Characterization of the Essential Activities of *Saccharomyces cerevisiae* Mtr4p, a 3′→5′ Helicase Partner of the Nuclear Exosome

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The processing of newly transcribed RNAs is the initial post-transcriptional series of events that culminates in an expressed gene product. Ribosomal, small nuclear, small nucleolar, and messenger RNAs are transcribed as long precursors (pre-RNAs) that must be fragmented and trimmed to yield functional RNAs (1). In particular, each ribosomal RNA, small nuclear RNA, and small nucleolar RNA must have its 3′-end exonucleolytically shortened to remove any deleterious or otherwise unnecessary extensions. Any byproducts of the conversion from extended transcripts to functional RNAs must be rapidly degraded, and defective RNAs must be efficiently removed from circulation by 3′→5′ exonucleolytic degradation. From an extensive analysis of nuclear RNA processing in *Saccharomyces cerevisiae* (2–4), it has become clear that this 3′-end maturation is mediated by the DExH-box helicase Mtr4p and the nuclear exosome, a collection of six RNase PH homologues (Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p, and Mtr3p) that are apparently inactive (5), three proteins (Rrp4p, Rrp40p, and Csl4p) that form a cap on the RNase PH hexamer (6), and two active 3′→5′ exonucleases (Rrp4p (5, 7) and Rrp6p (8)). Mtr4p is an indispensable partner of the exosome that presumably maintains the momentum of exonucleolytic decay/processing as it moves through structured regions of its substrates. The action of Mtr4p on some RNAs occurs in the context of the TRAMP complex, which consists of a poly(A) polymerase (Tyf4p or Trf5p), a zinc-knuckle protein (Air1p or Air2p), and Mtr4p (9, 10). Addition of poly(A) to substrates to help maintain the momentum of 3′→5′ exonucleolytic decay is prevalent in bacteria (11, 12) and might reflect the ancestral purpose of 3′ poly(A) extensions on RNAs. Experimental evidence has just begun to emerge that polyadenylation can promote pre-mRNA decay in mammalian cells, an indication that this is an evolutionarily conserved feature (13).

Recent experiments have begun to shed light on the role of Mtr4p in nuclear RNA processing, demonstrating that Mtr4p impacts a diverse array of pre-RNA substrates. Mtr4p in conjunction with the nuclear exosome plays a key part in ribosome biogenesis via the production of the 5.8 S ribosomal RNA, an essential component of the 60 S ribosomal subunit, from its precursor 7 S ribosomal RNA (2). Mtr4p also has a role in ensuring ribosome function by processing small nucleolar RNAs (4). In addition, Mtr4p helps scrutinize mRNAs before they exit the nucleus. Pre-mRNAs that exhibit adenylation or splicing abnormalities are efficiently degraded by Mtr4p and the exosome (14, 15), and read-through transcripts are either degraded or rescued as functional messenger ribonucleoproteins (16). Mtr4p also participates in the 3′-end transcripts of small nuclear RNAs such as the U4 small nuclear RNA, which is...
part of the U4/U5/U6 trimer recruited during spliceosome assembly. In the context of the TRAMP complex, Mtr4p helps remove defective tRNAs before they are exported to the cytoplasm to assist in translation. Specifically, hypomodified tRNA$^{\text{Met}}$ has been recently identified as a substrate of the TRAMP-exosome complex (10). Degradation is highly specific for the hypomodified form of tRNA$^{\text{Met}}$ and might involve multiple rounds of polyadenylation and exonucleolytic decay (10).

In addition to its role in post-transcriptional processing, recent evidence has emerged that the TRAMP-exosome complex is involved in regulation of gene expression at the transcriptional level (17). The gene NRD1 is autoregulated by premature transcription termination with the aid of the protein Nab3p. Surprisingly, the TRAMP complex is likely required for the termination event and not just for degrading prematurely terminated transcripts as elevated levels of the full-length NRD1 transcript appear in both TRAMP and exosome mutant strains (17). In addition, other genes regulated by Nrd1p and Nab3p are also processed by the TRAMP-exosome complex (17, 18), indicating that the premature termination regulation scheme might be more prevalent than previously envisioned.

Based on the content of conserved helicase sequence motifs (Q, I, Ia, Ib, II, III, IV, V, and VI, see Fig. 1A), Mtr4p belongs to superfamily 2, which includes the DEAD-, DEAH-, DExH-, and DExD-box families of helicases. Mtr4p is typically grouped with Ski2p-like helicases because of its functional relationship to the cytoplasmic exosome partner Ski2p (19). It has been proposed that both of these DEVH-box-containing proteins partner with the exosome (Mtr4p in the nucleus, Ski2p in the cytoplasm) to ensure the momentum of 3′→5′ exonuclease activity is maintained. Recent genetics experiments have demonstrated that the putative helicase motifs of Mtr4p are critical for its function in vivo. In particular, mutation of the conserved lysine of motif I (Lys-177) results in a dominant negative growth defect, as does mutation of the conserved serine of motif III (Ser-293) (20). Surprisingly, mutation of the conserved aspartate of the DExH-box motif (motif II, Asp-262) to alanine has no obvious yeast phenotype (20). Motif II is thought to impact both NTP binding and hydrolysis (21), and it is unclear why this mutation fails to impair yeast growth. In addition to the above helicase motifs, Mtr4p contains ~500 amino acids at its C terminus that have no known function. This region is well conserved among Mtr4p and Ski2 homologues, yet is uncharacterized. What role the C-terminal portion of Mtr4p plays in its various activities is at present unclear.

Here we examine the biochemical activities of recombinant S. cerevisiae Mtr4p. We demonstrate that it has nucleic acid-dependent nucleotide triphosphatase activity that is specific for either ATP or dATP. We also demonstrate that Mtr4p can, in the presence of ATP or dATP, unwind duplex RNA in the 3′→5′ direction. Mtr4p also possesses single-stranded RNA-binding activity that is greatly influenced by both the presence and nature of bound nucleotide. Finally, Mtr4p preferentially binds to a short poly(A) substrate, adopting a unique binding mode that might be well suited for promoting degradation of RNA substrates.

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### EXPERIMENTAL PROCEDURES

**Cloning, Expression, and Purification of Full-length S. cerevisiae Mtr4p**—The Mtr4 gene was amplified from S. cerevisiae genomic DNA using the Herculase (Stratagene) DNA polymerase and PCR primers for the sense (ATCGTGCAGGTACCCATGGATTCCTACTGATCTGTTGATG; the initiating ATG codon is underlined) and antisense (TGCAATCGGAATTCCTATAAATCAAAGAACCAGCAGATACG) strands. The resulting PCR product was then cloned into a dual His$_6$-maltose-binding protein (MBP) fusion vector. The sequence of the cloned gene was then verified before undertaking subsequent experiments. Rosetta2(DE3) (Novagen) cells transformed with the Mtr4p expression vector were grown to an A$_600$ of 0.6, and expression of the recombinant protein was induced by addition of isopropyl-$\beta$-D-thiogalactopyranoside to a final concentration of 0.1 mM. The cells were grown for 16 h at 25 °C and harvested by centrifugation. Following high pressure disruption of the cells and removal of the cell debris by centrifugation, the protein was purified as described under "Results."

**Creation of the K177A Mutant**—To change the lysine in Motif I to alanine (K177A), the wild-type Mtr4p expression plasmid was mutagenized using the QuikChange XL mutagenesis kit (Stratagene). The integrity of the entire open reading frame and the presence of the desired mutation were confirmed by automated DNA sequencing.

**Nucleotide Hydrolysis Assays**—(d)NTPase activity was evaluated by quantifying the release of inorganic phosphate (P$_i$) from (d)NTP hydrolysis using an acidic ammonium molybdate solution containing malachite green (22). In each experiment, the micromolar amount of P$_i$ released was determined by comparing the experimental change in A$_650$nm with the absorbance change generated by addition of a phosphate standard (0.045 μmol) to a mock reaction lacking (d)NTP. This assay was used to determine both the nucleotide and substrate specificities of Mtr4p. A typical assay testing nucleotide specificity (50 μl) contained 25 mM Tris, pH 7.5, 10 mM magnesium acetate, 2 mM dithiothreitol, 10 μg poly(A) RNA (GE Healthcare), 2 μM Mtr4p, and 2 μM (d)NTP. The (d)NTPase reactions were incubated for 30 min at 37 °C. 750 μl of a malachite green-molybdate reagent was then added, and the reactions were incubated at room temperature for 5 min. Following the incubation, the A$_650$nm for each sample was measured. Each set of measurements included an appropriate control reaction lacking protein. In assays designed to determine the substrate specificity of Mtr4p, the conditions were modified to include 40 nM substrate (ssRNA, dsRNA, tRNA, ssDNA, or dsDNA) and 0.3 μM Mtr4p. Each of the model substrates used for these reactions (i.e. ssRNA, dsRNA, ssDNA, and dsDNA) consisted of short oligonucleotides of random sequence (see Table 1). The tRNA used was type X-SA tRNA from S. cerevisiae (Sigma-Aldrich).

We also employed the coupled pyruvate kinase-lactate dehydrogenase assay to assess the Michaelis-Menten kinetics of ATPase activity as described (23) with minor modifications. Briefly, Mtr4p (100 nM) was incubated with varying concentrations of ATP (0–3.5 mM) and 250 nM of a 40-nucleotide ssRNA (substrate number J in Fig. 6A) at 37 °C in a reaction volume of 0.5 ml containing 50 mM Hepes-Na, pH 7.5, 50 mM NaCl, 5 mM...
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TABLE 1

| Substrate (40 nM, normalized) | Nucleotide | -Fold stimulation a |
|-------------------------------|-----------|---------------------|
| UUGAAUACAUUUUUGACA            | ATP       | 8.6 ± 1.1           |
| dATP                          | 6.6 ± 1.8 |
| ATP                           | 1.9 ± 2.0 |
| dATP                          | 2.3 ± 1.7 |
| ATP                           | 12.0 ± 3.8|
| dATP                          | 37.3 ± 13.1|
| ATTACTCTCCACAGCATGTCGGGTACTGTTCAGA | ATP      | 3.2 ± 1.0 |
| dATP                          | 4.3 ± 2.9 |
| ATP                           | 2.8 ± 1.5 |
| dATP                          | 5.4 ± 3.1 |
| ATP                           | 1.1 ± 1.0 |
| dATP                          | 2.2 ± 1.3 |
| ATP                           | 1.6 ± 0.7 |
| dATP                          | 0.6 ± 1.6 |
| ATP                           | 9.9 ± 3.1 |
| dATP                          | 14.6 ± 4.8|
| ATP                           | 3.0 ± 0.5 |
| dATP                          | 1.9 ± 0.5 |

a Concentration of 20-nucleotide units, except for the 40-nucleotide ssRNA (see footnote e).

b Relative to a reaction lacking substrate, each calculated in micromoles of P min⁻¹ µmol Mtr4p⁻¹; expressed as the mean value ± its standard error.
c Based on the sedimentation coefficient for that lot of poly(A) (GE Healthcare).
d Average molecular mass of 7 MDa (Fluka).
e ATP 1.6
f ATP 1.1 ± 0.1

MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 3.5 units of pyruvate kinase, and 5 units of lactate dehydrogenase. In a parallel experiment, Mtr4p was incubated with amounts of substrate 1 varying from 0 to 250 nM, 4 mM ATP under the same conditions as outlined above. Oxidation of NADH to NAD⁺, which is coupled to ATP hydrolysis, was continuously monitored at 338 nm in a Beckman DU-640 spectrophotometer. Initial rates were calculated from the absorbance data using an extinction coefficient (ε338) of ~6220 M⁻¹ cm⁻¹. A blank rate omitting ATP was subtracted from the initial rate at each ATP or RNA concentration. Kinetic parameters were determined by fitting the rates as a function of ATP or RNA concentration to the Michaelis-Menten equation using the KaleidaGraph software package (Synergy Software).

Preparation of Radiolabeled RNA Substrates—RNA substrates were radiolabeled at the 5’-end using T4 polynucleotide kinase (New England Biolabs). 10 pmol of RNA was incubated with ~20 µCi of [γ-32P]ATP (>6000 Ci/mmol stock) and T4 polynucleotide kinase in a final volume of 10 µl for 10 min at 37 °C. Following incubation, the reaction was brought to a final volume of 60 µl, and EDTA was added to a final concentration of 20 mM. The reaction was then heat-inactivated at 70 °C for 15 min. Labeled RNA was extracted once with phenol:CHCl₃:isoamyl alcohol, and unincorporated nucleotides were removed by passing the extracted RNA through a G-25 spin column (Ambion).

RNA Binding—RNA binding affinity was measured using a fluorescence anisotropy-based assay according to the method of Brewer and colleagues (24, 25). The anisotropy measurements were conducted using a Beacon 2000 variable temperature fluorescence polarization system equipped with fluorescence excitation (490 nm) and emission (535 nm) filters. A typical reaction (100 µl) contained a limiting concentration (0.2 nM) of an RNA substrate labeled with fluorescein at its 5’-end, Mtr4p (0–2000 nM), binding buffer (25 mM Tris-HCl, pH 8.0, and 25 mM NaCl), 2 mM adenosine 5’-(β,γ-imido)triphosphate (AMP-PNP) or ADP, 5 mM dithiothreitol, and 5 mM MgCl₂. The polarimeter was operated in static mode with each sample (including all components other than the substrate RNA) read as blank prior to addition of fluorescein-labeled RNA. Following substrate addition, samples were incubated for a time increment empirically determined to allow the system to reach equilibrium (see “Results” and below), before anisotropy was measured. Binding isotherms were obtained by plotting the measured anisotropy (each datum represents the average of multiple independent measurements) versus the concentration of Mtr4p. The total intensity of emission was monitored concurrently with anisotropy to ensure that interactions between Mtr4p, and the RNA substrate did not affect the quantum yield of the fluorophore. In cases where the total fluorescence emission varied as a function of added protein concentration, an appropriate correction factor (26–28) was applied to the measured anisotropies. The equilibrium association constant was calculated from the binding isotherm using GraphPad Prism version 3.03 and Equation 1 (29),

[A total] = [A RNA] + [A complex][P]/[1 + [A complex][P]]

where [A total] represents the total anisotropy, [A RNA] is the intrinsic anisotropy of the RNA, [A complex] is the anisotropy of the saturated protein-RNA complex, [P] is the protein concentration, and Kₐ is the association constant. This binding model assumes that one binding site exists on the substrate RNA. The appropriateness of the single binding site model was evaluated by the agreement between the observed and calculated binding isotherms, the random distribution of residuals, and a lack of multiple bound species observed in an electrophoretic mobility shift assay (EMSA). In cases where the single-site model was deemed inappropriate, binding isotherms were analyzed using a variant of the Hill equation (30),

[A total] = [A comp] + [(A comp - [A RNA])(P/Pₘ)ᵇ]/[1 + (P/Pₘ)ᵇ]

where [A total] is the measured anisotropy, [A comp] is the anisotropy...
of the saturated protein–RNA complex, $A_{RNA}$ is the inherent anisotropy of the RNA substrate, and $h$ is the Hill coefficient. This equation also returns an estimate of the midpoint of the binding isotherm ($[P]_m$), which, in the case of a single binding site (i.e., $h$ is constrained to be 1.0), is equivalent to the $K_d$.

To qualitatively assess RNA binding, and in particular to determine the number of bound species, EMSAs were performed as follows. Mtr4p at the indicated concentrations was incubated with 5 nm of each $^{32}$P-labeled RNA substrate (prepared as described above) in a low ionic strength buffer (10 mM Tris–HCl, pH 8.0, 50 mM KCl, 2.5 mM AMP–PNP, 5 mM MgCl$_2$, 2 mM dithiothreitol, and 6% glycerol) for 30 min at 25 °C, followed by incubation for 15 min on ice. A 6% native polyacrylamide gel containing 10% glycerol was prepared and run at 90 V for 30 min in 1× Tris–Borate–EDTA buffer at 4 °C after loading samples. The samples were then electrophoresed at 150 V for 3 h at 4 °C, and the results were evaluated by autoradiography using a Storm 860 PhosphorImager.

**RNA-Protein Complex Dissociation Kinetics**—To determine how fast Mtr4p releases single-stranded substrates, we incubated a fixed amount of protein (150 nm) with the fluorescein-labeled ssRNA and measured the decrease in anisotropy over time in response to a competitive challenge with unlabeled ssRNA added in 5000-fold excess. The solution conditions were identical to those employed in the equilibrium binding experiments. Complex lifetimes were measured for a 20-nucleotide ssRNA with a random sequence (substrate 2 in Fig. 6A) and a 20-nucleotide poly(A) (substrate $A_{20}$), each in the presence and absence of AMP–PNP. The time-dependent decrease in anisotropy was fit to an exponential equation,

$$ A = A_0 e^{-kt} + B $$

(Eq. 3)

where $A$ is the measured anisotropy at time $t$, $A_0$ is the measured anisotropy in the absence of unlabeled competitor RNA, $k$ is the rate constant, $t$ is time, and $B$ is the minimum anisotropy (i.e. the plateau of the reaction).

**Competition Assay**—The ability of the $A_{20}$ substrate to compete with substrate 2 for binding to Mtr4p was determined as follows. Mtr4p (150 nm) was incubated with a mixture of 0.2 nm 5'-fluorescein-labeled $A_{20}$ (Fl-$A_{20}$) mixed with unlabeled competitor RNA (either substrate 2 or $A_{20}$) at concentrations ranging from 0 to 1000 nm. The mixture was incubated at 25 °C for 30 min, a time determined empirically to be sufficient for the system to reach equilibrium (see Fig. 7A). The anisotropy was then measured and analyzed as a function of competitor RNA concentration. The apparent inhibition constant ($K_{app}$) of the competitor RNA, used as an estimate of the relative preference of Mtr4p for that substrate, is estimated from plots of anisotropy versus competitor concentration via Equation 4,

$$ A = A_{RNA} + \frac{A_{max}}{1 + ([I]/K_{app})} $$

(Eq. 4)

where $A$ is the measured anisotropy, $A_{RNA}$ is the anisotropy of the RNA alone, $A_{max}$ is the maximum observed anisotropy shift (i.e. the anisotropy in the absence of competitor RNA), and $[I]$ is the competitor RNA concentration.

**Helicase Assay**—Helicase activity was measured by monitoring the displacement of a $^{32}$P-labeled, single-stranded product from a duplex substrate. In all five RNA substrates were used: 1) a 20-bp duplex region with a 20-nucleotide single-stranded 3’ overhang; 2) a 10-bp duplex region with a 30-nucleotide single-stranded 3’ overhang; 3) a 10-bp duplex region with a 20-nucleotide poly(A) 3’ overhang, 4) a 20-bp duplex region with a 20-nucleotide single-stranded 5’ overhang; and 5) a 20-bp duplex. The shorter strands were $^{32}$P-labeled at the 5’-end as described above. The annealing reactions were performed in the presence of 10 mM Tris and 75 mM NaCl, 50 nm labeled RNA, and 250 mM unlabeled RNA. Annealing was facilitated by rapid heating to 95 °C followed by slow cooling to 25 °C. A typical helicase reaction (20 μl) contained 5 nm RNA, RNasin (20 units), 2 mM (d)ATP (where indicated), 5 mM MgCl$_2$, 25 mM NaCl, 10 mM Tris, pH 8.0, 0.1–5 μM Mtr4p (or 50 nm Mtr4p for the time course experiments), and a large molar excess of an unlabeled ssRNA trap (to prevent substrate re-annealing). The helicase reactions were incubated for 30 min at 37 °C and then quenched with 5 μl of stop solution (32% glycerol, 3.5% SDS, 10× DNA loading dye (Ambion), 20 mM EDTA, 4 units of Proteinase K) on ice. The quenched reaction was electrophoresed on a 12% native polyacrylamide gel in 1× Tris–Borate–EDTA buffer at 25 °C and analyzed by autoradiography using a Storm 860 PhosphorImager. For the substrate with the 10-bp duplex, the reaction buffer was identical to the above but with 65 mM NaCl and 50 mM KCl. The reaction was incubated for 1 h at 30 °C, quenched as above, and electrophoresed on a 20% native polyacrylamide gel.

**RESULTS**

**Overexpression and Purification of S. cerevisiae Mtr4p**—S. cerevisiae Mtr4p was expressed in *Escherichia coli* with a His$_6$-MBP tag fused to its N terminus. A PreScission Protease (GE Healthcare) cleavage site was incorporated to facilitate removal of the tag. Mtr4p was isolated from the soluble fraction via nickel–Sepharose affinity chromatography (Fig. 1B, lane 2). The partially-purified His$_6$-MBP-Mtr4p was then incubated with PreScission Protease according to the manufacturer’s instructions, resulting in complete removal of the tag (Fig. 1B, lane 3). This procedure yielded intact Mtr4p with a four-amino acid (GPVP) linker remaining at its N terminus. Mtr4p was further purified using heparin–agarose affinity chromatography (Fig. 1B, lane 5). To remove a persistent nucleic acid contaminant, we employed centrifugal anion exchange membrane spin columns (Vivascience). To find the optimal buffer storage conditions for the protein, we examined the solubility and solution properties of Mtr4p under a variety of buffer conditions (31). Briefly, the pure protein was incubated overnight at 25 °C in 1 of 24 buffers ranging from pH 3.0 to 10.5. Buffer conditions that lacked visible precipitate were tested for the presence of a homogenous preparation via dynamic light scattering using a Zetasizer Nano S (Malvern Instruments). The results of this experiment identified CHES, pH 9.5 as the optimum storage buffer for Mtr4p (Fig. 1C). The recombinant Mtr4p was snap frozen in liquid nitrogen and stored at −80 °C. The identical procedure was employed to purify the K177A mutant (not shown).
Mtr4p Exhibits RNA-dependent (d)ATPase Activity—To determine the nucleotide specificity of Mtr4p we assayed the ability of Mtr4p to hydrolyze each ribo- and deoxyribonucleotide in the presence of poly(A) RNA using a colorimetric indicator of released Pi (see under “Experimental Procedures”). We performed an additional assay lacking RNA as a means to determine the influence of RNA on (d)NTPase activity. In the absence of RNA, Mtr4p exhibited little hydrolysis activity toward any of the nucleotides tested (Fig. 2). The presence of poly(A) RNA caused a ~3-fold increase in the hydrolysis of ATP and dATP, to 13.6 ± 0.8 μmol P_i min⁻¹μmol Mtr4p⁻¹ and 12.1 ± 0.5 μmol P_i min⁻¹μmol Mtr4p⁻¹, respectively. For the other nucleotide substrates, Mtr4p did not show statistically significant differences in the amount of P_i produced in the presence and absence of RNA. The (d)NTPase assays demonstrated that both ATP and dATP are the preferred nucleotide substrates for Mtr4p and that (d)ATPase activity is stimulated by RNA. Specificity for adenine nucleotides has been observed for other RNA helicases, most notably eIF4A (32, 33). We also examined the Michaelis-Menten kinetics of ATP hydrolysis and found that Mtr4p has Michaelis-Menten parameters that are comparable to other superfamily 2 helicases. It should be noted that, although the solution conditions differ slightly for the Michaelis-Menten kinetics experiment and malachite green colorimetric assay, Mtr4p has equal ATPase activity in both conditions (not shown). The turnover number of Mtr4p (120–134 min⁻¹, Fig. 3) far exceeds that of, for example, human eIF4A (3 min⁻¹ (34)), but is approximately 3- to 5-fold less than more proficient ATPases such as Ded1p (35), DbpA (36), and Prp22p (37). The Michaelis constant (K_m) for ATP is 0.39 mM,
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ssDNA, tRNA, dsRNA, and dsDNA. Because the single-stranded RNA and DNA substrates are 20-nucleotides in length, all substrates were used at equimolar concentrations of 20-nucleotide units. For each substrate, we evaluated its -fold stimulation of (d)ATPase activity relative to an identical reaction lacking substrate (Table 1). For some substrates, there appears to be a far greater stimulation of dATPase activity than ATPase activity. In most cases, this simply reflects the fact that there is less noise (i.e. substrate-independent production of 
P)
in the dATPase reactions. However, for tRNA, there was a legitimate increase in the amount of 
P produced with dATP versus ATP. The significance of this preferential hydrolysis (if any) is not clear. These experiments yielded some results that were largely expected, namely that potential substrates (e.g. tRNA) or components thereof (e.g. ssRNA) were effective stimulators of (d)ATPase activity, stimulating (d)ATP hydrolysis from 6.6- to 37-fold. A recent study by another group also demonstrated that Mtr4p exhibits tRNA-stimulated ATPase activity (38). In addition, ssRNA was a more potent stimulator of (d)ATPase activity than ssDNA. Finally, a 20-bp duplex RNA was ineffective in stimulating the (d)ATPase activity of Mtr4p, a result foreshadowed by the inability of Mtr4p to bind this substrate in the presence of AMP-PNP (data not shown). As a control, we tested a mutant protein known to have a dominant negative in vivo effect (Mtr4p-K177A (20), see “Experimental Procedures” and Fig. 1A) for its ability to hydrolyze ATP in the presence of a 40-nucleotide single-stranded RNA. As expected, we saw no stimulation of ATPase activity with this mutant (Table 1). We also observed some unexpected behavior in our (d)ATPase experiments. Intriguingly, poly(A) is a poor stimulator of the (d)ATPase activity of Mtr4p. Each poly(A) substrate only weakly stimulated the (d)ATPase activity of Mtr4p, with the exception that the A20 substrate showed some stimulation of ATP hydrolysis (Table 1). The reasons why the ATPase activity of Mtr4p is stimulated less than half as effectively by A20 relative to substrate 2 are not entirely clear but could be due to the dynamics of the Mtr4p-poly(A) interaction. These points are considered under “Discussion.”

Mtr4p Unwinds Duplex RNA with 3’ to 5’ Polarity in a Concentration-dependent Manner—To determine whether Mtr4p possesses helicase activity, we monitored the displacement of a radioactively labeled RNA strand from a partial duplex substrate as a function of Mtr4p concentration. To determine the polarity of Mtr4p unwinding activity, two substrates were used, each with a 20-bp duplex region and a 20-nucleotide single-stranded tail on either the 3’- or 5’-end. Densitometric analysis showed that, in the presence of ATP and in response to increasing concentrations of Mtr4p, the labeled strand is concurrently released from the substrate containing a single-stranded 3’-end, to a maximum of 31.4% unwound in the presence of 5 μM Mtr4p (lane 10, Fig. 4), a 26-fold increase in product relative to that seen in the absence of Mtr4p (i.e. residual product strand that failed to anneal). In the presence of the substrate with a single-stranded 5’-end, Mtr4p was unable to displace the labeled strand (2.55–2.63% from 0–2.5 μM Mtr4p; lanes 2–5, Fig. 4). Mtr4p was unable to unwind a 20-bp duplex substrate without a single-stranded end (data

which is modest but still far below the cellular concentration of ATP. The K_{d} for substrate 1 is ~15 nm, which is similar to its K_{d} for that substrate (Table 2) and the K_{d} observed for Prp22p using a 40 nucleotide poly(A) RNA (37).

To examine the nucleic acid substrate specificity of Mtr4p, (d)ATPase assays were conducted in the presence of ssRNA,
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![Image of a diagram showing RNA unwind reactions](image)

FIGURE 4. Assessment of the polarity of Mtr4p helicase activity. 

Labeled RNA (*, 5 nM) was incubated in the presence of Mtr4p and 2 mM ATP, 5 mM MgCl₂, at 37 °C for 30 min. Following the incubation, stop buffer was added, and the mixture was electrophoresed on a non-denaturing 12% polyacrylamide gel. The substrates used in the two unwinding reactions are displayed above the gel. The polarity of unwinding for each substrate is displayed below the gel. The lane marked “ss” is a 20-nucleotide ssRNA standard. Lanes 2–5 contain a substrate with a single-stranded overhang on the 5’-end and increasing concentrations of Mtr4p. Even at 2.5 μM Mtr4p, no displacement of the labeled strand was observed. Lanes 6–10 contain a substrate with a single-stranded overhang on the 3’-end and increasing amounts of Mtr4p. The labeled strand is displaced in a concentration-dependent manner, up to a maximum of 31% unwound in the presence of 5 μM Mtr4p. The Mtr4p concentrations in lanes 6–10 are 0, 0.1, 1, 2.5, and 5 μM.

not shown). These results demonstrate that Mtr4p can unwind duplex RNA in the presence of ATP and a single-stranded RNA tail in the 3’→5’ direction. The fourth substrate tested was a 10-bp duplex region with a 30-nucleotide single-stranded 3’-end. Increasing the length of the single-stranded region and decreasing the size of the duplex region greatly increased the amount of product generated by Mtr4p (Fig. 5A). We were also able to demonstrate for this substrate that both ATP and dATP are competent to promote the unwinding activity of Mtr4p only when the single-stranded region is on the 3’-end (see lanes 5–8 and 9–12, and lane 13 for the 5’-tailed control, Fig. 5A). In these experiments, controls were performed in which nucleotide was omitted altogether and AMP-PNP replaced ATP. These experiments show that Mtr4p is unable to unwind duplex RNA in the absence of a hydrolyzable ATP source (see lanes C1 and C2, Fig. 5A). In accord with our findings, Wang et al. showed that, in the presence of ATP, Mtr4p produced a significant amount of unwinding product (i.e. displaced strand) in a time-dependent manner when incubated with a substrate containing a free 3’-end, but no product when incubated with a similar substrate containing a free 5’-end (38).

Given the observation that complexes between Mtr4p and poly(A) were distinct in both (d)ATP hydrolysis proficiency and binding behavior (see following section) from complexes with substrates containing a random sequence, we investigated whether or not Mtr4p could unwind a partial duplex substrate containing a poly(A) tail. Mtr4p can, in fact, unwind a partial duplex substrate containing a 20-nucleotide poly(A) tail in a concentration-dependent manner in the presence of either ATP or dATP (Fig. 5B). However, when tested as a function of time, we observed no significant differences in unwinding proficiency for the poly(A) or random partial duplex substrates (Fig. 5C).
Mtr4p Binds ssRNA in a Length- and Nucleotide-dependent Manner—To determine the binding affinity of Mtr4p for RNA we examined the measured anisotropy over a range of concentrations of Mtr4p. Four model substrates were tested: 1) a 40-nucleotide ssRNA of random sequence (substrate 1); 2) a 20-nucleotide ssRNA of random sequence (substrate 2) and two partial duplex RNA substrates each with a 20-nucleotide single-stranded region; 3) one containing a single-stranded 3’-end (substrate 3), and the other 4) a single-stranded 5’-end (substrate 4) (see Fig. 6A). Where appropriate, each binding isotherm was fit to the single site binding model described under “Experimental Procedures.” For those substrates and conditions with which we observed multiple complexes by EMSA (i.e. substrate 1 and substrate 4 in the absence of nucleotide, Fig. 6C), we compared the fits of the cooperative binding model (Equation 2) and Equation 1 to the data to determine the appropriate binding model. To determine nucleotide dependence of RNA binding we measured the anisotropy of Mtr4p-RNA complexes in the absence of nucleotide, in the presence of the non-hydrolyzable ATP analogue AMP-PNP, and in the presence of ADP. We examined these three states of Mtr4p because they reflect the stages of the (d)ATPase and thus the unwinding reaction. In the absence of nucleotide, which under our experimental design represents a point in between cycles of

FIGURE 6. Evaluation of Mtr4p binding to a series of RNA substrates. A, substrates used for evaluation of binding by fluorescence anisotropy. The location of the fluorescein label in each substrate is indicated by a star. B, representative binding isotherm for the association of Mtr4p with a single-stranded 40-nucleotide RNA (substrate 1 in A) in the presence of the non-hydrolyzable ATP analogue AMP-PNP. Binding was analyzed by fluorescence anisotropy as described under “Experimental Procedures” and plotted as a function of Mtr4p concentration. The curve overlaid on the data (solid black line) represents the best fit of the data to the single site binding model described by Equation 1. Each datum represents the average of three independent measurements. A plot of the residual values (lower panel) for the curve fit (i.e. the difference between the calculated anisotropy from the curve fit and the experimental data) shows no bias for data subsets, indicating a good fit to the single site model. C, electrophoretic mobility shift assay using 32P-labeled substrates 1–4. Unbound RNA is marked “Free RNA.” Where multiple shifted species are observed, each species is marked by the ◊ symbol.
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ATP hydrolysis, Mtr4p binds the tested substrates in the low (i.e. <40) nanomolar range and appears to discriminate only based on the size of the single-stranded RNA footprint (Table 2). The orientation of the single-stranded region (i.e. 3' versus 5'-overhang) has little effect on binding affinity (26 nm versus 35 nm). Although the EMSA experiments show two distinct shifted species for substrate 4, there is no evidence of cooperativity as judged by the quality of curve fits for Equation 1 versus Equation 2. This is consistent with the fact that the second shifted species is a minor one and doesn’t increase in prominence with increasing concentration (e.g. 100 versus 2000 nm, Fig. 6C). We interpret this as weak interactions with the duplex region. Doubling the ssRNA size to 40 nucleotides (substrate 1) more than doubled the binding affinity (17 nm versus 7 nm). Mtr4p binding to substrate 1 in the absence of nucleotide exhibits significant apparent cooperativity with a Hill coefficient of 2.3 ± 0.3 and multiple bound species in an EMSA (Fig. 6C). This binding behavior is only observed for substrate 1 in the absence of nucleotide (see below; contrast with the A20 substrate, discussed in the next section). Because substrate 2 (20 nucleotides) shows only one bound species by EMSA (Fig. 6C) and lacks cooperative behavior, it is likely that the minimal RNA binding site (random sequence) for Mtr4p is 20 nucleotides or less. In general, the tightest overall binding occurs in the absence of nucleotide (with the exception that the affinity for substrate 2 does not change in the presence of ADP). In the presence of AMP-PNP, which mimics the ATP-bound state, the binding affinity for all tested substrates is markedly reduced. To ensure that contaminating ATP was not artificially reducing the observed affinity of Mtr4p for RNA in the presence of AMP-PNP, we treated our AMP-PNP stock with hexokinase and measured binding to substrate 2. Although the hexokinase treatment does alter the binding affinity (63 nm treated versus 89 nm untreated, see supplemental Fig. S1A), the observed affinity is still markedly less than in the absence of nucleotide (21 nm). Therefore, we still conclude that the tightest binding is observed in the absence of nucleotide. In addition, both the length of the single-stranded region and placement of the duplex region affect the affinity of Mtr4p for the RNA substrate in the presence of AMP-PNP. Mtr4p binds substrate 1 the tightest, substrates 2 and 3 with roughly equivalent affinities, and substrate 4 (the 5'-tailed substrate) weakly (457 nm). Thus, ATP appears to provide discrimination between productive and non-productive Mtr4p-RNA complexes. In the presence of ADP, which would represent the enzyme in the act of ATP hydrolysis and/or translocation, Mtr4p shows a marked preference for a single-stranded stretch of RNA. Mtr4p binds the 20-nucleotide ssRNA ~7-fold more tightly than the partial duplex with a 20-nucleotide single-stranded 3' tail (146 nm versus 21 nm). The placement of the duplex region confers some specificity to Mtr4p binding in the presence of ADP (366 nm for substrate 4 versus 146 nm for substrate 3). This effect is less pronounced than that observed in the presence of AMP-PNP, in which case there was an ~5-fold difference in affinity between the two substrates (457 nm versus 92 nm). Strikingly, increasing the length of the ssRNA from 20 to 40 nucleotides had no effect on the binding affinity of Mtr4p in the presence of ADP (19 nm versus 17 nm).

Mtr4p Preferentially Binds Poly(A) RNA—The activity of Mtr4p is physically and functionally coupled to that of the Trf4p and Trf5p poly(A) polymerases via the TRAMP complex. Therefore, we examined the ability of Mtr4p to interact with poly(A). For these experiments, we used a chemically synthesized 20-nucleotide poly(A) (substrate A20). It is clear from an examination of the dissociation kinetics of Mtr4p-ssRNA complexes that the interaction of Mtr4p and poly(A) RNA is strikingly less dynamic than with substrate 2. In the presence of AMP-PNP, the Mtr4p-substrate 2 complex is highly dynamic, as evidenced by a swift decrease of the anisotropy of a pre-formed complex when challenged with unlabeled RNA. The off-rate (k_{off}) for the Mtr4p-A20 complex is 4-fold longer in the absence of AMP-PNP than in its presence, whereas the lifetimes of the two Mtr4p-substrate 2 complexes are statistically indistinguishable. B, A_{20} is a superior competitor for Mtr4p binding when directly compared with substrate 2. Mtr4p was incubated with a mixture of Fl-A20 and unlabeled competitor RNA both in the presence and absence of AMP-PNP as described under “Experimental Procedures,” and the anisotropy versus competitor concentration was plotted. The decrease in anisotropy as a function of competitor concentration was used to assess the relative preference of Mtr4p for A_{20} and substrate 2.

FIGURE 7. Preferential binding of Mtr4p to a short poly(A) oligonucleotide, A, the lifetime of the Mtr4p-A_{20} complex is significantly longer than that of a complex between Mtr4p and substrate 2. A pre-formed complex of Mtr4p and either Fl-A_{20} (left panel) or Fl-substrate 2 was challenged with a 5000-fold molar excess of unlabeled RNA and the anisotropy measured as a function of time. Each datum represents the mean value of multiple measurements. The off rate (k_{off}) of each complex was determined by fitting the anisotropy versus time curves to Equation 3. The lifetime of each complex was determined both in the presence and absence of AMP-PNP. The lifetime of the Mtr4p A_{20} complex is 4-fold longer in the absence of AMP-PNP than in its presence, whereas the lifetimes of the two Mtr4p-substrate 2 complexes are statistically indistinguishable. B, A_{20} is a superior competitor for Mtr4p binding when directly compared with substrate 2. Mtr4p was incubated with a mixture of Fl-A_{20} and unlabeled competitor RNA both in the presence and absence of AMP-PNP as described under “Experimental Procedures,” and the anisotropy versus competitor concentration was plotted. The decrease in anisotropy as a function of competitor concentration was used to assess the relative preference of Mtr4p for A_{20} and substrate 2.
structure/conformation confers this extended lifetime to the complex with A20 RNA. In the absence of nucleotide, the lifetime of the Mtr4p-A20 complex is markedly extended ($k_{off} \sim 0.006 \text{ s}^{-1}$, $t_{1/2} \sim 108 \text{ s}$, Fig. 7A, left panel). This decrease in off rate is consistent with our observation that Mtr4p binds A20 most avidly in the absence of nucleotide. In contrast, the lifetime of the Mtr4p-substrate 2 complex is not substantially different in the absence of nucleotide ($k_{off} \sim 0.07 \text{ s}^{-1}$, $t_{1/2} \sim 10 \text{ s}$, Fig. 7A, right panel) than in the presence of AMP-PNP. Statistical examination of the lifetime data of the two Mtr4p complexes indicates that the statistical significance of the differences is marginal for the Mtr4p-substrate 2 complexes ($p$ value $= 0.06$), whereas the difference between the $k_{off}$ of the two Mtr4p-A20 complexes is highly significant ($p$ value $< 0.0001$).

Based on the above dissociation kinetics, we determined that a preincubation time of 30 min was required to ensure that the system reached equilibrium in studies using the A20 substrate. It is clear from evaluation of the binding isotherms that Mtr4p binds A20 with tighter affinity than substrate 2 (see also Table 3), both in the presence and absence of AMP-PNP. In addition to solution binding experiments, we examined the preference of Mtr4p for poly(A) RNA directly in solution via competition experiments. In these experiments, a pre-formed Mtr4p-A20 complex was challenged with varying concentrations of competitor RNA (either substrate 2 or A20). Plots of observed anisotropy versus competitor RNA concentration indicate that A20 RNA competes far more effectively for Mtr4p binding than substrate 2. Using Equation 4, we can estimate an apparent $K_i$ of each competitor RNA, thereby providing an estimate of the relative preference of Mtr4p for poly(A) RNA. Examination of the apparent inhibition constants shows that A20 competes 60-fold more effectively in the presence of AMP-PNP and 28-fold more effectively in its absence (Fig. 7B). This further corroborates the preference of Mtr4p for poly(A) RNA. Upon examination of the binding isotherms and EMSA experiments for the A20 substrate, it became evident that the single-site model is inappropriate for the poly(A) system (Fig. 8A). The EMSA experiments clearly show two distinct bound poly(A) species in all three conditions (no nucleotide, ADP, and AMP-PNP; see Fig. 8B). An analysis of the curve fits by the $F$ test shows that the decrease in the sum of squared residuals obtained by using the cooperative binding model is statistically significant ($p$ values $< 0.01$ for all three nucleotide bound states), indicating that the Hill model better describes the poly(A) binding data. In contrast, the same analysis of the curve fits to the data for substrate 2 gives a $p$ value of 0.14. The midpoint concentration, which approximates the concentration at which half-maximal binding is achieved, is 11 nM in the absence of nucleotide, 47 nM in the presence of AMP-PNP, and 80 nM in the presence of ADP (see Table 3). Compared with substrate

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### TABLE 3

Apparent cooperativity observed in the association of Mtr4p with the A20 substrate and substrate 1 as a function of nucleotide

| Substrate | Nucleotide | $P_{max}$ | $h$ |
|-----------|------------|-----------|-----|
| A20       | None       | 11 ± 2    | 1.52 ± 0.04 |
| A20       | ADP        | 80 ± 5    | 1.89 ± 0.13 |
| A20       | AMP-PNP    | 48 ± 15   | 1.69 ± 0.31 |
| 1         | None       | 7 ± 1     | 2.31 ± 0.33 |
| 1         | ADP        | 17 ± 1    | N/A   |
| 1         | AMP-PNP    | 28 ± 6    | N/A   |

* The Mtr4p concentration (in nM) resulting in half-maximal binding ($P_{max}$) and Hill coefficients ($h$) were calculated from binding isotherms using Equation 2. Both resolved constants are expressed as the mean ± S.E. of the constant.

* Calculated using the single-site model; the cooperative model does not provide additional information as indicated by a $p$ value of $> 0.05$ ($F$ test).

* N/A, not applicable because the Hill model did not provide a better fit to the binding data.

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**FIGURE 8.** Binding of Mtr4p to the A20 substrate in the absence of nucleotide and in the presence of AMP-PNP or ADP. A, association of Mtr4p with A20 in the absence of nucleotide (left), in the presence of ADP (center), or presence of AMP-PNP (right) was analyzed by fluorescence anisotropy as described under “Experimental Procedures.” Binding isotherms were fit to a cooperative binding model using Equation 2 (solid lines). For comparison, the fit to the single-site binding model is also displayed (dashed lines). In all three cases, the cooperative binding model provided a statistically-superior fit to the poly(A) binding data. Residual plots (lower panels) are presented as described in Fig. 6B. Electrophoretic mobility shift assay using $^{32}$P-labeled A20 in the absence of nucleotide (left), presence of ADP (center), and presence of AMP-PNP (right). Unbound RNA is marked “Free RNA.” In all three panels, two distinct shifted species (marked by †) appear as the $^{32}$P-labeled A20 substrate is titrated with Mtr4p.
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2, Mtr4p binds A$_{20}$ with ~2-fold higher affinity, with the exception that binding in the presence of ADP is weaker. The apparent cooperativity of binding in the Mtr4p-A$_{20}$ complex is striking. Unlike substrate 1, the apparent cooperative behavior is observed regardless of nucleotide-bound state (i.e. nucleotide-free, ADP-bound, and AMP-PNP-bound). The Hill coefficient is >1.5 in each condition, indicating that the binding cooperativity is positive. Given the sizes of Mtr4p (123 kDa) and the A$_{20}$ RNA (6.7 kDa), it is unclear how the two complexes observed in the EMSA experiment differ. However, our data strongly suggest that sequence-specific interactions between Mtr4p and poly(A) evidence themselves as apparent cooperativity and markedly slowed dissociation kinetics and, furthermore, that these interactions are functionally important.

**DISCUSSION**

Recent biochemical and cellular data have shown that Mtr4p is necessary for processing a variety of RNAs and that its presence can stimulate the exonucleolytic activity of the exosome (9), perhaps by altering RNA structure (3). In this work, we have begun to characterize the essential activities of Mtr4p as a means to better understand its role in nuclear RNA processing. We have shown that Mtr4p hydrolyzes both ATP and dATP in an RNA-dependent manner, unwinds duplex RNA with 3'→5' polarity in the presence of either ATP or dATP, and binds ssRNA and partial duplex substrates with affinities that vary depending on both the nature of bound nucleotide and the RNA sequence, with a marked preference for short poly(A) tails.

**Nucleotide Hydrolysis Activity**—Mtr4p can hydrolyze only ATP or dATP, and requires an RNA substrate for effective hydrolysis. Although most substrates tested produced the expected effect, poly(A) was an unexpectedly anemic stimulator of the (d)ATPase activity of Mtr4p. Commercially available poly(A) substrates (see Table 1 and Fig. 2) stimulated the (d)ATPase activity of Mtr4p at extremely high concentrations (~29 μM of 20-nucleotide units), yet the amount of P$_1$ produced was less than that as a result of stimulation by 40 nM substrate 2. However, the A$_{20}$ substrate at a concentration of 40 nM can stimulate the ATPase activity of Mtr4p, albeit less efficiently than substrate 2. If poly(A) is such a poor stimulator of ATPase activity, why couple Mtr4p to a poly(A) polymerase as a means to promote exonucleolytic decay? Our RNA binding studies (discussed below) indicate that Mtr4p forms a unique complex with poly(A) RNA, thereby providing a likely explanation as to why these activities are coupled. Thus far experiments have shown that the polyadenylation activity of Trf4p and the presence of Mtr4p are required to stimulate the exosome *in vitro* (9, 10, 39). It has not yet been demonstrated that Mtr4p activity is required for TRAMP-stimulated exosome degradation, although recent data suggest that this is almost certainly the case (38, 39). It is possible that the inefficiency with which poly(A) stimulates the ATPase activity of Mtr4p is designed to limit Mtr4p activity to those circumstances in which the TRAMP complex is required to complete stalled degradation and/or processing. Thus, Mtr4p would only actively hydrolyze ATP when physically associated with both the polyadenylation machinery and the short poly(A) tail that marks the bound substrate for degradation or processing, avoiding normally polyadenylated mRNAs. This idea is consistent with two observations: first, the A$_{20}$ substrate can stimulate the ATPase activity of Mtr4p; second, the length of poly(A) added by Trf4p and Trf5p in *vitro* is typically between 7 and 20 adenosines (40, 41).

**Helicase Activity**—In this study, we have shown that purified Mtr4p is a *bona fide* RNA helicase and that, like other DEXH-box helicases such as NPH-II (42) and NS3 (43), Mtr4p unwinds duplex RNA in the 3'→5' direction in a concentration-dependent manner. The fact that a vast molar excess of Mtr4p is required to displace a 20-nucleotide strand from a partial duplex substrate suggests that Mtr4p, unlike NPH-II (44), does not efficiently unwind duplex RNA, at least not in the absence of other protein factors. Recently another group observed comparably limited activity when testing the helicase activity of Mtr4p on substrates containing similar lengths of duplex RNA (38). This is not surprising given that nuclear RNA processing will not likely require Mtr4p to unwind long stretches of duplex RNA in a single pass. The RNA processing reactions are instead tailored to yield discrete functional 3’-ends on their target RNA substrates. Mtr4p also participates in the degradation of aberrant RNAs in the nucleus, a function that could conceivably involve unwinding longer stretches of RNA. Perhaps in these instances, a cofactor (i.e. Trf or Air proteins, exosome subunits, etc.) enhances the amount of strand displacement by Mtr4p similar to the manner that eIF4B and eIF4H stimulate unwinding by eIF4A (45), and NS4A stimulates unwinding by NS3 (46). Surprisingly, the change in binding affinity and dynamic behavior associated with the interaction of Mtr4p with the A$_{20}$ substrate do not result in a detectable enhancement of unwinding proficiency. The modest response of the (d)ATPase activity of Mtr4p to poly(A) substrates appears to attenuate the effect of complex dynamics on helicase activity. Perhaps, because the hydrolysis of nucleotide requires significant conformational changes, the nature of the poly(A)-Mtr4p interaction is such that the energetic costs of these conformational changes are increased to make nucleotide hydrolysis and subsequent unwinding less likely. If so, this would represent a remarkable scheme for self-regulation by Mtr4p. This scenario might reflect the need for efficient use of ATP when parallel processes (i.e. Trf4/5 polyadenylation) increase demands for ATP. More specifically, for the large expenditure of energy in the form of ATP to be worthwhile, each ATP hydrolysis event needs to have associated with it a high probability that it will result in RNA structure modification that promotes exonucleolytic decay. Thus, the trigger for stimulating the unwinding activity of Mtr4p would not entail a simple dramatic increase in activity in response to limited polyadenylation. Rather, Mtr4p would only respond to this signal in the presence of other protein factors. Clearly the Trf and Air proteins are likely candidates for proteins that stimulate the unwinding activity of Mtr4p. However, a more intricate level of regulation could be achieved if interactions with exosome components, either in the context of the TRAMP complexes or alone, constituted the necessary stimulus to promote unwinding by Mtr4p.

By establishing the polarity with which Mtr4p unwinds RNA, we can gain some insight into how the helicase acts in the context of the RNA processing machinery in the nucleus. Mtr4p
interacts with the same strand that is subject to exonucleolytic cleavage by the exosome (most likely by the Rrp44p subunit (5)). Thus, there exists the possibility of a direct handoff of the newly formed single-stranded RNA to the RNA processing machinery. Consistent with this idea, a recent study demonstrated that tandem affinity-purified TRAMP complex promotes the degradation of hypomethylated tRNA<sub>Met</sub> by purified recombinant Rrp44p alone, an effect that is enhanced upon supplementing Rrp44p with the remaining exosome subunits (39).

**RNA Binding Is Influenced by Nucleotide**—The affinity of Mtr4p for various RNA substrates is dependent both on the composition of the substrate itself and the status of bound nucleotide. We have found for each substrate tested that Mtr4p binds tightest in the absence of nucleotide. It is unusual, but not unprecedented, for a helicase to bind a nucleic acid substrate more tightly in the absence of bound nucleotide. The hepatitis C virus NS3 helicase is one notable example of a helicase that binds RNA more tightly in the absence of nucleotide (47, 48). In contrast, the differences in affinity observed for those substrates with ADP versus AMP-PNP are of dubious statistical significance as judged by an unpaired t test.

**RNA Binding as a Function of Substrate**—Our RNA binding studies have also given insight into how Mtr4p discriminates potential unwinding substrates from non-substrate RNAs. We chose model substrates in which variations were introduced to three parameters: first, the length of ssRNA available for Mtr4p binding; second, the presence or absence of a region of duplex RNA; and third, placement of the duplex RNA (Table 2 and Fig. 6A). In the absence of nucleotide, discrimination between substrates appears to be solely based on the length of ssRNA presented to Mtr4p. These data suggest that, in the absence of nucleotide, Mtr4p does not “sense” the presence of the duplex region. In the presence of AMP-PNP, Mtr4p binds the substrates tested with markedly different affinities, discriminating now on the basis of length of the ssRNA region and the placement of the duplex region. Mtr4p binds the 5′-tailed substrate weakly (~0.5 μm), whereas the 3′-tailed substrate is bound more tightly (~90 ms). This behavior suggests that there is some structural transition affected by ATP binding that causes Mtr4p to reject the 5′-tailed substrate as being non-productive. The simplest explanation would be that a structural expansion causes part of Mtr4p to clash with the 5′ duplex region, leading to dissociation from the RNA. However, recent studies of the archaeal DEXH-box helicase Hel308 (49) and the related DEAD-box helicases (50–55) have failed to demonstrate that nucleotide binding leads to a less compact conformation. This suggests that the preference of Mtr4p for 3′-tailed partial duplex substrate in the presence of AMP-PNP might not be simply explained by an increase in binding footprint and requires further investigation. In the presence of ADP, Mtr4p displays yet a different preference for RNA substrates, strongly favoring ssRNA over the partial duplex substrates. In particular, the preference for the 20-nucleotide ssRNA over the 3′-tailed substrate is ~7-fold, despite the fact that the same length of ssRNA is present in both substrates. These two observations suggest that Mtr4p responds to elements 5′ to the current binding site.

**Poly(A) Binding**—The most intriguing results of our RNA binding experiments are that Mtr4p forms a complex with the A<sub>20</sub> substrate that is of higher affinity than the complex containing a counterpart with a random sequence (substrate 2) and that the dynamics of this complex are entirely different than those exhibited by the Mtr4p-substrate 2 complex. There is a precedent in bacteria for adding short stretches of poly(A) to RNAs flagged for degradation as a means of providing a foothold for the associated helicase, thus maintaining the momentum of decay (56). Our results indicate that this “foothold” in the yeast system is not just a convenient landing pad for the helicase. Rather, Mtr4p binds to a short poly(A) stretch in such a manner as to severely retard its dissociation from the RNA substrate as evidenced by the dramatically slowed off rates both in the presence and (especially) absence of AMP-PNP. The tenacious hold that Mtr4p has on the poly(A) substrate indicates that the coupling of the helicase activity of Mtr4p with the Trf poly(A) polymerases was specifically designed such that, once an RNA substrate is flagged for degradation, Mtr4p does not allow that substrate to be released when degradation is slowed by secondary structure or bound proteins. The fact that active Trf4p is required for degradation of hypomethylated tRNA<sub>Met</sub> (39), combined with our observations, suggest that the helicase activity of Mtr4p is carefully coordinated with the poly(A) polymerase activity of the Trf proteins to ensure degradation of highly structured substrates. The lack of propensity of poly(A) to form secondary structure suggests that some aspect of the structure of Mtr4p accounts for this unique mode of binding. Perhaps the C-terminal 500 amino acids that reside outside the superfamily 2 helicase motifs, which have no known function, specifically interact with poly(A) RNA to prevent dissociation. The effects of poly(A) binding on helicase activity in the context of the TRAMP complex and exosome subunits (in particular Rrp44p) and how these effects impact processing and degradation require further investigation.

Taken together, these RNA binding experiments suggest the existence of two RNA binding modes that correspond to the two functional modes of Mtr4p (i.e. processing and degradation). Mtr4p binds to ssRNA in a variety of contexts in a manner that is controlled by the status of bound nucleotide. Based on the rapid dynamics of the interactions with the model substrates composed of random sequences, Mtr4p appears to be in “processing” mode when in complex with these substrates. In contrast, Mtr4p avidly binds to poly(A) RNA in a positively cooperative manner and the Mtr4p-poly(A) complex exhibits drastically slower dynamics. This reluctance to release bound poly(A) RNA suggests that Mtr4p is interacting with poly(A) in “degradation” mode. Thus, Mtr4p might maintain its complex with poly(A) RNA to ensure degradation and prevent release of that substrate into the nuclear pool of RNAs.

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