A conditional yeast mutant deficient in mRNA transport from nucleus to cytoplasm (mRNA stability/in situ hybridization)

TATSHIKO KADOWAKI*, YANMING ZHAO†, AND ALAN M. TARTAKOFF‡‡

*Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, OH 44106; and †Department of Hematology, University of Alabama, Birmingham, AL 35294

Communicated by George E. Palade, November 18, 1991

ABSTRACT Transport of mRNA from nucleus to cytoplasm is critical for eukaryotic gene expression; however, the mechanism of export is unknown. Selection and screening procedures have therefore been used to obtain a family of temperature-sensitive conditional mutants of Saccharomyces cerevisiae that accumulate poly(A)⁺ RNA in the nucleus when incubated at 37°C, as judged by in situ hybridization. In one such mRNA transport mutant, mtr-ts, RNA synthesis continues, the export of poly(A)⁺ RNA is inhibited, intranuclear poly(A)⁺ is remarkably stable, and protein synthesis gradually stops. Thus, there is no tight coupling between RNA synthesis and export. The export lesion is reversible. Although mRNA export is clearly not a default option, neither inhibition of protein synthesis, inhibition of mRNA splicing, nor inhibition of poly(A)-binding protein function blocks export of the average poly(A)⁺, as judged by in situ hybridization. Further analysis of the family of mtr mutants should help map the path of mRNA transport.

Gene expression in eukaryotes requires the rapid and selective export of mRNA from the nucleus to the cytoplasm. Most pre-mRNAs acquire a 5' m⁵G cap and are cleaved and polyadenylated to generate their 3' end. mRNAs have few other common structural features; however, specific proteins are associated with both extremities of intranuclear pre-mRNAs (1, 2), and intranuclear mRNAs associate with a number of additional proteins (3, 4) as well as the karyoskeleton (5).

Export of SS RNA and rRNAs requires association with specific proteins (6–8) and the export of tRNA is highly sensitive to base changes, possibly for this reason (9). mRNA export is facilitated by its 5' m⁵G cap (10, 11). Exit of tRNAs and ribosomal subunits appears to be receptor mediated (9, 12).

Because colloidal gold coated with poly(A), tRNA, or 5S RNA microinjected into the nucleus exits via nuclear pores (13) and because exit of tRNA and 5S RNA is inhibited by antibodies that react with pore complexes (14), it is likely that mRNA also exits via pores. Maximal mRNA release from isolated animal cell nuclei requires ATP (15–17).

In Saccharomyces cerevisiae several features of processing of pre-mRNA are relatively simple: only the 5' extremity of mRNA is methylated (18), only few mRNAs undergo splicing (19, 20), and the 3' poly(A) tail of yeast mRNA is somewhat shorter than in animal cells (1, 21). Although nuclear pores of yeast have been characterized (22–24), and although temperature sensitive (ts) splicing mutants (prp mutants) have been produced (19, 20, 25–27), only a single ts mutant has been claimed to affect mRNA export (28). This mutant, rnu1, has multiple defects in covalent processing of RNA. The corresponding gene product is cytoplasmic (29).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

METHODS

Selection of mtr Mutants. The YPH1/2 and YPH258/9 strains (wild type; ref. 30) were treated with 3% ethyl methanesulfonate. Two selection procedures were then used. (i) Selection A. Cells were incubated 3 hr at 37°C in SD medium (31) and labeled 30 min at 37°C with [³H]lysine in lysine-free SD medium. The cells were then washed and stored in 20% glycerol/YPAD medium (31) at −80°C. When survival was <1%, replica plates were prepared to screen for ts growth on SD medium. (ii) Selection B. Cells were incubated 3 hr at 37°C in lysine-free SD medium supplemented with the lysine analogue S-2-aminoethyl-L-cysteine and the proline analogue L-azetidine-2-carboxylate. The incubation was continued at 37°C for 24 hr; then cells were inoculated to YEPD plates. The survival fraction of the cells after this treatment was 0.1–0.2%. Replica plates were made to screen for ts growth on SD medium.

To evaluate protein synthesis, individual colonies were labeled in complete SD medium supplemented with [³H]-labeled amino acid mixture. Aliquots were spotted onto filter paper and processed by trichloroacetic acid precipitation.

Genetic Procedures. The original mutant, ts17a, was backcrossed twice with YPH47 and then once with BJ2664 to yield the YTK strain series (mtr-1) (31). The double mutant YTK105 (rpl1-l mtr-1) was constructed by a cross between the two ts parents. Tetrad s were selected that included two ts and two Ts⁺ spores. The ts spores were further crossed to YTK103, YTK104 (mtr-1) and RY260, RY262 (rpl1-1; ref. 32) to verify their genotypes.

In Situ Hybridization and 4',6-Diamidino-2-Phenylindole (DAPI) Staining. Cells were fixed in suspension with formaldehyde, washed in 1.2 M sorbitol/0.1 M potassium phosphate, pH 7.5 (solution A), “spheroplasted,” spotted onto poly(I-lysine)-coated glass slides, dehydrated through 100% ethanol, and dried. Ten microliters of hybridization solution consisting of 3'-biotinylated (dT)₂₅-₃₀, yeast tRNA, salmon sperm DNA, 2x SSC (1 x SSC = 0.15 M NaCl/15 mM sodium citrate), vanadyl complex, bovine serum albumin, and dextran sulfate was then added. Each preparation was then washed with 2x SSC, 1x SSC, and, briefly, 4x SSC/0.1% Triton X-100. Fluorescein isothiocyanate-avidin was added for 30 min at room temperature. The preparation was then washed with 4x SSC, 4x SSC/0.1% Triton X-100, and 4x SSC, mounted in 90% glycerol, 1 mg of p-phenylenediamine per ml, 45 ng of DAPI per ml, and PBS, and examined.

Effect of Inhibition of ATP Production on Poly(A)⁺ RNA Distribution. YTK101 was cultured in SD medium at 23°C. An equal volume of 55°C SD medium was added and incubated at 37°C for 2 hr. The cells were washed once with 37°C water and then washed with glucose-free SD medium con-
containing cycloheximide and suspended in the same medium at 37°C. Part of the cell dispersion was fixed. The rest received an equal volume of 4°C 4% glucose SD medium containing cycloheximide or 4°C glucose-free SD medium containing dinitrophenol, deoxyglucose, and cycloheximide. The incubation was continued at 23°C until the cells were fixed for in situ hybridization.

Reversibility of Protein Synthesis. YTK102 was grown overnight in SD medium at 23°C. An equal volume of 55°C SD medium was then added and the incubation was continued at 37°C for 1–3 hr. After the appropriate interval, an equal volume of 4°C SD medium containing 6 µg of thiolutin per ml and 3H-labeled amino acid mixture was added. Culture at 23°C was continued for 0–6 hr followed by determination of acid-insoluble cpn.

Analysis of β-Galactosidase Expression. Strains YTK100 and YTK102 were transformed with pLGS5 (33) and grown in uracil-free medium containing 2% raffinose to an OD 600 nm of ~1. Galactoside induction was achieved by adding an equal volume of 23°C or 55°C 4% galactose in uracil-free medium.

To evaluate the reversibility of lacZ mRNA transport at 23°C, galactose induction of cells transformed with pLGS5 was performed for 2.5 hr at 37°C, as above. Control samples remained in raffinose-containing medium at 37°C. For the 1- to 7-hr reinduction interval at 23°C, uracil-free medium was then added. During this interval all samples contained 1.5% raffinose, 1% galactose, and 2% glucose. β-Galactosidase activity was measured and expressed as in ref. 31.

RNA Analysis. Total RNA was extracted (34), dot-blotted to nylon membrane, and UV cross-linked. Oligo(dT) end-labeled with [α-32P]TTP was used for hybridization in 2X SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), 5× Denhardt’s solution, 1% SDS, and 0.1 mg of salmon sperm DNA per ml at 37°C. The membrane was then washed with 2× SSC/1% SDS at room temperature, 2× SSC/1% SDS at 37°C, and then 1× SSC/1% SDS at room temperature.

To evaluate synthesis of poly(A)+ RNA, tRNA, and rRNA, wild type (wt) and mtrl-1 were grown in low phosphate YEPD medium (35) at 23°C and then incubated 0–90 min at 23°C or 37°C in low phosphate YEPD medium prior to addition of 32P. After 2 min and 10 min samples were washed by filtration using 0.1% Na2HPO4/100 µg of cycloheximide per ml in 50 mM sodium acetate, pH 5.3/10 mM EDTA. Total RNA was extracted and analyzed on 1% formaldehyde/agarose and 10% acrylamide/7 M urea gels after elimination of polyphosphate (36) or fractionated on oligo(dT)-cellulose (32, 37).

RESULTS

Selection of mRNA Transport (mtrl) Mutants. To enrich for mutants that cannot export mRNA at 37°C, we have used "suicide" selection procedures based on the supposition that cells that have not exported mRNA for 3 hr will tolerate incubation in the presence of high concentrations of 3H-labeled amino acids or toxic amino acid analogues. In a typical experiment, 3H-labeled amino acid suicide selection of cells at 37°C, incubation with non-nutrient cell-grown spheroplasts, and cell lysis using lysozyme, allowed isolation of a temperature-sensitive mutant, ts17a, that exhibited progressive slowing of protein synthesis at 37°C and intranuclear accumulation of poly(A)+ RNA at 37°C. A dozen complementation groups of recessive mutants have been obtained. The data presented below concern one of several alleles of one such ts mutant, ts17a, that was backcrossed to yield the YTK series of strains (mtrl-1). These strains exhibited 2:2 cosegregation of ts growth and accumulation of poly(A)+ RNA in the nucleus after 3 hr at 37°C upon dissection of 20 tetrads from the last backcross.

Basic Phenotype of mtrl-1. Fig. 1A illustrates the kinetics of protein synthesis of mtrl-1 by comparison to wt at 23°C and 37°C. Synthesis is quasilinear except for mtrl-1, which stops after 1–2 hr at 37°C. Comparable kinetics are seen for a mutant in which RNA polymerase II is ts (rpbl-1; Ry260; ref. 32) and for rna1 (not shown).

In situ hybridization to detect poly(A)+ RNA revealed a uniform distribution of fluorescence in wt cells, rpbl-1, or mtrl-1 grown at 23°C (Fig. 1B, panels 1 and 3). In rpbl-1, incubation at 37°C leads to a progressive disappearance of fluorscence (panel 2). Moreover, in all cases fluorescence was eliminated by pretreatment with nonspecific RNase (not shown). When mtrl-1 cells were incubated for increasing periods at 37°C, the cytoplasmic signal faded and the nuclear signal increased for at least 3 hr [compare panel 3 (23°C) with panels 4 and 7 (3 hr, 37°C)]. During this period, the intranuclear signal grows from a focal spot (40 min) to a more complex often multilobed structure (+80 min) (not shown), which ultimately fills the entire nucleoplasm (panel 6). The accentuated nuclear signal was not seen with a double mutant, rpbl-1 mtrl-1 (panel 5; 3 hr, 37°C), or when the 37°C incubation of mtrl-1 was in the presence of 3 µg of thiolutin per ml, an RNA polymerase inhibitor that has little effect on RNA turnover (37, 38). Since the production of the intranuclear signal requires active RNA polymerase II and is sensitive to RNase we consider that it is poly(A)+ RNA.

To learn whether poly(A)+ RNA export is interrupted at 37°C, mtrl-1 was incubated 2 hr at 37°C to yield a strong intranuclear signal and then maintained at 37°C with 3 µg of thiolutin per ml for 1–3 hr. The intense nuclear signal persisted during this period (not shown). Thus, the intranuclear poly(A)+ RNA is surprisingly stable in mtrl-1 at 37°C. Such a result would not have been obtained if the steady-state distribution of poly(A)+ RNA in mtrl-1 at 37°C (bright nuclei with dark cytoplasm) resulted primarily an acceleration of turnover of cytoplasmic poly(A)+ RNA.

After 3 hr and 24 hr at 37°C, followed by replating at 23°C, recovery of mtrl-1 was essentially 100% and 10%, respectively. After incubation for 1–3 hr at 37°C, a heterogeneous mixture of unbudded and budded cells was seen.

Steady-State Levels, Synthesis, and Turnover of RNA. The impact of the mtrl-1 mutation on RNA levels and turnover has been evaluated by comparison to rpbl-1 and a mtrl-1 rpbl-1 double mutant. In the latter two cases, upon shift to 37°C one can directly follow the turnover of preexisting—i.e., >90% cytoplasmic—mRNA (39). As illustrated in Fig. 2 A1 and A2, mtrl-1 cells at 37°C maintain ~45% of their initial (23°C) levels of total poly(A)+ RNA for 3 hr at 37°C. Northern analysis of actin mRNA shows a comparable persistence of normal-sized transcripts (not shown). For the mtrl-1 rpbl-1 double mutant, turnover of cytoplasmic poly(A)+ RNA is accelerated relative to rpbl-1.

Synthesis of poly(A)+ RNA and synthesis and processing of rRNAs were evaluated in 2-min and 10-min 32P-labeling experiments, respectively. Synthesis of poly(A)+ RNA and labeling of 35S, 27S, 25S, 20S, 18S, 5.8S, and 5S rRNAs and tRNA (4S) by mtrl-1 continued at levels similar to wt after 30 min at 37°C but were severely inhibited within 90 min (not shown).

To evaluate turnover of intranuclear poly(A)+ RNA, cells incubated 1–2 hr at 37°C were reincubated 0–3 hr at 37°C with thiolutin. Fig. 2B shows that by comparison to wt cells, turnover is severely slowed in mtrl-1. The slow turnover seen for rpbl-1 (Fig. 2B) pertains to that poly(A)+ RNA that remains after the preincubation at 37°C.

Reversibility of RNA Accumulation. When mtrl-1 cells were incubated 3 hr at 37°C and then returned to 23°C, the in situ hybridization signal returned to normal over 2–3 hr (not shown). Normalization was dependent on ATP production.
Protein after 1, 2, and galactose under enzyme of grown mtrl-1 examine 23°C Cell of thiolutin presence in 3 hr occurred presence of 3 hr at 37°C (Fig. 1). panels 1 and 2 compare the in situ signal in rpb1-1 at 23°C (panel 1) with that seen after 3 hr at 37°C (panel 2). Panel 3, mtrl-1 at 23°C. Panels 4 and 7, mtrl-1 after 3 hr at 37°C. In panels 4 and 6, several corresponding nuclei are designated. Panel 5, mtrl-1 rpb1-1 double mutant after 3 hr at 37°C.

(Fig. 3) but was not altered by inclusion of 100 µg of cycloheximide per ml. To evaluate whether RNA that was accumulated in the nucleus at 37°C can pass to the cytoplasm at 23°C, mtrl-1 cells incubated 1–3 hr at 37°C were returned to 23°C in the presence of thiolutin and mixed 3H-labeled amino acids. As illustrated in Fig. 4A, protein synthesis does resume at 23°C. To examine reversibility of export of a single mRNA, we transformed mtrl-1 with pLGSD5 (33), which codes for β-galactosidase under GAL10 control. When these cells were grown in raffinose at 23°C, no enzyme activity was seen; however, growth on galactose at 23°C (but not 37°C) rapidly induced activity. Fig. 4B illustrates an experiment in which transformed mtrl-1 cells were incubated 2.5 hr at 37°C in the presence of galactose or raffinose. In each case, the subsequent 23°C incubation included 1% galactose, 2% glucose, and 1.5% raffinose. For galactose (but not raffinose) a major increase in enzyme activity was seen at 23°C. Thus, most activity seen with galactose is due to transcription that occurred at 37°C. When transformed wt was exposed to galactose after 1, 2, and 3 hr at 37°C, 95, 280, and 460 units of enzyme activity were produced.

Protein Synthesis, mRNA Splicing, and the Poly(A)-Binding Protein (PABP) Are Not Critical for Export of the Average Poly(A)⁺ RNA. Neither protein synthesis, mRNA splicing, nor a normal titer of functional PABP is essential for synthesis of most mRNAs (1, 3, 20, 25). We therefore have used in situ hybridization to evaluate the importance of these parameters for RNA export: when wt cells were incubated for 3 hr at 37°C in the presence of cycloheximide, the uniform fluorescent signal was more intense than in controls, probably due to stabilization of mRNA (37). Incubation for 3 hr at 37°C of a mutant that is ts for translation initiation (ts 187; ref. 25) or any of four ts splicing mutants (prp2, prp5, prp8, and prpl1; refs. 20 and 27) did not modify the uniform in situ hybridization signal. The importance of the PABP for poly(A)⁺ RNA export was evaluated by shifting a strain that harbors a ts allele of the PABP (YAS120; ref. 1) to 37°C for 3–12 hr and by shifting a strain in which expression of PABP is under galactose control (YAS352; ref. 1) to glucose medium for up to 30 hr. In neither case was an obvious change in the in situ hybridization pattern seen (not shown).

The rnal Mutant Is Distinct from mtrl-1. We have constructed heterozygous +/mtrl-1 +/ral-1 diploids at 23°C and shown that they grow at 37°C. We have also inquired whether rnal-1 gives a nuclear in situ hybridization signal comparable to that of mtrl-1. This is not the case, although some nuclear signal is seen after shorter incubation at 37°C.
After polyadenylylation, the export of mRNA from the nucleus of yeast is rapid and efficient (39). By analogy to animal cells, the intranuclear pre-mRNA is likely to be bound to the karyoskeleton. Export must involve targeting to the sites of exit and translocation through them, probably as a RNA–protein complex.

Our strategy to identify components responsible for mRNA export would not identify lesions that block polyadenylylation, inhibit RNA polymerase II, cause rapid turnover of intranuclear mRNA, or stabilize cytoplasmic mRNA. Judging from our observations on prp2, prp5, prp8, and prp11, they also would not identify lesions that primarily block pre-mRNA splicing.

The intranuclear accumulation of poly(A)+ RNA in conjunction with the persistence of intranuclear poly(A)+ RNA in the absence of RNA synthesis and the data on reversibility indicate that export has indeed been interrupted at 37°C. Since the kinetics of protein synthesis in mtr1-1 at 37°C are very similar to rpb1-1, the arrest of export must occur in no more than ~10 min. We have also used subcellular fractionation to document inhibition of export of [3H]uridine-labeled poly(A)+ RNA in mtr1-1 at 37°C vs. 23°C. These observations are striking: after 30 min labeling, only 2–3% of total [3H]-labeled poly(A)+ RNA is recovered in the nuclear fraction of wt at 23°C or 37°C or mtr1-1 at 23°C. By contrast, >40% is in the nuclear fraction of mtr1-1 at 37°C.

The reversibility of inhibition of translation and the stability of poly(A)+ RNA in mtr1-1 show that poly(A)+ RNA in the mtr1-1 nucleus at 37°C is considerably more stable than the average intron or cytoplasmic mRNA of wt cells (19, 37). The volume of the nucleus is only about 1/50 of the cell volume. Since the total amount of poly(A)+ RNA in mtr1-1 after several hours at 37°C is ~1/2 that of wild type and since most of this poly(A)+ RNA is in the nucleus, the intranuclear concentration of poly(A)+ RNA must be much higher in
Transcripts that accumulate in the nucleus of mtr1-1 at 37°C are translated at 23°C. TCA, trichloroacetic acid. (A) mtr1-1 cells were incubated 1–3 hr at 37°C and then returned to 23°C in the presence of 3 μg of thiolitin per ml and 3H-labeled amino acids. Protein synthesis resumes at 23°C. (B) mtr1-1 cells transformed with pLGDS were incubated 2.25 hr at 37°C with galactose to induce lacZ transcription (a) or with raffinose (b). The cells were then supplemented with glucose and normalized with regard to sugar composition to terminate transcription for 0–7 hr at 23°C. A burst of β-galactosidase activity is seen after a lag.

mtr1-1 after 3 hr at 37°C than in normal cells. This exaggeration may stop further RNA synthesis after 30 min and may contribute to the lag period that precedes translation of accumulated mRNAs once the temperature is reduced to 23°C. The lag may also reflect the progressive depletion at 37°C of factors that are essential for protein synthesis.

Why does mRNA export stop in mtr1-1 at 37°C? Our data argue that neither the interruption of protein synthesis, inhibition of splicing, nor lesions in the PABP are responsible. The 5' cap methylation and poly(A) length distributions of the average transcript in mtr1-1 at 37°C are as expected (T.K., Y.Z., A.M.T., P. Narayan, and F. Rottman, unpublished). Thus, the mutation might affect a critical protein that accompanies mRNA from the nucleus to the cytoplasm. Alterations in its structure could stop export and account for accelerated mRNA turnover in the cytoplasm.

We thank S. Lemmon, P. Hieter, R. Young, A. Sachs, and L. Guarente for yeast strains and plasmids, N. Belcher for thiolitin, S. Lemmon and Y. Kitagawa for advice, S. Meyale, G. Tucker, and D. Cai for technical help, M. Ward for preparing the manuscript, and the National Science Foundation for Grant DCB90-02365 and the National Institutes of Health for Grant GM-46569.