbl-1, mtr1-1 and mtr1-1rpbl-1.
The fluorescent signal in wild type cells is uniform and strong at 23°C and 37°C (A and E). The signal is diminished in rpbl-1 at 37°C (B and F), suggesting that it is derived from RNA pol II transcripts. In mtr1-1 at 23°C the fluorescence is uniform (C), while after 3 hr at 37°C the nucleus is greatly accentuated (G). The nuclear signal is not visible in mtr1-1rpbl-1 (D and H), indicating the requirement of active RNA pol II for polyA⁺RNA accumulation in the nucleus of mtr1-1.
active RNA polymerase II. The "thiolutin block test" was applied to mtrl-1 and as shown in Figure 3, polyA•RNA is stably maintained in the presence of thiolutin. It suggests mRNA export is indeed blocked in mtrl-1.

The mRNA levels and turnover of mRNA

To evaluate the impact of the mtrl-1 mutation on mRNA levels and turnover, RNA dot blots of wt, mtrl-1, rpbl-1, and mtrl-1rpbl-1 incubated for 0-3 hr at 37°C have been probed with labeled oligo-dT. In the latter two cases, upon shift to 37°C one can directly follow the turnover of pre-existing, i.e. >90% cytoplasmic, mRNA (Groner and Phillips, 1975).

As illustrated in Figure 4A, mtrl-1 cells maintain ~45% of their initial (23°C) levels of total polyA•RNA after 3 hr at 37°C. The turnover of cytoplasmic polyA•RNA is accelerated in mtrl-1rpbl-1 cells relative to that of rpbl-1, suggesting the mtrl-1 mutation has effects on the turnover of cytoplasmic mRNA.

To evaluate the turnover of intranuclear polyA•RNA in mtrl-1, cells have been incubated 1-2 hr at 37°C and then maintained for 0-3 hr at 37°C with thiolutin. Wt and rpbl-1 were analyzed in parallel. Total RNA was isolated after increasing periods at 37°C and dot blots were processed as above. Figure 4B shows that by comparison to wt cells, turnover is severely slowed in mtrl-1.
Figure 3  The "thiolutin block test" of mtr1-1
The intranuclear signal seen in mtr1-1 after 2 hr at 37°C is stable in the absence of continued mRNA synthesis.
After 2 hr preincubation the cells were maintained at 37°C in the presence of 3 μg/ml thiolutin to block RNA synthesis. This dose of the drug totally blocks RNA synthesis. As shown, the intranuclear signal persists for at least 2 hr. This observation makes it unlikely that the characteristic intranuclear signal seen after 1-3 hr at 37°C is simply the result of accelerated degradation of cytoplasmic polyA+RNA.
Figure 4  PolyA-RNA levels and polyA-RNA turnover in wild type, mtrl-1, rpbl-1 and mtrl-lrpbl-1.

Panel A  The levels of polyA-RNA. Each strain was incubated for 0-3 hr at 37°C followed by RNA isolation. Equal amounts of total RNA were dot blotted then probed with $^{32}$P-oligo-dT. The quantitative evaluation of dot blots is shown. The mtrl-1 mutant maintains ~45% of the initial levels of polyA-RNA after 3 hr at 37°C. Accelerated loss of (cytoplasmic) polyA-RNA is seen in rpbl-1. A still more rapid loss is seen in mtrl-lrpbl-1.

Panel B  Turnover of polyA-RNA. Wild type and mtrl-1 were incubated at 37°C for 1 hr then recultured in the presence of thiolutin for 0-3 hr. Total RNA was isolated and analyzed as described above. As shown, the turnover of polyA-RNA is much slower in mtrl-1 than in wild type.
A

Total Cell-Associated Poly A+ RNA

37°C throughout

![Graph showing cpm hybridized vs. hr.](image_url)
B

Turnover of PolyA+ RNA

thiolutin

-1h  0h  +1h  +2h  +3h

37°C throughout

% of initial cpm

wt

mtr1

hr + thiolutin
Since at steady state ~90% of yeast polyA⁺RNA is in the cytoplasm (Groner and Phillips, 1975), it is estimated that when mtr1-1 has been incubated 1-2 hr at 37°C (i.e. under the conditions of the experiment described in the preceding paragraph), ~85% of the total polyA⁺RNA of the cell is in the nucleus. This is because the total polyA⁺RNA content of mtr1-1 remains roughly constant during this period, but ~85% of cytoplasmic polyA⁺RNA turns over (Figure 4A).

Reversibility of mRNA accumulation

The reversibility of mRNA accumulation was judged both by FISH and by measurement of protein synthesis. When mtr1-1 cells were incubated 3 hr at 37°C to cause intranuclear accumulation of polyA⁺RNA and were then returned to 23°C, the FISH signal returned to normal over 2-3 hr (data not shown). When cells were simply maintained at 37°C for the additional 3 hr, the signal did not change (not shown). Normalization was dependent on ongoing ATP production (Figure 5) but was not altered by 100μg/ml cycloheximide to inhibit protein synthesis.

The experiment described in the preceding paragraph shows that the distribution of polyA⁺RNA in mtr1-1 can be normalized by transfer of a 37°C culture to 23°C. These experiments do not however prove that the mRNA which was accumulated in the nucleus has passed to the cytoplasm. To
Figure 5  Reversibility of polyA+RNA accumulation and its requirement for ATP in mtr1-1

The mtr1-1 was incubated 3 hr at 37°C and subsequently returned to 23°C for increasing periods. At each time point 200-300 cells were examined and the fraction of cells exhibiting a distinct nuclear signal was counted. The FISH pattern rapidly returns to normal at 23°C. As illustrated, when the 23°C incubation is in the presence of 0.1 mM dinitrophenol and 0.5% deoxyglucose in glucose-free medium the rate of normalization of the pattern is greatly retarded.
check this possibility, mtrl-1 cells were incubated 1-3 hr at 37°C and then returned to 23°C in the presence of thiolutin and mixed ³H-amino acids. As illustrated in Figure 6A, protein synthesis does occur at 23°C. When the preincubation at 37°C is for 2-3 hr there is a lag; however, accumulated mRNA is ultimately translated. The mRNA structure must therefore have been substantially normal.

To examine reversibility in the context of a single defined mRNA, mtrl-1 was transformed with pLGSD5 which carries lacZ gene under GAL control (Guarente et al., 1982). When these cells were grown in raffinose at 23°C, no β-galactosidase activity was seen; however, growth on galactose at 23°C rapidly induced abundant activity (not shown). When the transformed cells were grown in the presence of galactose at 37°C, little enzyme activity was seen; however, if they were grown for 3 hr at 37°C with galactose and were subsequently incubated with glucose at 23°C, to block lacZ transcription, a major increase in enzyme activity was seen (Figure 6B). As in Figure 6A, a lag of several hours preceded the burst of activity. This burst was not observed in uninduced cells, indicating the dependency of lacZ transcription during 37°C incubation.

RNA processing in mtrl-1

To understand the basis of the mRNA export defect
Figure 6  Transcripts which accumulate in the nucleus of 
mtri-l at 37°C exit and are translated when the temperature 
is subsequently returned to 23°C

Panel A  The mtri-l was incubated 1-3 hr at 37°C and then 
returned to 23°C in the presence of thiolutin and $^{3}$H-amino 
acids to block further transcription and monitor protein 
synthesis. As illustrated, protein synthesis occurred at 
23°C. When the 37°C preincubation was for 2-3 hr, there 
was a lag period. When thiolutin was included both in the 
37°C preincubation and in the 23°C incubation, essentially 
no incorporation was seen.

Panel B  The mtri-l transformed with pLGSD5, a plasmid 
encoding lacZ under GAL10 control, was incubated at 37°C in 
the presence of galactose to induce lacZ transcription. 
After 3 hr, the cells were transferred to glucose-
containing medium to terminate transcription for 0.7 hr at 
23°C. As illustrated, a burst of $\beta$-galactosidase activity 
was seen after a lag (a). Little activity was seen if 
galactose was not added during the 37°C incubation (b).
observed in the mtrl-1 mutant, the structures of the 5' cap
and 3' polyA tail of the average polyA•RNA were analyzed.

Wt and mtrl-1 mutant cells were labeled with ³H-
methionine before or after temperature shift, then
polyA•RNA was isolated, enzymatically hydrolyzed and the
products were fractionated by HPLC. As shown in Figure 7,
the polyA•RNA synthesized at 23°C and at 37°C has a m⁷G cap
in both wt and mtrl-1.

The polyA tail length of total polyA•RNA in wt and
mtrl-1 was analyzed by 3'end labelling with ³²pCp and RNA
ligase followed by RNase A digestion and gel
electrophoresis. In wt cells, the distribution of polyA
tail length remains roughly constant during 37°C
incubation. In mtrl-1 at 37°C the average length increases
with time (Figure 8). This result is consistent with the
fact that the polyA tail of mRNA progressively shortens in
the cytoplasm (Sachs, 1990). It also strongly suggests
that 3'end formation of mRNA in mtrl-1 is normal at 37°C
and that mRNA is indeed accumulated in the nucleus where
its polyA tail does not shorten. Taken together, the
results suggest that mutation of MTR1 does not affect the
structure of mRNA (some mRNAs have extended sequence at
both 5' and 3' ends as described below) but rather the
machinery of mRNA transport.

The extent of pre-mRNA splicing was analyzed in mtrl-1.
Figure 7  Analysis of 5' cap structure

Both wild type and mtrl-1 strains were grown 10 min at 23°C or 37°C then labeled 40 min at the same temperature. PolyA-RNA was isolated, enzymatically hydrolyzed and the products were resolved by HPLC. $A_0$ and $G_0$ designate the labeled capped dinucleotides with A and G in the second position. $X$ is unidentified. Clearly, the same capped dinucleotides are present in each sample.
Figure 8  Analysis of polyA tail length

Both wild type and mtrl-l strains were incubated 0, 1, 2, or 3 hr at 37°C. PolyA\(^+\)RNA was recovered, labeled at its 3' terminus and digested with RNase A to remove but the polyA tail. The products were analyzed on a 12% polyacrylamide-8M urea gel. The average tail length increases progressively at 37°C for mtrl-l.
Using CRY1 (Larkin et al., 1987) as a probe on Northern blots (CRY1 is a ribosomal protein gene that contains intron), an accumulation of pre-mRNA was observed in prp5-1 (known to be ts for pre-mRNA splicing) at 37°C but not in mtr1-1. Moreover, two "oversized" CRY1 transcripts were specifically synthesized in mtr1-1 at 37°C, but not in prp5-1. Similar oversized transcripts are produced in prp20-1 (see following sections), where they reflect incorrect transcriptional initiation and termination (Figure 9). When pre-mRNA splicing was checked using an actin intron as a probe, an accumulation of actin pre-mRNA was not detected in mtr1-1 over 3 hr at 37°C (data not shown).

The synthesis and processing of rRNA in mtr1-1 was analyzed by pulse-chase labeling with 3H-methionine. As shown in Figure 10A, synthesis of rRNA in mtr1-1 is severely reduced after 30 min at 37°C and processing of 35S pre-rRNA to 27S and 20S rRNA is very slow, judging from the persistence of 35S pre-rRNA throughout the chase period. Furthermore, mature 25S and 18S rRNA do not accumulate during the chase period, indicating a defect of processing from 27S to 25S and 20S to 18S rRNA. The rRNA processing defect is not simply due to the alteration of rRNA methylation since comparable defects were observed in 3H-uridine labeling experiments (data not shown).

3H-uridine labeled RNA in mtr1-1 was also analyzed on
Figure 9  Detection of CRY1 transcripts in wild type, mtr1-1 and prp5-1 strains

Both normal sized (cry) and oversized CRY1 transcripts are detected in mtr1-1 at 37°C. The oversized transcripts are indicated with arrowheads pointing to the right. Only in the prp5-1 strain is the pre-mRNA seen (arrowhead pointing to the down). s designates a small RNA, snRNA189, which is also detected by the probe containing both CRY1 and snRNA189.
Figure 10  Analysis of rRNA and tRNA processing

Panel A  Wild type and mtr1-1 strains were preincubated at 37°C then labeled for 3 min with ^3H-methionine followed by 0, 5 or 10 min chase. The labeled RNA was electrophoresed on a 1% formaldehyde-agarose gel. Lanes 1-3 and 4-6 show RNA isolated from mtr1-1 and wt, respectively. Lane 1 and 4 are samples withdrawn at the end of the pulse. Lanes 2 and 5: 5 min chase. Lanes 3 and 6: 10 min chase. In the mutant, by comparison to wild type, all processing steps appear to be slowed.

Panel B  Wild type (w, lane 1 and 4), mtr1-1 (m, lane 2 and 5) and rna1-1 (r, lane 3 and 6) strains were preincubated at 25°C or 37°C for 30 min then labeled for 10 min with ^3H-uridine at the same temperature. The labeled RNA was electrophoresed on a 10% polyacrylamide-8M urea gel. The positions of 5.8S and 5S rRNA are indicated by arrowheads. Pre-tRNAs and mature tRNAs are designated by p and t, respectively.
a 10% polyacrylamide-8M urea gel to monitor the processing of small RNAs. Synthesis of 5.8S rRNA is severely reduced and 5S rRNA synthesis is also moderately inhibited at 37°C. The processing of pre-tRNA in mtrl-1 is inhibited. Pre-tRNA species accumulate, as in rna1-1 at 37°C (Figure 10B).

Identification of MTRl gene

To clone the MTRl gene, YTK102 (mtrl-1) was transformed with a YCp50 yeast genomic DNA library (Rose and Broach, 1991) and plasmids were rescued from several TS+ transformants. All plasmids were identical judging from their restriction enzyme maps. Subcloning of the original complementing DNA fragment yielded 5.3 kb SalI-XbaI DNA fragment that retained mtrl complementing activity. Its restriction enzyme map is identical to that of SRM1/PRP20 gene which is essential for growth (Aebi et al., 1990; Clark and Sprague, 1989). Both prp20-1 and srm1-1 ts mutants grow at 37°C after transformation with the plasmid containing the MTRl gene. None of the five strains (including mtrl-1, mtrl-2, and mtrl-3) complement each other (all pairwise combinations have been tested). These results show that each mutant has a mutation in the same gene. The lack of intragenic complementation may suggest that MTRl acts as monomer. The mammalian, Drosophila and S. pombe homologs of MTRl are RCC1 ("rapid chromosome condensation") (Ohtsubo et al., 1987; Uchida et al., 1990),
BJ1 (Frasch, 1991), and pim1* ("premature initiation of mitosis") (Matsumoto and Beach, 1991), respectively. These genes were first identified as regulators of cell cycle checkpoint control. Checkpoint control is a mechanism that prevents cells to enter mitosis without completion of DNA synthesis. It is a system to couple DNA synthesis and mitotic entry. Although the previously known functions of these genes may seem different from the S. cerevisiae homolog, the essential functions of RCC1, BJ1, and MTR1 are largely interchangeable (Clark et al., 1991; Fleischmann et al., 1991; Ohtsubo et al., 1991).

Distribution of polyA+RNA in five different alleles of mtr1 mutants

Since prp20-1 and srml-1 mutants were originally isolated in investigations of pre-mRNA splicing (Vijayraghavan et al., 1989) and pheromone responsiveness (Clark and Sprague, 1989), we asked whether, like mtr1-1 they also accumulate polyA+RNA in the nucleus when incubated at 37°C. As shown in Figure 11, only mtr1-1, mtr1-2, mtr1-3, and prp20-1 exhibit nuclear polyA+RNA accumulation, when analyzed by FISH. The cytoplasmic fluorescent signal of srml-1 remains uniform and strong at 37°C and nuclear staining is not increased. Thus, mtr1-1, mtr1-2, mtr1-3, and prp20-1, but not srml-1, appear to be defective in mRNA export at 37°C.
Cell cycle progression in mtr1 mutants

The smnl-1 mutant was originally characterized as showing accumulation in G1 and transcribing mating factor-specific genes at the restrictive temperature, even in the absence of mating factor (Clark and Sprague, 1989). The appearance of cells with projections was also observed at 33°C. We checked whether other mtr1 alleles also show cell cycle arrest at 33°C or 37°C by tabulating their budding index. No specific cell cycle arrest is seen at 37°C, but the fraction of unbudded cells increases notably at 33°C (74-87%) in mtr1-1, mtr1-2, mtr1-3 and prp20-1 mutants. This result suggests that partial loss of function of MTR1 gene slows entry into or progression through G1.

We have inquired whether incubation of mtr1 mutants at 37°C may lead to loss of checkpoint control by incubating each mutant +/- hydroxyurea at 37°C and then estimating survival upon plating at 25°C. The survival of mtr1 mutants, like the sec1 mutant used as a control, is reduced only ~2X due to the presence of hydroxyurea (not shown).

Comparison of under and over expression of MTR1

The pleiotropic effects of mutation of the MTR1 gene make it difficult to understand the function of this gene. We therefore looked at the effects of total loss-of-function of MTR1 gene by creating a yeast strain in which MTR1 gene is under control of GAL1 promoter. One copy
Figure 11  Distribution of polyA+RNA in five different mtr1 mutants

The indicated strains were incubated at 37°C for 3 hr and then fixed and processed for FISH. As illustrated, in all alleles except srml-1, a conspicuous signal is seen. It coincides with DAPI stain of nuclei. No nuclear intensification was seen when the strains were examined after growth at 23°C.
of MTR1 genes in diploid strain is disrupted by the insertion of LEU2 marker followed by transformation with plasmid carrying URA3 and MTR1 and another plasmid carrying TRP1 and MTR1 that is driven by GAL1 promoter. After sporulation and tetrad dissection, LEU-URA-TRP+ spores are recovered then they are streaked on 5-FOA plate containing raffinose and low amount of galactose to eliminate URA3 plasmid. Resulting LEU-TRP+ strains have disrupted MTR1 on chromosome and plasmid carrying MTR1 under GAL1 promoter. These cells will not grow on 2% glucose or on 2% galactose, but will grow in the presence of low amounts of galactose supplemented with an additional carbon source. As shown in Figure 12A, cell growth ceases at 12 hr after shifting from 2% raffinose + 0.0075% galactose to 2% glucose. During glucose incubation, cells enlarge and aggregate. Both large budded and un budded cells come to dominate the population. Figure 12B shows the distribution of polyA+RNA in cells incubated for 24 hr in glucose. As obvious accumulation of polyA+RNA in the nucleus is observed in 40-50% of the cells. Thus, depletion of MTR1 blocks mRNA export.

Overexpression of MTR1 by transfer to 2% galactose is toxic. It does not appear to arrest at a single point in the cell cycle. It does reduce the titer of polyA+RNA throughout the cell (data not shown), indicating that MTR1
Figure 12 The effects of MTR1 depletion on cell growth and polyA-RNA distribution

Panel A Growth kinetics of YTK110 in medium with a mixture of raffinose and galactose or 2% glucose. The MTR1 depletion slows cell growth after ~10 hr.

Panel B The distribution of polyA-RNA in cells in which MTR1 transcription has been stopped for 24 hr. Roughly half of the cells give an obvious nuclear signal (coincident with DAPI), with very little residual cytoplasmic staining.
overexpression may inhibit mRNA transcription. These results suggest that the level of MTRl may be tightly regulated in the cells.

**Identification of point mutations in mtrl alleles**

The *S. cerevisiae* MTRl resembles mammalian, *Drosophila*, and *S. pombe* homologs in including seven approximate glycine-rich repeats (Figure 13). Only the N and C terminal regions are closely conserved among seven units and only these regions are highly conserved in different species (Ohtsubo et al., 1991).

To understand structure-function relationships of this protein, the sites of mutation in *mtrl-1, l-2, l-3*, and *srml-1* have been identified. As shown in Figure 13, *mtrl-1, l-2*, and *l-3* each changes a glycine to serine or aspartate and these glycine residues are all located in the conserved N or C terminal regions of a repeat unit. Those which are mutated in *mtrl-1, l-2*, and *srml-1* (as well as for *prp20-1*) (Fleischmann et al., 1991) are also conserved among different species. As indicated, the *mtrl-3* glycine to aspartate is toward the end of a repeat but does not occur at a conserved glycine. The mutation in *tsBN2* (serine to phenylalanine) is near the end of the fourth repeat (Uchida et al., 1990). The sites of mutation in *piml-46* are not known. These results clearly indicate the importance of glycine for function of MTRl.
Figure 13  Identification of mutations in the MTR1 gene
The amino acid sequences of the seven approximate repeats of all known MTR1 homologs are illustrated. All sequences are continuous. The boxes enclose the mutated residues, which are, with one exception, in glycine residues near the ends of the repeat regions. The mutations in the second repeat are mtr1-2 (3rd residue) and srml-1 (11th residue), in the fourth repeat tsBN2, in the fifth repeat mtr1-1, in the seventh repeat prp20-1 and mtr1-3. The vertical arrowheads designate additional residues: In S. cerevisiae 67-68, NK; 135-163, SSDDEGDNLNELESTPAKIPRESFPPLAE; 366-379, KDQLPEYTYKDVHG; 430, P; 465, G. In S. pombe 158-174, LLEGTPSKVEGALSHL; 372-385, DNALPETVVKDEKG; 447, D; 472, G. The dashed regions are non-conserved amino acid sequences between different species.
Analysis of mammalian and *S. pombe* mutants

The mammalian BHK-21 ts mutant, tsBN2, and *S. pombe* pim1-46 ts mutant have mutations in MTR1 homologs. The tsBN2 mutant was originally identified as a mutant which arrested in G1 and ceased DNA synthesis at the restrictive temperature (Nishimoto et al., 1978). Additionally, when tsBN2 cells are synchronized at G1/S before temperature shift, many cells undergo rapid premature chromosome condensation. The *S. pombe* pim1-46 mutant also undergoes chromosome condensation at 37°C, even when DNA synthesis is inhibited (Matsumoto and Beach, 1991).

Since ts mutations in the *S. cerevisiae* homolog of RCCL and pim1* cause nuclear accumulation of polyA⁺RNA, we asked whether tsBN2 and pim1-46 also accumulate polyA⁺RNA at the restrictive temperature. When tsBN2 cells were analyzed by FISH, nuclear accumulation of polyA⁺RNA became striking after 12 hr at 39.5°C, as shown in Figure 14A. During the 39.5°C incubation, the nuclear signal progressively increased while the cytoplasmic signal faded (data not shown). When actinomycin D or 6-amanitin was added during the 39.5°C incubation, the nuclear signal decreased, as expected (data not shown). The tsBN2 cells which undergo chromosome condensation at 39.5°C did not exhibit polyA⁺RNA accumulation in "micronuclei" (Figure 14B). The polyA⁺RNA distribution in wt BHK-21 cells did
not change during 39.5°C incubation. When tsBN2 cells incubated 12 hr at 39.5°C were reincubated at 33°C, the distribution of polyA•RNA gradually returned to normal over the following 12 hr (data not shown).

Analysis of S. pombe piml-46 is shown in Figure 15. In wt S. pombe at 25°C or 37°C and in piml-46 at 25°C, the FISH differed in two respects from that of S. cerevisiae. First, cytoplasmic polyA•RNA was not uniformly distributed. Instead, there were bright spots in the cytoplasm which did not obviously correspond to cytoplasmic DAPI stain (mitochondria). Second, the nuclear region that was not stained by DAPI exhibited a relatively weak fluorescent signal, suggesting a low polyA•RNA content in nucleolus. In most piml-46 cells after 2 hr at 37°C one or two bright regions were visible in the nucleus. The FISH signal and DAPI signal did not always overlap, suggesting that polyA•RNA may preferentially accumulate in a chromatin-poor region of nucleus (most likely nucleolus). A comparable nuclear signal was seen when the 37°C incubation includes cycloheximide. When piml-46 is incubated for longer at 37°C, chromosome condensation is seen (~15% at 3 hr, 50% at 3.5 hr) (Matsumoto and Beach, 1991). For example, after 4 hr at 37°C, most piml-46 cells have condensed chromosomes and a septum (indicating continued progression through the cell cycle).
Figure 14  PolyA-RNA distribution in BHK-21 and tsBN2 cells

Panel A  Both BHK-21 and tsBN2 cells were incubated 12 hr at 33°C or 39.5°C then fixed and processed for FISH. In tsBN2 the accumulation of polyA-RNA in the nucleus was striking at 39.5°C.

Panel B  The tsBN2 cells were incubated 12 hr at 39.5°C then analyzed as above. The cell with micronuclei (designated by an arrowhead) that had committed pre-mature mitosis did not accumulate polyA-RNA in the nucleus. The identical field is shown by DIC, FITC (polyA-RNA) and DAPI (DNA).
Figure 15  PolyA'-RNA distribution and chromosome condensation in *S. pombe* *piml*-46 mutant

Top: Wild type (SP6) was fixed and processed for FISH after 0 or 2 hr at 37°C.

Bottom: The *piml*-46 (SP1032) was fixed and processed for FISH after 0, 2 or 4 hr at 37°C.

Nuclear polyA'-RNA was easily detected at 2 hr, i.e., before chromosome condensation. At 4 hr when chromosome condensation was evident, the nuclear FITC signal persists only in those cells in which condensation had not occurred (arrow).
These cells have lost much of their nuclear FITC signal. One of the rare cells in Figure 15 (arrow) that still exhibits nuclear and relatively high cytoplasmic signal has non-condensed chromatin. These results suggest that commitment to mitosis may stop mRNA transcription or induce mRNA breakdown in *S. pombe*.

**The effects of mutations affecting protein synthesis and the cell cycle on the mRNA export defect**

In tsBN2 and *piml-46* at the restrictive temperature, p34<sup>cdc2</sup> kinase is activated before chromatin condensation (Nishitani et al., 1991; Matsumoto and Beach, 1991). Genetic studies with double mutants show that kinase activation is required for chromosome condensation in *piml-46* (Matsumoto and Beach, 1991). Protein synthesis is also necessary for kinase activation in tsBN2. This is also true for *piml-46*, since when *piml-46* is shifted to 37°C with cycloheximide, chromatin does not condense (data not shown). Thus, by two criteria, the consequences of loss of function of RCC1 and *piml* on chromosome condensation are indirect.

To check the possible involvement of p34<sup>cdc28</sup> kinase activation and protein synthesis in the mRNA export block in *mtr1-l*, we constructed *mtr1-lcdc28-l*, *mtr1-lcdc28-lN* and *mtr1-lprtl-l* double mutants. The *cdc28-l* and *cdc28-lN*
mutant arrests at G1 and G2 at 37°C, respectively (Surana et al., 1991). The prtl-1 is a ts mutation for translation initiation (Hartwell and McLaughlin, 1969). As shown in Figure 16, the double mutants exhibit nuclear polyA-RNA accumulation at 37°C. Thus, the mRNA export block does not require p34\(^{CDC28}\) kinase activation or protein synthesis.

In addition, we have recently checked polyA-RNA accumulation in *S. pombe* piml-46cdc2 and piml-46cdc25 double mutants. As shown in Figure 17, these double mutants also have an mRNA export defect at 37°C.
Figure 16  Distribution of polyA+RNA in *S. cerevisiae*

single and double mutants

Each cell type was incubated 3 hr at 37-38.5°C then
processed for FISH. A and B: *mtr1-l*, C and D: *prt1-l*, E
and F: *mtr1-lprt1-l*, G and H: *cdc28-l*, I and J: *mtr1-lcdc28-
1*, K and L: *cdc28-1N* and M and N: *mtr1-lcdc28-1N*. A-M and
B-N represent polyA+RNA and corresponding DAPI images,
respectively. Clearly neither CDC28 activation nor PRT1
function is required to give a strong nuclear FISH signal.
Cells maintained at 23°C showed uniform cytoplasmic and
nuclear fluorescence. In the FISH images, the relatively
dark area corresponds to vacuoles.
Figure 17  Distribution of polyA•RNA in S. pombe single and double mutants

Each cell type was incubated at 37°C for 2 hr followed by FISH analysis. A-E show polyA•RNA distributions and F-J are the corresponding DAPI staining patterns. The single mutants, cdc2 (A and F) and cdc25 (B and G), do not exhibit nuclear polyA•RNA accumulation at 37°C. The double mutants, piml-46cdc2 (D and I) and piml-46cdc25 (E and J), accumulate polyA•RNA in the nucleus at 37°C like piml-46 (C and H). Thus, the functions of cdc2 and cdc25 are not involved in the nuclear accumulation of polyA•RNA in piml-46.
DISCUSSION

The phenotypes of mtrl-1 mutant

Several lines of evidence strongly suggest that mtrl-1 is defective in mRNA export; i) polyA·RNA accumulation in the nucleus is visible by FISH, ii) the kinetics of protein synthesis at the restrictive temperature is similar to rpbl-1 and rnal-1 mutants, iii) translation of single and bulk mRNA can be re-initiated upon shift to a permissive temperature, iv) polyA tail length is increased. As shown by dot blots and the "thiolutin block test" experiments, nuclear polyA·RNA is very stable relative to cytoplasmic polyA·RNA. This feature enabled us to isolate mtr mutants by examining nuclear polyA·RNA accumulation using FISH.

Consistent with data on reversibility of mRNA export in mtrl-1, the structure of mRNA (5' cap and polyA tail) synthesized at the restrictive temperature is normal. This suggests that mtrl-1 mutation affects mRNA export machinery instead of mRNA structure.

The effects of the mtrl-1 mutation on RNA processing are pleiotropic. The production of "oversized" CRY1 transcripts is similar to that observed in prp20-1 and rnal-1 (Forrester et al., 1992). Those transcripts are produced by the alteration of transcriptional initiation and termination, indicated by the extension in both 5' and 3' ends. In addition, our data suggest that the mtrl-1
mutation accelerates the turnover of cytoplasmic polyA+RNA. It is not clear how MTR1 is able to affect the stability of cytoplasmic mRNA because it is a nuclear protein. The recent report showing the cytoplasmic localization of MTR1 in mtrl mutant alleles at the restrictive temperature suggests that MTR1 might re-cycle between nucleus and cytoplasm (Amberg et al., 1990). If this is true, MTR1 could have effects on cytoplasmic mRNA. Interestingly, the phenotypes of mtrl and rnal-l are almost identical (RNA processing and transport defects) but RNA1 is known to be a cytoplasmic protein (Hopper et al., 1990). These results may imply the processing of RNA in nucleus and cytoplasm is regulated by proteins in both compartments (ex. chapter 4). Not only mRNA processing and transport but also rRNA and tRNA processing are inhibited in the mtrl-l mutant at 37°C. This is again similar to the rnal-l mutant. In summary, MTR1 is suggested to have multiple roles on the processing and transport of RNA in nucleus and cytoplasm.

Identity and structure of the MTR1 gene

The result of cloning of MTR1 gene shows it is identical to the SRM1/PRP20 gene. As described, the phenotypes of prp20-l are similar to those of mtrl-l. The phenotypes of srml-l is extremely different: At the non-permissive temperature, srml-l appears to continue to synthesize and export mRNA, but accumulates in G1.
transcribes mating specific genes in the absence of mating
factor and exhibits chromosome instability (Clark et al.,
1991). The phenotypes of srml-1 at 33-37°C may result from
partial loss-of function of this gene product.

The mapping of mtr1-1, l-2, l-3, prp20-1 and srml-1
mutations revealed that the conserved extremities of the
repeat domains are important for function. The point
mutation in tsBN2 is also near the end of a repeat domain.
In each of these mutants, except for mtr1-3, glycine
residues which are particularly well conserved have been
changed to either aspartate, glutamate or serine. Glycine
residues at different positions clearly have different
roles judging from the difference of phenotype of mtr1 and
prp20-1 v.s. srml-1 mutants. It will be interesting to
know how these glycine residues are aligned in three
dimensions.

Since the MTR1 gene product is involved in mRNA export
in S. cerevisiae, we inquired whether tsBN2 and piml-46
mutants also accumulate polyA⁺ RNA in the nucleus at 37-
39.5°C. Judging from FISH data, this is the case. For
tsBN2, this accumulation of nuclear polyA⁺ RNA requires
active transcription and can be observed with or without
pre-synchronization. Since those tsBN2 cells which undergo
premature mitosis and produce "micronuclei" show only a
weak uniform signal throughout cell, we suggest that once
cells commit to mitosis, they can not accumulate polyA·RNA in the nucleus. Data from pim1-46 (where the possible issue of integrity of the nuclear envelope is not a concern) support this hypothesis: After 2 hr at 37°C, almost all pim1-46 cells show a nuclear polyA·RNA accumulation. By 4 hr they lose both nuclear and cytoplasmic signals and exhibit condensed chromosomes and septa. Thus, the mRNA export defect is detected relatively early, occurring before p34cdc2 kinase activation (which increases ~80% after 3 hr in pim1-46 at 37°C and then declines) (Matsumoto and Beach, 1991) and chromosome condensation.

**Is there a causal relation between the interruption of RNA processing and transport and effects on cell cycle progression?**

Several observations indicate that MTR1 is involved in RNA processing and export with some specificity: 1) polyA·RNA accumulates in the nucleus in mtr1-1, l-2, l-3 and prp20 mutants at 37°C, upon MTR1 depletion and in related mutants in two other species, and 2) polyA·RNA accumulation in mtr1-1 and pim1-46 does not require protein synthesis or p34cdc2/cdc28 kinase function. Nevertheless, the MTR1 gene product clearly is important for multiple nucleoplasmic events.

Since RCC1 associates with chromatin, and since
elimination of RCC1 from Xenopus egg extracts blocks formation of DNA replication complexes, but not DNA elongation per se (Dasso et al., 1992), RCC1 may be a part of or regulate an abundant structural unit in the nucleoplasm. Such a structural unit may also interact with components of the transcriptional machinery, or equip polyadenylated RNA with factors which are essential for their transport. The importance of MTR1 for rRNA and tRNA processing may reflect comparable dependence on this structural unit.

Alternatively, the mRNA export lesion might be primary and lead to progressive depletion of nuclear proteins which are required for multiple events. For example, it is conceivable that mere depletion of critical cytoplasmic RNAs at the restrictive temperature in piml-46 and tsBN2 leads to activation of p34\(^{cdc2}\) kinase and chromosome condensation. Arguing against this possibility is the observation that G1/S synchronized BHK-21 cells treated at 39.5°C with actinomycin D or α-amanitin do not show chromosome condensation (data not shown). Moreover, the observation that premature chromatin condensation in tsBN2 does not absolutely require RNA synthesis (Nishimoto et al., 1981) argues against the possibility that nuclear accumulation of high levels of polyA\(^+\)RNA itself causes cell cycle progression. It therefore is most likely that--in
those organisms in which mutations of MTR1 homologs affect chromosome condensation--these effects are not mediated by gross alteration of RNA metabolism.

Chromosome condensation in tSBN2 and piml-46 at 37-39.5°C requires protein synthesis and (in S. pombe at least) p34cdc2 kinase activation (Matsumoto and Beach, 1991; Nishitani et al., 1991), unlike the nuclear accumulation of polyA+RNA. We therefore conclude that the consequences of loss of function of MTR1 homologs for RNA processing and transport are relatively direct. Also supporting these considerations is our observation that, in piml-46 at 37°C, accumulation of polyA+RNA in the nucleus does not require active p34cdc2 kinase. Furthermore, since in S. cerevisiae (and apparently in S. pombe -- S. Sazer, personal communication) loss of MTR1 (or piml+) function does not cause loss of checkpoint control, the functions of this gene, although affecting several classes of RNA, appear more limited than for its homolog in animal cells.

RCC1 promotes guanine nucleotide exchange by a small nuclear GTPase of the ras superfamily, RAN/TC4 (Bischoff and Ponstingl, 1991), which is highly homologous to S. pombe spil (Matsumoto and Beach, 1991), the product of an extragenic suppressor of piml-46.

The S. cerevisiae homologues of these GTPases have also been identified by us and others (Kadowaki et al.,
1993; Belhumeur et al., 1993). The *S. cerevisiae CNR1/CNR2* genes encode products which are extremely similar to RAN/TC4 and spil and appear to interact with MTR1. They are able to suppress the temperature sensitivity of *mtr1* mutants in an allele specific manner.

We therefore suggest that MTR1/RCC1/pim1 act as guanine nucleotide exchange factors for small nuclear GTPases, CNR1&2/RAN&TC4/spil and their activation affects one or more factors which are important for RNA processing and transport in all three species.
MATERIALS AND METHODS

FISH

The FISH was carried out as previously described. The BHK-21 and tsBN2 cells grown on coverslips are washed twice with PBS then fixed in PBS containing 4% paraformaldehyde at room temperature for 15 min.

Dot blot analysis of polyA+RNA

10 μg of total RNA was used for dot blot analysis. The oligo-dT was end-labeled with Δ³²P-TTP and TdT. The hybridization was done in 2X SSPE, 5X Denhardt’s, 1% SDS, and 0.1 mg/ml salmon sperm DNA at 37°C. The membrane was then washed 2X with 2X SSC, 1% SDS at room temperature (10 min each), 3X with 2X SSC, 1% SDS at 37°C (10 min each), then 3X with 1X SSC, 1% SDS at room temperature (10 min each).

The effect of inhibition of ATP production on polyA+RNA distribution

YTK102 was cultured in SC medium at 23°C to 1 OD₆₀₀nm. An equal volume of 55°C prewarmed SC medium was added and incubated at 37°C for 2 hr. The cells were washed once with 37°C water and then in glucose-free SC medium containing 100μg/ml cycloheximide and suspended to the same medium at 37°C. Part of the cell suspension was fixed. The rest received an equal volume of 4°C SC medium containing 100 μg/ml cycloheximide or 4°C glucose-free SC
medium containing 0.2mM dinitrophenol, 1% deoxyglucose, and cycloheximide. The incubation was continued at 23°C and the cells were fixed after 1.5, 3.0 and 4.5 hr. The fixed cells were analyzed by FISH. Cycloheximide was included in these incubations since it did not influence the speed of normalization of mRNA distribution and since interruption of ATP production blocks protein synthesis.

**Reversibility of protein synthesis**

YTK102 was grown overnight in SD medium at 23°C to an OD$_{600nm}$ of 0.5-0.7. An equal volume of 55°C SD medium was then added and the incubation continued at 37°C for 1, 2 or 3 hr. After the appropriate interval, an equal volume of 4°C SD medium containing 6 μg/ml thiolutin and 0.2 mCi/ml $^3$H-amino acid mixture was added. Culture at 23°C was continued for 0-6 hr followed by determination of TCA insoluble radioactivity.

**Analysis of β-galactosidase activity**

The strains YTK100 and YTK102 were transformed with pLGSD5. Transformed cells were grown in SC-URA medium containing 2% raffinose to an OD$_{600nm}$ of ~1 at 23°C.

Galactose induction was achieved by adding an equal volume of 23°C or 55°C SC-URA medium with 4% galactose.

To evaluate the reversibility of lacZ mRNA transport at 23°C, galactose induction of cells transformed with pLGSD5 was performed for 2.5hr at 37°C as described above.
Control sample was remained in raffinose containing medium at 37°C. During 1-7 hr reincubation at 23°C, all samples contained 1.5% raffinose, 1% galactose and 2% glucose. 8-galactosidase activity was measured and expressed as described in (Legrain and Rosbash, 1989).

**DNA manipulation**

The `MTR1` gene was cloned by complementation of YTK102 with a yeast YCp50 genomic DNA library. Five independent `TS+` clones had the same insert DNA. The complementing DNA fragment was further defined by subcloning the insert DNA. The `mtr1-2` and `srml-1` alleles were rescued by introduction of BamH1-gapped pTK1-9 carrying the `MTR1` SalI-XbaI DNA fragment on pRS316 (Sikorski and Hieter, 1989) into YTK107 and SY1115. The `mtr1-1` and `mtr1-3` alleles were rescued by introduction of MluI and PstI-gapped pTK1-10 carrying the `MTR1` BgIII-SalI DNA fragment on pRS314 (Sikorski and Hieter, 1989) into YTK106 and YTK108. Mutations were identified by sequencing the original gapped regions of the plasmids described above. For `mtr1-1` the second position of codon 338 (G) was found to be A, for `mtr1-2` the first position of codon 109 (G) was found to be A, for `mtr1-3` the second position of codon 464 (G) was found to be A, and for `srml-1` the second position of codon 117 (G) was found to be A, showing that all are transition mutations.

In order to place expression of `MTR1` under control of
a GAL1 promoter, plasmids pTK1-11 and pTK1-12 contain the
MTR1 ORF DNA fragment made by PCR with KpnI-digested pTK1-9
as the template and the following primers,
GGAGATCTAATGGTCAAAAGAACAGTCG and GGAGATCTCTTAATCATCCATTCCA
TCC. After BglII digestion, PCR product was cloned into
the BamHI site between the GAL1 promoter and PGK terminator
on pRS314 (pTK1-11) and YEp357 (Myers, 1988) (pTK1-12).

For disruption of the MTR1 gene, pTK1-13 was
constructed as follows: the MTR1 BglII-SalI DNA fragment
was cloned into pBluescript SKII+ and then the internal
BamHI fragment (extending from -40 to +798) was replaced by
a LEU2 BglII-BglII DNA fragment.

**Yeast strains and tissue culture**

YTK110 was constructed as follows: One of the MTR1
genesis was disrupted by one-step gene replacement using XbaI
digested pTK1-13 in a diploid strain YPH501 (Sikorski and
Hiete, 1989). Gene replacement was confirmed by Southern
hybridization, using the PstI-XhoI MTR1 DNA fragment to
probe genomic DNA digested with PstI and XhoI. The
disruptant was then transformed with pTK1-9, followed by
sporulation and tetrad dissection. A LEU+ URA+ spore was
then transformed with pTK1-11 and a resulting LEU+ URA+ TRP+
clone was inoculated to a 5-FOA plate containing 2%
raffinose and 0.1% galactose. A 5-FOA resistant colony was
picked up and grown in SC-LEU, TRP medium containing 2%
raffinose and 0.0075% galactose.

The strains containing two ts mutations were constructed by crossing two parental ts mutants. After sporulation, tetrads containing two ts and two TS+ spores were selected. The ts spores were backcrossed to parental strains to verify their genotypes.

*S. pombe* strains were grown in YES medium (Guthrie and Fink, 1991). For synchronization, tsBN2 or BHK-21 cells were inoculated at 10⁴ cells/well (24 well dish), cultured 24 hr at 33.5°C in 0.25% serum in DMEM and then shifted to leucine-free DMEM with 5% dialyzed serum for 30 hr. They were then shifted to 10% serum-DMEM-2.5mM hydroxyurea for 24 hr at 33.5°C and finally transferred to 39.5°C in 10% serum-DMEM-2.5mM hydroxyurea.

**Hydroxyurea treatment**

Yeast strains were treated with 0.2M hydroxyurea (HU) in YEPD medium at 25°C (4hr, until they were synchronized with large buds). The temperature was then shifted to 37°C in the presence of HU for 6 hr. Serially-diluted samples were then inoculated to YEPD plates (lacking HU) at 25°C for several days and the number of colonies was counted.

**RNA analysis**

For Northern hybridization, 10μg of total RNA was electrophoresed on a 1.5% formaldehyde-agarose gel. The 2.2kb HindIII fragment of the CRY1 gene (including the
snr189 gene) and the XhoI-ClaI actin intron fragment were used as probes.

Cap methylation was evaluated by labeling YNZ1 and YNZ4 with \(^{3}H\)-methionine, isolating polyA'RNA, digesting it with RNase T2 and P1 nuclease and fractionating the products by HPLC (Camper et al., 1984).

PolyA tail length was evaluated according to (Sachs and Davis, 1989).

\(^{3}H\)-methionine and \(^{3}H\)-uridine labeling of rRNA and tRNA

YTK100 and YTK102 were grown in SD medium at 25°C then an equal volume of 48°C SD medium was added to yield a 3 ml culture of each. The incubation was continued at 37°C for 30 min, followed by addition of 60μCi/ml of \(^{3}H\)-methionine. After 3 min, 1 ml of cells was poured onto crushed ice in an Eppendorf tube and then centrifuged at 4°C. The rest of culture was adjusted to 0.5 mg/ml methionine and 1 ml samples of cells were collected as above after 5 and 10 min of continued incubation at 37°C. The extracted RNA was electrophoresed on a 1% formaldehyde-agarose gel and blotted to Genescreen membrane. The labeled RNA was detected by fluorography using EN\(^{3}\)HANCE.

EE1b(rnaI-l), pRS316 transformed YTK100 and YTK102 were grown in SC-URA medium at 25°C. An equal volume of 48°C SC-URA medium was added and the incubation was
continued at 37°C for 30 min, followed by labeling with 0.5 mCi/ml of ³H-uridine. After 10 min, cells were collected as above and total RNA was extracted. The labeled RNA was electrophoresed on a 1% formaldehyde-agarose gel or a 10% polyacrylamide-8M urea gel then processed for fluorography using EN³HANCE.
Yeast strains

RY260       MATa  ura3-52  rpbl-1
SY1115  MATa  leu2-3,112  ura3  trpl  his4  srml-1
prp20-2A     MATa  ura3-52  ade2-101  his3-Δ200  prp20-1
187        MATa  adel  ade2  ural  his7  lys2  tyr1  gall
            prtl-1
108        MATa  adel  ade2  ural  his7  lay2  tyr1  gall
            prp5-1
EE1b(rna1-1) MATa  rnh1::URA3  ura3-52  tyr1  his4  his7
            adel  gal-  rna1-1
185-3-4     MATa  adel  ade2  ural  his7  lys2  tyr1  gall
            leu2  cdc28-1
cdc28-1N  MATa  ade2-1  trpl-1  canl-100  leu2-3,112  ura3
            his3-11,15  GAL+  [psi+]  cdc28-1N
YTK100  MATa  ura3-52
YTK102     MATa  ura3-52  mtr1-1
YTK105     MATa  ura3-52  lys2-801  ade2-101  his3-Δ200
            leu2-Δ1  mtr1-1
YTK106     MATa  ura3-52  trpl-Δ1  mtr1-1
YTK107     MATa  ura3-52  leu2-Δ1  lys2-801  ade2-101
            mtr1-2
YTK108     MATa  ura3-52  trpl-Δ1  lys2-801  ade2-101
            mtr1-3
YTK109     MATa  ura3-52  pTK1-12
YTK110  MATα  ura3-52 ade2-101 lys2-801 his3-Δ200
        trpl-Δ63 leu2-Δ1 ΔMTR1::LEU2  pTK1-11
YTK111  MATα  ade1 ade2 his7 lys2 tyr1 mtr1-1 cdc28-1
YTK112  MATα  ade2 trpl-1 his3-11,15 ura3 mtr1-1
        cdc28-1N
YTK113  MATα  ade2 his7 lys2 tyr1 ura3 mtr1-1 prt1-1
YNZ1   ura-  ade-  met10
YNZ4   ura-  ade-  met10  mtr1-1
S. pombe
SP6    h-  leu1-32
SP32   h-  cdc25
SP353  h-  cdc2
SP1032  h-  pim1-46
SP1034  h-  pim1-46  cdc2
SP1036  h-  leu1-32  ade6-210  pim1-46  cdc25
CHAPTER 4

MTR2, An essential nuclear protein which governs mRNA transport within the nucleus and the distribution of nucleolar components in *Saccharomyces cerevisiae*

**SUMMARY**

One of the MTR genes, *MTR2*, has been cloned and shown to encode a novel 21 kD nuclear protein which is essential for vegetative growth. In a *mtr2-1* strain at 37°C, RNA polymerase II remains detectable throughout much of the nucleoplasm, but polyA⁺RNA accumulates within the nucleus in 2-3 foci. This is accompanied by an increase of polyA tail length. Pre-mRNA splicing, tRNA processing and rRNA export continue at 37°C. Thus, the *MTR2* gene product defines an intermediate along the path of mRNA export. At 37°C nucleolar antigens also redistribute to 2-3 foci in the nucleoplasm. Surprisingly, these foci coincide with the sites of polyA⁺RNA accumulation. We conclude that components of the nucleolus depend on factors which govern RNA export in the nucleoplasm.
INTRODUCTION

In this chapter, we characterize a second recessive mutant, mtr2-l, and the MTR2 gene. The consequences of mutation of MTR2 with regard to RNA processing appear to be much more specific than for MTR1. Only rRNA processing is slightly inhibited at 37°C. Moreover, in mtr2-l at 37°C, polyA RNA accumulates in multiple foci which are distinct from the DNA and RNA polymerase II stained regions. It is consistent with that mRNA transport has been interrupted at an intermediate step prior to arrival at the nuclear pores. Nucleolar antigens also accumulate in the same foci but other nuclear proteins are unaffected at 37°C. Similar fragmentation of nucleolus has been observed in mtr1-l, rna1-l and RNA polymerase I deficient strains. The relationship of the mRNA export block and nucleolar fragmentation is discussed in detail. The MTR2 is predominantly localized in the nucleus.
RESULTS

Distribution of polyA⁺RNA in the mtr2-1 mutant

The mtr2-1 mutant is one of the mtr mutants described in Chapter 2. At the restrictive temperature, this mutant exhibits the most rapid accumulation of nuclear polyA⁺RNA of all sixteen mtr mutants. As shown by FISH with biotinylated oligo-dT (Figure 18), an accumulation of polyA⁺RNA in the nucleus is evident after 20 min and this signal remains unchanged for up to 3 hr at 37°C. The polyA⁺RNA preferentially accumulates as two or three foci in the nucleus, as indicated by arrowheads in Figure 18. Such a signal is not visible when hybridization is performed with a biotinylated oligo-dA probe (sense probe). To learn whether this nuclear polyA⁺RNA accumulation is dependent on active RNA polymerase II, a mtr2-1rpbl-1 double mutant has been constructed. The rpbl-1 is a temperature sensitive mutation of RPBl that encodes the largest subunit of RNA polymerase II. This double mutant does not show this phenotype at the restrictive temperature (Figure 18). Furthermore, once polyA⁺RNA has been accumulated in the nucleus by incubation at 37°C, the FISH signal persists upon continued incubation for several hours at 37°C with the RNA polymerase inhibitor, thiolutin ("thiolutin block test"). This proves that the nuclear signal is not primarily a reflection of destabilization of
Figure 18  Distribution of polyA•RNA in wt, mtr2-1 and mtr2-1lrpb1-1

Distribution of polyA•RNA in YPH499 (wt), YTK200 (mtr2-1) and YTK202 (mtr2-1lrpb1-1) was analyzed by FISH at 23°C and after incubation at 37°C. Panel A, E and I illustrate the polyA•RNA distributions at 23°C. Panel B, F and J are the corresponding DAPI staining patterns. Panel C, G and K illustrate the polyA•RNA distributions after 30 min at 37°C. Note the foci of polyA•RNA visible in the mtr2-1 strain at 37°C (arrowheads in panel G). Panel D, H and L are the corresponding DAPI staining patterns.
cytoplasmic polyA RNA.

Protein synthesis of mtr2-1 gradually stops at 37°C, as expected if the pool of cytoplasmic mRNA is progressively reduced.

**The polyA tail length distribution in mtr2-1**

The polyA tail length increases in mtr1/prp20/srml and rna1-1 mutants at 37°C (Forrester et al., 1992; Kadowaki et al., 1993). This is presumably because the trimming of polyA tails occurs in the cytoplasm. As shown in Figure 19, an increase in average polyA tail length is also seen in mtr2-1 within 30 min at 37°C. The kinetics of nuclear polyA-RNA accumulation, judged from FISH (Figure 18), and polyA tail lengthening are roughly parallel, indicating they may both be satisfactory indicators of an inhibition of polyA-RNA transport.

**Pre-mRNA splicing in mtr2-1**

To learn whether the mtr2-1 mutation affects pre-mRNA splicing at 37°C, actin mRNA was analyzed by an RNase protection assay. As shown in Figure 20A, accumulation of actin pre-mRNA is not detected in mtr2-1 after 0.5 and 2 hr at 37°C, by contrast to prp5-1 which is ts for splicing. Splicing of a newly-transcribed pre-mRNA at 37°C was also checked by transforming wt and mtr2-1 with a plasmid (Acc⁰) which carries a galactose-inducible lacZ gene containing an out-of-frame synthetic intron (Legrain and Rosbash, 1989).
Figure 19  PolyA tail length distribution in mtr2-1
Total RNA isolated from YPH499 (wt; lane 1, 2 and 3) and
YTK200 (mtr2-1; lane 4, 5 and 6) after incubation at 37°C
for 0, 0.5 and 1 hr was 3' end labeled with 32pCp followed
by RNase A/T1 digestion. The products were analyzed on a
12% polyacrylamide-8M urea gel. As seen, the length of the
polyA tail increases with time at 37°C for the mtr2-1
strain.
Upon splicing, the mature mRNA codes for active β-galactosidase. As shown in the primer extension experiment illustrated in Figure 20B, after incubation for 2 and 4 hr at 37°C with galactose, equal amounts of mature lacZ mRNA (and pre-mRNA) are detected in wt and mtr2-1. Thus, the mtr2-1 mutation does not stop pre-mRNA splicing.

When β-galactosidase activity is measured in Acc° transformed cells or in cells transformed with its parent plasmid which lacks an intron, pLGSD5 (Guarente et al., 1982), mtr2-1 transformants incubated with galactose show very little activity at 37°C compared to 23°C. The ratio of β-galactosidase activity at 37°C to 23°C is ~40X higher in wt cells (Table 2). This is consistent with the expectation that lacZ mRNA synthesized in mtr2-1 mutant is not exported at 37°C.

The tRNA and rRNA processing in mtr2-1

The tRNA processing was analyzed in ³H-uridine pulse labeling experiments (Figure 21A). After preincubation at 37°C, the labeling of tRNA species in the mtr2-1 mutant is comparable to that of a wt strain and no accumulation of pre-tRNA species is observed, unlike rna1-1 and mtr1-1 mutants. As expected from the data on rRNA processing (below), the synthesis of 5.8S rRNA is reduced at 37°C.

The rRNA processing was analyzed in ³H-[methyl]methionine pulse-chase experiments after
Figure 20  Pre-mRNA splicing in mtr2-1

Panel A  Detection of actin pre-mRNA and mature mRNA by RNase protection assay. Total RNA isolated from YPH499 (wt; lane 1 and 2), 108 (prp5-1; lane 3 and 4) and YTK200 (mtr2-1; lane 5 and 7) at 23°C and after 2 hr incubation at 37°C was analyzed to detect pre-mRNA and mature mRNA. Lane 6 is total RNA isolated from YTK200 after 30 min incubation at 37°C. Yeast tRNA is used as a hybridization control (lane 8). MW designates $^{32}$P-labeled HaeIII fragments of pBR322.

Panel B  Detection of lacZ pre-mRNA and mature mRNA by primer extension. Total RNA was isolated from yeast transformed with a plasmid encoding a lacZ with a synthetic intron (Acc$^\circ$): SL7 (wt; lane 1, 2 and 3) and YTK203 (mtr2-1; lane 4, 5 and 6) at 0, 2 and 4 hr after galactose induction at 37°C followed by primer extension analysis. MW indicates $^{32}$P-labeled HaeIII fragments of pBR322. Both panels document the continued production of spliced mRNA in mtr2-1 at 37°C.
<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>prp5</th>
<th>mtr2-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mw</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>0hr</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>2'</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

↑ pre-mRNA

↓ mRNA
Table 2  
β-galactosidase activity in SL7 and YTK203 transformants

<table>
<thead>
<tr>
<th></th>
<th>pLGSD5</th>
<th></th>
<th>Acc&lt;sup&gt;o&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23°C</td>
<td>37°C</td>
<td>23°C</td>
</tr>
<tr>
<td>SL7</td>
<td>891.6</td>
<td>156.4</td>
<td>84.6</td>
</tr>
<tr>
<td>YTK203</td>
<td>63.4</td>
<td>0.24</td>
<td>7.5</td>
</tr>
</tbody>
</table>

β-galactosidase was measured in SL7 (wt) and YTK203 (mtr2-1) transformed with pLGSD5 or Acc<sup>o</sup> plasmid. The cells were preincubated for 20 min at 23°C or 37°C followed by galactose induction for 3 hr at the same temperature.
preincubation at 37°C. Accumulation of 35S pre-rRNA and reduced production of 27, 25, 20, 18S rRNA is evident in mtr2-1 after 3 min pulse labeling. After 10 min chase, mature 25 and 18S rRNA are visible but their appearance is slow relative to wt cells (Figure 21B). Similar defects of rRNA processing are observed with 3H-uridine labeled RNA, indicating that the illustrated inhibition is not due to alteration of rRNA methylation (data not shown). Since processing of 20S to 18S rRNA takes place in the cytoplasm (Udem and Warner, 1973) and 18S rRNA is produced at 37°C in this mutant, the mtr2-1 mutation certainly does not completely block pre-rRNA transport.

**Nucleolar structure in mtr2-1**

The yeast nucleolus forms a crescent in contact with the nuclear envelope. It is thought to accomplish rRNA processing and ribosome assembly (Smitt et al., 1973; Warner, 1989; Woolford, 1991). In the electron microscope, it is visualized as an electron-dense argentophilic crescent apposed to the inner aspect of the nuclear envelope (Potashkin et al., 1990).

Since we and others (Oakes et al., 1993) have observed that nucleolar structure is severely disorganized in the mtr1/prp20/srml mutants at 37°C, we have studied the organization of the nucleolus in mtr2-1 using monoclonal antibodies which recognize yeast nucleolar proteins. The
Figure 21  tRNA and rRNA processing in mtr2-l

Panel A  tRNA processing in mtr2-l. YPH499 (wt; lane 1 and 4), YTK200 (mtr2-l; lane 2 and 5) and EElb (rnl-l) (rnl-l; lane 3 and 6) were labeled with $^3$H-uridine for 10 min at 23°C or at 37°C after 20 min pre-incubation at 37°C. The labeled RNA was analyzed on a 10% polyacrylamide-8M urea gel.

Panel B  rRNA processing in mtr2-l. YTK200 (mtr2-l) and YPH499 (wt) were pulse-labeled with $^3$H-methionine for 3 min (lane 1 and 3; P) followed by 5 min chase (lane 2 and 4; +5) at 23°C. YTK200 and YPH499 were also pulse-labeled for 3 min (lane 5 and 8; P) after 20 min pre-incubation at 37°C followed by 5 min (lane 6 and 9; +5) and 10 min (lane 7 and 10; +10) chase at 37°C. The labeled RNA was analyzed on a 1% formaldehyde-agarose gel.
typical nucleolar morphology is unchanged in wt cells at 37°C, but it is grossly altered in mtr2-1 at 37°C (Figure 22). In most cells the nucleolus fragments to yield two or three foci. Even more extensive nucleolar fragmentation is observed in rna1-1 at 37°C. Thus, the mRNA transport defect is accompanied by disorganization of the nucleolus. Since comparable changes are not seen upon incubation with cycloheximide (Oakes et al., 1993), the observed changes are not due to the progressive inhibition of protein synthesis.

Other antibodies recognizing histone H2B and nuclear pore proteins were also used to analyze the nuclear structure of mtr2-1. The staining patterns obtained with them are comparable to those with wt cells at 37°C, suggesting that the mtr2-1 mutation specifically affects nucleolar structure.

Co-localization of polyA+RNA and nucleolar proteins

As shown in Figure 18, after 30 min at 37°C, mtr2-1 accumulates polyA+RNA as discrete foci in the nucleus which are reminiscent of the nucleolar foci detected by indirect immunofluorescence (Figure 22). Figure 23A shows that the accumulated polyA+RNA co-localized with these foci, while the chromatin-rich region of the nucleoplasm (DAPI stained region) has less polyA+RNA. Comparable observations were made with an antibody which recognizes a second nucleolar
Figure 22  Distribution of nucleolar antigens in mtr2-l

The nucleolar structure of YPH499 (wt; panel A, B, C and D), YTK200 (mtr2-l; panel E, F, G and H) and EE1b(rnal-l) (rnal-l; panel I, J, K and L) was monitored by indirect immunofluorescence using a monoclonal antibody recognizing nucleolar antigen (YN9C5). Cells were incubated at 23°C (left) or for 30 min at 37°C (right). Panels B, F and J and D, H and L are the corresponding DAPI images.
Figure 23  Co-localization of polyA-RNA and nucleolar proteins

Panel A  PolyA-RNA and nucleolar antigen were simultaneously detected in YPH499 (wt), YTK200 (mtr2-1) and YTK102 (mtr1-1) after 30 min (YPH499 and YTK200) and 80 min (YTK102) incubation at 37°C. DNA is visualized by DAPI. Comparable observations of colocalization with polyA-RNA have been made with a monoclonal antibody recognizing NOP1.

Panel B  PolyA-RNA and RPB3 are simultaneously detected in Z277 (expressing an epitope-tagged RPB3; labeled wt) and YTK204 (an mtr2-1 strain including the tagged RPB3; labeled mtr2-1) after 30 min incubation at 37°C. DNA is visualized by DAPI.
antigen, NOP1 (Aris and Bolobel, 1988). The polyA-RNA overlaps less extensively with RPB3, the third subunit of RNA polymerase II (Kolodziej et al., 1990) (Figure 23B). RPB3 staining is identical in wt and mtr2-1 at 37°C, as is histone staining. Thus, the sites of polyA-RNA accumulation in mtr2-1 at 37°C are distinct from the presumed sites of mRNA transcription.

**Identification of the MTR2 gene**

To clone the MTR2 gene, YTK200 was transformed with a YCp50 yeast genomic DNA library. A complementing 3.0kb EcoRI-XbaI DNA fragment was recovered (Figure 24A). Integration of this fragment into YPH259 (Sikorski and Hieter, 1989) followed by cross with YTK201 and analysis of tetrads proves that the complementing DNA encodes the MTR2 gene. The sequence of the 1.8kb DNA reveals one open reading frame which encodes a protein of 184 amino acids (Figure 24B). When this ORF is expressed from the GAL1 promoter in a mtr2-1 strain, the transformant is able to grow in galactose medium at 37°C but not in glucose. The predicted molecular weight of MTR2 is 21kD. It is a novel largely hydrophilic protein without significant homology to known proteins by BLAST search (Altschul et al., 1990). It does not have an obvious nuclear localization signal or RNA-binding motifs. When one MTR2 gene is disrupted (by insertion of LEU2) in a diploid strain and the diploid is
Figure 24  Sequence of MTR2 gene

Panel A  Restriction map of the 3 kb EcoRI-XbaI DNA fragment containing the MTR2 gene. The ORF of MTR2 is shown by the open box and the transcriptional direction is indicated by the arrow.

Panel B  The nucleotide and predicted amino acid sequence of MTR2. The two BamHI sites used for gene disruption are underlined. The site of the HA-epitope insertion is designated by arrowhead.
then sporulated, two spores are recovered from each tetrad. All viable spores are leu-, indicating that the MTR2 gene is essential.

**Localization of MTR2**

To determine the intracellular localization of MTR2, a construct encoding an HA-epitope tagged MTR2 was made. A nine-amino acid peptide sequence (YPYDVPDYA) was attached to the last amino acid (isoleucine) of MTR2. A centromeric plasmid carrying this construct can restore the viability of spores in which the endogenous MTR2 gene is disrupted, indicating that epitope tagged MTR2 is functional. At this low level of expression, detection of HA-epitope tagged MTR2 protein in YTK206 was not possible. After overexpression, however, MTR2 is detected by Western blotting in the crude nuclear fraction, just as is the nucleolar protein, NOP1. In order to detect epitope-tagged MTR2 by immunofluorescence it was necessary to drive its expression from GAL1 promoter. Thus, although the overexpression of this protein might affect its intracellular localization, MTR2 is likely to be concentrated in the nucleus.
DISCUSSION

Inhibition of mRNA export

The mtr2-1 mutant was recovered by use of suicide selection enrichment and screening by FISH to detect nuclear accumulation of polyA+RNA at 37°C. The nuclear polyA+RNA signal and the polyA tail length increase in mtr2-1 at 37°C strongly argue that mtr2-1 is defective in mRNA export. Further evidence of the mRNA export block comes from the observation of inhibition of protein synthesis and the lack of β-galactosidase activity in Acc0 and pLGSD5 transformants at 37°C, despite ongoing lacZ mRNA synthesis.

In mtr2-1 the focal accumulation of polyA+RNA in the nucleus is detected by FISH within 20 min at the restrictive temperature and its pattern does not change during 3 hr at 37°C. This is in contrast to mtr1-1, for which accumulated polyA+RNA ultimately appears to fill the nucleus. Moreover, mtr2-1 allows transcripts to be spliced, unlike ts allele of mtr1 (prp20-1). Both for mtr1-1 and for mtr2-1 mRNA accumulated at 37°C can be translated when the temperature is returned to 23°C. These observations suggest that mtr1-1 and mtr2-1 interrupt distinct steps of mRNA transport and that each may define an intermediate along the path.

The mtr2-1 mutation does not affect pre-mRNA splicing nor tRNA processing at 37°C. In addition, the synthesis of
mRNA, tRNA and rRNA continue at the restrictive
temperature. On the other hand, rRNA processing is slowed,
possibly as an indirect secondary consequence, considering
that the synthesis and processing of rRNA is under many
controls (Warner, 1989; Woolford, 1991). For example, the
rRNA processing defect could be due to the reduction of
protein synthesis in mtr2-1 mutant. The fact that 18S rRNA
is synthesized at 37°C indicates that rRNA transport to the
cytoplasm continues.

**Intranuclear distribution of polyA*RNA**

We postulate that the focal accumulation of polyA*RNA
in mtr2-1 corresponds to an exaggeration of a normally-
occurring less conspicuous intermediate along the path of
transport. Possibly the normal counterpart is finely
distributed throughout much of the nucleoplasm.

The sites of nuclear polyA*RNA accumulation in mtr2-1
at 37°C coincide with two nucleolar antigens, which are
concentrated in 2-3 foci per nucleus and show minimal
overlap with RNA polymerase II. There are several ways to
explain this focal co-distribution of polyA*RNA and
nucleolar antigens. For example, 1) newly synthesized
polyA*RNA is transported to the same foci to which
nucleolar antigens redistributed, 2) the yeast nucleolus
normally is on the path of mRNA transport and its functions
and organization are closely linked to polyA*RNA transport.
We are not able to distinguish these possibilities, but several considerations argue against the idea that nucleolar organization and polyA-RNA distribution/processing are normally closely linked: (A) an anatomically normal nucleolus is absent in a RNA polymerase I deficient strain which, nevertheless, is viable (Oakes et al., 1993), (B) extensive depletion of the multifunctional nucleolar protein, NOP1 (Tollervey et al., 1993), does not lead to nuclear accumulation of polyA-RNA, (C) interruption of rRNA processing in ts mutants KS7-1D (Shuai and Warner, 1991) and G1/7-5-13C (Fabian and Hopper, 1987) does not block mRNA export.

Our observations thus are best interpreted according to our first hypothesis, i.e., in mtr2-1 at 37°C the accumulated mRNA is transported to the same foci as nucleolar antigens. Possibly, the proteins which we detect, like certain proteins of animal cells (Borer et al., 1989; Pinöl-Roma and Dreyfuss, 1992) normally shuttle between the nucleus and cytoplasm. In this case, their redistribution to foci may reflect on interruption in their return from the cytoplasm to the nucleus.

A possible functions of the nucleolus in pre-mRNA splicing is, however, suggested by several observations: 1) 2,2,7-trimethylguanosine capped RNA (a determinant which is shared by snRNAs involved in mRNA and rRNA processing)
(Fournier and Maxwell, 1993) is concentrated in the nucleolus of yeast (Potashkin et al., 1990), 2) a protein of the U1 snRNP is concentrated in the nucleolus of yeast (Potashkin et al., 1990), and 3) the rex protein, which controls splicing of HTLV-1 pre-mRNA, is functional only when it includes a nucleolar targeting sequence (Nosaka, 1989). Nevertheless, most yeast PRP6, which is required for spliceosome assembly, is detected in the nucleoplasm (not the nucleolus), like spliceosomal components of animal cells (Elliott et al., 1992). In animal cells, most mRNAs are detected in the nucleoplasm but some mRNAs (ex. myc and myoD) are concentrated in the nucleolus (Bond and Wold, 1993). This may suggest that the nucleolus has functions in processing and transport of specific mRNA populations in animal cells. As mentioned, fragmentation of nucleoli is characteristically linked to inhibition of RNA polymerase I. The example of mtr2-1 demonstrates that other factors also control nucleolar coherence.

**Identification of MTR2 and its possible functions**

MTR2 is a novel 21 kD essential protein which is concentrated in the nucleus. The precise intranuclear localization of MTR2 is unknown but low abundance of MTR2 may suggest that it is not a structural protein of the nucleus nor an RNA binding protein. Thus, MTR2 might function as a regulator which controls mRNA export and
nucleolar structure, hence rRNA processing. In addition, the nucleolar fragmentation and polyA-RNA co-localization with nucleolar proteins are observed in both mtr2-1 and in mtr1-1, which has defect in guanine nucleotide release protein which acts on small nuclear GTPases (CNR1/2). MTR2 could be a down-stream effector or a GAP (GTPase activating protein) of these GTPases, but no suppression of temperature sensitivity was observed in mtr2-1 transformed with high copy number plasmids carrying either MTR1 or CNR1/2. Similarly, transformation of mtr1-1 with a high copy number plasmid carrying MTR2 did not rescue its temperature sensitive growth. To elucidate the function of MTR2, we will look for extragenic suppressors of the mtr2-1 mutant and determine precise intranuclear localization of MTR2.
MATERIALS AND METHODS

RNase protection assay and primer extension

The XhoI-BgII DNA fragment of the yeast actin gene (Ng and Abelson, 1980) was cloned in pBluescript SKII+ (pTACTL) and linearized with XhoI. The 32P-labeled probe was prepared using linearized plasmid and T3 RNA polymerase. 10 µg of total RNA isolated from YPH499, YTK200 and 108 incubated 0, 0.5 or 2 hr at 37°C was used for hybridization and digested with RNase T1. The products were analyzed on a 5% polyacrylamide-8M urea gel. Pre and mature actin mRNA correspond to the 487 and 241 nt bands, respectively.

10 µg of total RNA isolated from Acc° transformed SL7 and YTK203 were used for primer extension. The induction of lacZ transcription by 2% galactose for 0, 2 or 4 hr was initiated after 20 min preincubation at 37°C. The methods and interpretation of extension products are based on (Legrain and Rosbash, 1989).

Analysis of rRNA and tRNA processing

3H-methionine: YPH499 and YTK200 were grown in SC-MET medium at 23°C and 4.5X10⁷ cells (2X10⁷ cells/ml) were preincubated at 23°C and 37°C for 20 min by adding equal volume of 23°C and 50°C prewarmed medium, respectively. 3H-methionine (0.1mCi/ml) was then added for 3 min followed by removal and chilling of 1 ml of cells. Cold methionine
(0.5mg/ml) was immediately added and further 1 ml samples of cells were collected after 5 and 10 min. The labeled RNA was analyzed as described in chapter 3.

3H-uridine: pRS316 transformed YPH499 and YTK200 were grown in SC-URA medium at 23°C and 1.5x10⁷ cells (1.5x10⁷ cells/ml) were preincubated at 23°C and 37°C for 20 min by adding an equal volume of 23°C and 50°C prewarmed medium, respectively. Cells were then labeled with 0.5 mCi/ml of 3H-uridine for 10 min and the labeled RNA was analyzed as described in chapter 3.

**Analysis of polyA tail length distribution**

1 µg of total RNA isolated from YPH499 and YTK200 was labeled with 32pCp and RNA ligase, followed by RNase A/T1 digestion, as described (Minvielle-Sebastia et al., 1991). Digestion products were ethanol precipitated with sonicated salmon sperm DNA as carrier and analyzed on a 12% polyacrylamide-8M urea gel.

**Indirect immunofluorescence**

The fixation, spheroplasting and attachment of cells to poly-L-lysine coated slide was done as for FISH. The rest of the procedures was carried out as described (Guthrie and Fink, 1991). The monoclonal antibody YN9C5 (from J. Broach) recognizing an unidentified yeast nucleolar protein, the anti-NOP1 antibody (from J. Aris) and 12CA5 (recognizing the HA-epitope) were diluted 200
fold (in PBS containing 2% BSA). A 100 fold dilution of FITC-conjugated anti mouse IgG was used for detection.

Detection of polyA'RNA and nuclear proteins

For combined detection of polyA'RNA and nuclear proteins, cells were first processed as for FISH. Primary antibodies were added to a solution containing 1X SSC, 1% BSA and 2μg/ml FITC-avidin as for indirect immunofluorescence. Samples were then washed 5 times (10 min each) with 1X SSC and reincubated with Lissamine-rhodamine B-conjugated anti mouse IgG in 1X SSC, 1% BSA. Samples were washed twice with 2X SSC for 10 min each, twice with 2X SSC, 0.1% TX-100 for 10 min each and finally twice with 2X SSC for 10 min each.

Identification, cloning and sequencing of MTR2 gene

YTK200 was transformed with a Ycp50 yeast genomic DNA library and plasmids were rescued from several TS' transformants. All plasmids carried a common DNA fragment and the complementing DNA fragment was identified by subcloning. The 3kb EcoRI-XbaI fragment was cloned into the integration vector, pRS306 (URA3) (Sikorski and Hieter, 1989) to yield pTK200 and then linearized upstream of the MTR2 gene with BglII. The linearized plasmid was used for transformation of YPH259 (ura3-52) (Sikorski and Hieter, 1989) and resulting URA' transformants were crossed with YTK201 (mtr2-1 ura3-52). All tetrads were parental ditype,
indicating that plasmid DNA was integrated at the MTR2 locus. The sequence of the MTR2 BglII-XbaI DNA fragment was determined using a series of nested deletions created by ExoIII/Mung bean nuclease.

**Disruption of MTR2 gene**

The 3 kb EcoRI-XbaI DNA fragment including MTR2 was cloned in pBluescript SKII+ to yield pTK201. The internal BamHI-BamHI fragment was then replaced with a BglII-BglII DNA fragment carrying the LEU2 gene from PS118 (Silver et al., 1988), to yield pTK202. pTK202 was cut in vector sequence with SacI and XhoI and used for transformation of YPH501 (Sikorski and Hieter, 1989). The disruption of MTR2 in LEU+ transformants was checked by Southern hybridization probing BglII/XbaI digested genomic DNA with the MTR2 BglII-XbaI DNA fragment.

**Epitope tagging of MTR1 and MTR2**

Epitope tagging of MTR2 was performed by PCR, using linearized pTK201 and the following primers, primer 1: GGAGATCTTTATGAACACCAATAGTAATA, primer 2: GGAGATCTACTAAGCGTAGTCTGGGACGTCGTATGGGTAAATTTTTAGCAGAGAATCCT, and primer 3: GGAGATCTACTAAATTTTTAGCAGAGAA. The combination of primer1/2 and 1/3 results in the production of C-terminal HA-epitope tagged and untagged MTR2, respectively. PCR products were digested with BglII followed by cloning in pGAP316 (Kadowaki et al., 1993). pTK203 (untagged MTR2) and pTK204
(tagged MTR2) were constructed by replacing the GAL1 promoter with the endogenous MTR2 promoter. The construction of YTK205 and YTK206 involved disrupting one copy of MTR2 genes in YPH501, transformation with pTK203 (or pTK204), followed by sporulation. URA+ LEU+ spores were recovered.

Yeast strains

The double mutant (mtr2-1rpb1-1) YTK202 was created by crossing YTK207 and RY260. For construction of YTK203 and YTK204, the mtr2-1 mutant allele was first rescued by introducing BglII and BamHI gapped pTK205 (which carries a 3kb MTR2 EcoRI-XbaI fragment in pRS316) into YTK200. The rescued DNA fragment containing the mtr2-1 allele was then cloned in pBluescript SKII+ to yield pTK206. pTK207 and pTK208 were then constructed from pTK206 by inserting a HindIII-HindIII fragment carrying URA3 from pFL20 and the BglII-BglII fragment carrying LEU2 from PS118 into the BglII site of pTK206, respectively. pTK207 and pTK208 were each digested with SacI and XhoI (which cut in vector sequence) then used for transformation of Z277 (Kolodziej et al., 1990) and SL7, respectively to yield strains YTK204 and YTK203. Gene replacement was checked by Southern hybridization probing genomic DNA restricted with EcoRI and XbaI with the MTR2 BglII-XbaI DNA fragment.
Plasmid list

pTACT1  S. cerevisiae ACT1 XhoI-BglII DNA fragment cloned in pBluescript SKII+
pTK200  S. cerevisiae EcoRI-XbaI DNA fragment including MTR2 cloned in pRS306
pTK201  The same DNA fragment as pTK200 cloned in pBluescript SKII+
pTK202  The BamHI-BamHI DNA fragment of MTR2 in pTK201 replaced by LEU2 BglII-BglII DNA fragment
pTK203  Wild type MTR2 ORF made by PCR expressed from its own promoter and terminated by PGK terminator. Based on pRS316
pTK204  Same as pTK203 except MTR2 is tagged with a HA epitope at its C-terminus
pTK205  The same DNA fragment as pTK200 cloned in pRS316
pTK206  The mtr2-1 allele EcoRI-XbaI fragment cloned in pBluescript SKII+
pTK207  URA3 HindIII-HindIII DNA fragment cloned in the BglII site of pTK206
pTK208  LEU2 BglII-BglII DNA fragment cloned in the BglII site of pTK206
pTK209  Same as pTK203 except MTR2 is under GAL1 promoter
pTK210  Same as pTK204 except MTR2 is under GAL1 promoter
pTK211  Same as pTK204 except plasmid is based on YEp357
Yeast strains

YPH259
MATΔ ura3-52 lys2-801 ade2-101
his3-Δ200 leu2-Δ1

YPH499
MATα ura3-52 lys2-801 ade2-101
his3-Δ200 trpl-Δ63 leu2-Δ1

YPH501
MATαΔ ura3-52/ura3-52
lys2-801/lys2-801 ade2-101/ade2-101
his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1
trpl-Δ63/trpl-Δ63

RY260
MATα ura3-52 rpbl-1

YTK102
MATΔ ura3-52 mtr2-1

108
MATα adel ade2 ura1 his7 lys2 tyr1
gal1 prp5-1

EE1b(rnal-1)
MATα rnh1::URA3 ura3-52 tyr1 his4
his7 adel rnal-1

YTK200
MATα mtr2-1 ura3-52 lys2-801
ade2-101 his3-Δ200 leu2-Δ1 trpl-Δ1

YTK201
MATα mtr2-1 ura3-52 lys2-801
ade2-101 trpl-Δ1

YTK202
MATα mtr2-1 rpbl-1 ura3-52

SL7
MATα ura3-52 leu2 GAL+

YTK203
MATα ura3-52 leu2 GAL+ mtr2-1::LEU2

Z277
MATα ura3-52 lys2-128Δ leu2-3,112
his4-912Δ RPB3-1::LEU2
YTK204  MATa  ura3-52 lys2-128Ø leu2-3,112
         his4-912Ø RPB3-1::LEU2 mtr2-1::URA3
YTK205  MATα  ura3-52 lys2-801 ade2-101
         his3-Δ200 leu2-Δ1 trp1-Δ63
         ΔMTR2::LEU2 [pTK203]
YTK206  MATα  ura3-52 lys2-801 ade2-101
         his3-Δ200 leu2-Δ1 trp1-Δ63
         ΔMTR2::LEU2 [pTK204]
YTK207  MATα  mtr2-1 ura3-52 lys2-801
         ade2-101 his3-Δ200 leu2-Δ1 trp1-Δl
YTK208  MATα/α ura3-52/ura3-52
         lys2-801/lys2-801 ade2-101/ade2-101
         his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1
         trp1-Δ63/trp1-Δ63 ΔMTR2::LEU2/MTR2
YTK209  MATα  ura3-52 lys2-801 ade2-101
         his3-Δ200 leu2-Δ1 trp1-Δ63 ΔMTR2::LEU2
         [pTK211]
CHAPTER 5

Concluding remarks

The study of RNA export in eukaryotes is still at primitive stage due to the lack of system to work with. Most of studies concerning RNA export have used microinjection of either DNA or RNA into Xenopus oocytes. Although this system gives information about the export kinetics and RNA sequence required for the efficient export, it does not reveal anything about the transport machinery. Possible in vitro RNA export system utilizing isolated nuclei also have problem in terms of the manipulation of nuclear interior.

Under these circumstances, genetics is only hope to reveal the molecular and cellular mechanisms of RNA export in eukaryotes. We isolated Saccharomyces cerevisiae temperature sensitive mutants that accumulated polyA-RNA in the nucleus at the restrictive temperature. All mutants appear to be defective in mRNA export judging from thiolutin block test. In addition, the increase of polyA tail length in most of mutants at the restrictive temperature supports this conclusion. The detailed phenotypic analysis of mtr mutants is in progress.
MTR1 and its homologs appear to be guanine nucleotide release proteins (GNRPs) on small nuclear GTPases. Thus, loss of function of MTR1 results in the incapability of GTPase activation. Judging from the phenotypes of mtr1-1, downstream effector(s) of these GTPases has influences on RNA transcription (initiation and termination), processing and export in budding yeast. In addition to these roles, they are also involved in check point control in animal and fission yeast. It is not known whether one or multiple effectors act on two quite different events. The identification of effector and GAP (GTPase activating protein) will be extremely interesting. Furthermore, question about the regulation of MTR1 is also interesting. It is well known that GNRPI is activated upon ligand binding to receptor tyrosine kinase at plasma membrane. It might be possible that MTR1 activation is regulated in a similar way. RNA1 is definitely a good candidate for regulator of MTR1 because of its cytoplasmic localization and extreme similarity of the phenotypes of mtr1 and rna1-1. If this is the case, events in the nucleus is clearly controlled by the factors in both cytoplasm and nucleus.

PolyA-RNA accumulates as foci in the nucleus in mtr2-1 and these foci coincide with fragmented nucleolus. Consistent with this, rRNA processing is also inhibited in mtr2-1. Since some mtr mutants show normal rRNA processing
at the restrictive temperature, it is unlikely that rRNA processing defect of mtr2-1 is a result of decrease of protein synthesis. The association of polyA•RNA and nucleolar proteins is also observed in mtr1-1 and mtr3-1. Furthermore, the nucleolus is morphologically disrupted in these mutants. It might be possible that nucleolar disorganization is the primary lesion in these mutants and it results in the inhibition of polyA•RNA export. Another possibility is that the co-incidence between polyA•RNA and nucleolar proteins is an artifact due to non-specific interaction between them. To distinguish these possibilities, further investigation of mtr mutants is necessary, for example, the identification of mutant that accumulates polyA•RNA in the nucleoplasm without nucleolar disorganization will be essential to support first hypothesis. What is the function of MTR2? It is an open question. The identification of high-copy number or extragenic suppressor genes of mtr2-1 will be helpful to answer this question.

The study of mRNA transport in eukaryotes by genetics has just begun. It will be my pleasure mtr mutants are appreciated among scientific community for understanding mRNA export.
LITERATURE CITED


