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In vitro Reconstitution and Characterization of the Yeast Mitochondrial Degradosome Complex Unravels Tight Functional Interdependence

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The mitochondrial degradosome (mtEXO), the main RNA-degrading complex of yeast mitochondria, is composed of two subunits: an exoribonuclease encoded by the *DSS1* gene and an RNA helicase encoded by the *SUV3* gene. We expressed both subunits of the yeast mitochondrial degradosome in *Escherichia coli*, reconstituted the complex *in vitro* and analyzed the RNase, ATPase and helicase activities of the two subunits separately and in complex. The results reveal a very strong functional interdependence. For every enzymatic activity, we observed significant changes when the relevant protein was present in the complex, compared to the activity measured for the protein alone. The ATPase activity of Suv3p is stimulated by RNA and its background activity in the absence of RNA is reduced greatly when the protein is in the complex with Dss1p. The Suv3 protein alone does not display RNA-unwinding activity and the 3′ to 5′ directional helicase activity requiring a free 3′ single-stranded substrate becomes apparent only when Suv3p is in complex with Dss1p. The Dss1 protein alone does have some basal exoribonuclease activity, which is not ATP-dependent, but in the presence of Suv3p the activity of the entire complex is enhanced greatly and is entirely ATP-dependent, with no residual activity observed in the absence of ATP. Such absolute ATP-dependence is unique among known exoribonuclease complexes. On the basis of these results, we propose a model in which the Suv3p RNA helicase acts as a molecular motor feeding the substrate to the catalytic centre of the RNase subunit.

Keywords: mitochondria; RNA degradation; mitochondrial degradosome; exoribonuclease; helicase

Introduction

Controlling RNA levels is one of the main prerequisites for the regulation of genome expression. The fate of any RNA in the cell is determined though an interplay of transcription and degradation.[1–4] RNA degradation is required to eliminate processing by-products and aberrant or defective molecules that form during synthesis and maturation of various RNAs (RNA surveillance).[5,6] The post-transcriptional mechanisms of genetic regulation, including RNA degradation, are of particular importance in mitochondria, because the transcriptional control is relatively simple,[7–11] and polycistronic RNAs require extensive processing.[12–14] Maintaining the balance between RNA synthesis and degradation is vital for the functioning of the mitochondrial genetic system.[15]

RNA degradation in prokaryotic and eukaryotic cells and organelles is executed by a number of very divergent enzymatic complexes and involves the action of exonucleases, both 3′ to 5′ and 5′ to 3′ endonucleases,[1–3,16–18] with a plethora of ancillary enzymatic activities, of which RNA helicases are a prominent group.[5,19–22] The 5′ to 3′ exoribonu-

Abbreviation used: dsRNA/DNA, double-stranded RNA/DNA.

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cleases were found to participate in RNA degradation only in eukaryotic cells, while 3′ to 5′ exonucleases are distributed universally in all types of cells and organelles.[1,3,23]

These exoribonucleases are often found in large multisubunit complexes, like the bacterial degradosome,[16,23] which is assembled around the endonuclease (RNase E), with associated activities such as an RNA helicase (RhlB) and phosphorolytic exoribonuclease (PNPase), and the exosome of the eukaryotes and Archaea.[2,5,24–26]

The enzymatic activity responsible for RNA turnover in all mitochondrial systems known so far is also that of a 3′ to 5′ processive exoribonuclease, although the actual enzymes show great evolutionary divergence.[3] The only potential 5′ to 3′ ribonuclease found in mitochondria is associated with the protein encoded by the yeast *PET127* gene,[27] although the ribonucleolytic activity of its product has not been demonstrated directly.

The first mitochondrial RNA degradation complex, named mtEXO or the mitochondrial degradosome, was described in the yeast *Saccharomyces cerevisiae*.[28,29] Yeast mitochondria, unlike bacterial cells and animal[30] or plant[31] mitochondria, lack the phosphorolytic polynucleotide phosphorylase (PNPase) activity, and mtEXO is the only known exoribonuclease participating in yeast mtRNA turnover.

The yeast mitochondrial degradosome, a very basic form of an RNA degradation enzymatic machinery, is composed of only two subunits; an RNR (RNase II-like) family exoribonuclease encoded by the *DSS1* (*MSU1*, YMR287C) gene;[29,32,33] and an NTP-dependent RNA helicase related to the DExH/D (Ski2p) superfamily, encoded by the *SUV3* (YPL029W) gene.[29,34]

RNases belonging to the RNR family, RNaseR and RNase II,[17,35–38] are involved in RNA turnover in bacteria. Even though the main exoribonuclease of the bacterial degradosome complex is the phosphorolytic PNPase,[16] hydrolytic exoribonucleases associate with the degradosome in some γ-proteobacteria.[39] In eukaryotes, a 3′ to 5′ exoribonuclease related to RNase II is a component of the nuclear and cytoplasmic exosome,[18] which plays a crucial role in RNA metabolism. It was demonstrated recently that the hydrolytic 3′ to 5′ exoribonuclease Dis3p is essentially responsible for the activity of the yeast exosome core.[26] In the chloroplasts of higher plants, an RNR-family exoribonuclease is involved in the maturation of rRNAs.[40] In yeast mitochondria, the 3′ to 5′ hydrolytic exoribonuclease associated with the mtEXO complex appears to be the main activity involved in RNA turnover.[15,29]

RNA helicases, like the protein encoded by the *SUV3* gene, are a common feature of protein complexes involved in RNA turnover in prokaryotes and eukaryotes.[5,19–22] Unlike *DSS1*, the *SUV3* gene of yeast has significantly conserved orthologues in all eukaryotes,[41–43] which encode proteins localized in mitochondria.

The activity of both Dss1p and Suv3p is essential for the functioning of the complex and their genetic inactivation leads to a similar phenotype, corresponding to a complete loss of the mitochondrial degradosome function. Such strains are strictly respiratory-deficient, and the stability of their mitochondrial genome is severely impaired.[15,29,32,34,44,45]

The molecular phenotype of the mitochondrial degradosome deficiency is consistent with impaired RNA turnover and surveillance, and includes overaccumulation of excised intronic sequences linked to the destabilization of transcripts,[44,46,47] accumulation of transcripts with abnormal 5′ and 3′ termini and of high molecular mass precursors, decreased steady-state levels of mature transcripts and disruption of translation.[15,29,44] Partial loss-of-function mutations in genes encoding the two subunits of the mitochondrial RNA polymerase that severely reduce the transcription rate in mitochondria partially rescue the phenotype associated with the degradosome deficiency, suggesting the vital importance of maintaining the balance between RNA synthesis and degradation.[15]

Initial characterization of the enzymatic activity of the mitochondrial degradosome complex purified from yeast cells demonstrated that it possessed the RNA helicase and 3′ to 5′ exoribonuclease activities. The two activities were found to be physically tightly associated and interdependent.[29,45]

In this work, we expressed both yeast mitochondrial degradosome subunits in *Escherichia coli* and reconstituted the functional complex. We compared the intrinsic RNase activity of purified Dss1p with that of the entire complex, and measured RNA binding capabilities of both degradosome subunits. We assayed the ATPase activity of Suv3p alone and in complex, and analyzed the stimulation of ATPase activity by the RNA substrates of the reconstituted complex and the Suv3 protein. Finally we found that only the reconstituted complex, and not the Suv3p subunit alone, showed the helicase activity in our assays *in vitro*.

Results

Heterologous expression and purification of the yeast mitochondrial degradosome and its components

Plasmids expressing full-length Suv3 and Dss1 proteins, both devoid of the N-terminal 26 amino acid residues encoding the mitochondrial targeting sequence, were prepared as described in Materials and Methods. The proteins were expressed as His_6MBP fusions,[48] and initially purified on a Ni-NTA column. The entire His_6MBP tag was subsequently removed by cleavage with TEV protease,[49] and the resulting protein preparation was finally purified on a Superdex 200 size-exclusion chromatography column (Figure 1(a)).

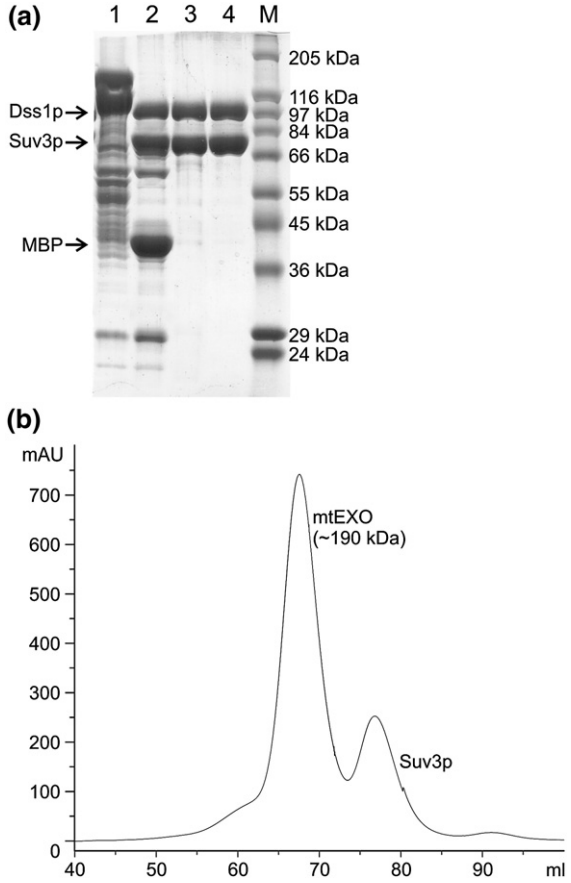


Figure 1. Purification of heterologously expressed mitochondrial degradosome (mtEXO) complex. (a) SDS-PAGE analysis of proteins in various stages of the preparation procedure (see Materials and Methods for details): 1, eluted proteins after the first round of purification on the Ni-NTA column; 2, proteins after cleavage with TEV protease; 3, proteins after the second round of purification on the Ni-NTA column; and 4, protein preparation after the final purification on the Superdex 200 size-exclusion column; M, molecular mass marker M4038 wide (Sigma). (b) Elution profile of the purified mtEXO complex on an externally calibrated Superdex 200 size-exclusion column.

In order to reconstitute the mitochondrial degradosome, bacterial cells expressing Suv3p and Dss1p were grown and frozen separately and, upon thawing, mixed at a 1:3 ratio to account for weaker binding of the Dss1-His_6MBP fusion protein to the Ni-NTA column, and the subsequent steps of the purification protocol were performed for this mixture. Analysis of the elution profile of the co-purified complex on an externally calibrated Superdex 200 size-exclusion chromatography column (Figure 1(b)) revealed a symmetrical peak at 190(±5) kDa, which corresponds to the sum of predicted masses of Dss1p and Suv3p (107.6 kDa and 81.7 kDa, respectively, for the expressed fragments), strongly suggesting a 1:1 heterodimer stoichiometry for the reconstituted complex. An additional peak corresponding to excess free Suv3p was also seen. Fractions corresponding to the mtEXO peak were collected and used in subsequent experiments.

In another experiment, separately purified Suv3p and Dss1p subunits were mixed just before the enzymatic assays. In all our assays, this mixture performed similarly to the co-purified reconstituted mtEXO complex (data not shown), which suggests that the two purified subunits bind each other easily and reconstitute the functional complex *in vitro*.

The exoribonuclease activity is enhanced greatly in the presence of the helicase subunit

The RNase activity of the yeast mitochondrial degradosome is associated with the Dss1 protein belonging to the RNR family of exoribonucleases.[29] We assayed the exoribonucleolytic activity of the heterologously expressed and purified Dss1p and the entire two-subunit mtEXO complex in the absence or in the presence of ATP. The helicase subunit Suv3p, which is not supposed to have any intrinsic ribonuclease activity, was included as a control.

Figure 2(a) shows the result of the assay using a radiolabeled, single-stranded polynucleotide RNA substrate. Reaction products were separated using thin-layer chromatography on polyethyleneimine (PEI)-cellulose plates. Migration of the degradation product corresponded to that of nucleoside monophosphates, which are released by ribonucleases belonging to the RNR family, like RNase II,[50–52] RNase R,[52] and Dis3.[26]

As expected, no ribonucleolytic activity was observed with the Suv3 protein or with the negative control. The Dss1 protein alone did exhibit some RNase activity, which was not dependent on ATP. The activity of the reconstituted mtEXO complex appeared, however, to be significantly higher than that of the ribonuclease subunit alone. Interestingly, when in complex with the helicase subunit, the ribonuclease was entirely ATP-dependent and showed no activity in the absence of ATP, even though the Dss1 protein alone did degrade the RNA substrate with a low efficiency regardless of the presence of ATP. Different polynucleotide substrates, either heterologous (a 341 nt control RNA from the T7 Transcription Kit, see Materials and Methods) or corresponding to fragments of yeast mitochondrial transcripts were used as substrates (data not shown) and no discernible substrate preference was ever observed.

Degradation of RNA by the reconstituted mtEXO complex was further analyzed using denaturing polyacrylamide gel electrophoresis to separate reaction products from substrates. In Figure 2(b), an 831 nt polynucleotide corresponding to the 3′ portion of the mature yeast mitochondrial *CYTB* transcript labeled uniformly with [α-^{32}P]UTP was used as a substrate. The mtEXO complex digested the substrate completely with no band corresponding to a partially degraded molecule apparent on the gel. When a shorter (30 nt) oligonucleotide labeled at the

5′ end was used as a substrate (Figure 3(c)) we were able to observe a residual oligoribonucleotide core left by the enzyme. Comparing its migration on the gel with the fragments obtained by limited digestion of the substrate with RNase T_1 we determined the size of this residual core to be four nucleotides (the 4 nt fragment generated by RNase T_1 migrated slightly faster due to the presence of an additional 3′ phosphate group). Residual cores of similar sizes are left by RNase II (3–5 nt),[50–52] RNase R (2–3nt),[52] and the yeast exosome subunit Dis3p (~3 nt).[26]

The increase of RNase activity observed for the entire complex could be attributed to stronger substrate binding by the Suv3 protein, or to increased catalytic activity of the complex in comparison with the Dss1 protein alone. In order to investigate these possibilities, we measured the Michaelis–Menten kinetics of RNA degradation by the degradosome complex and the Dss1 protein alone using a labeled, single-stranded RNA oligonucleotide 5W (see Materials and Methods) as substrate. Non-linear regression was used to fit theoretical curves to the data and estimate K_m and V_{max}. The results shown in Figure 3 and Table 1 indicate that the V_{max} of the entire complex (55(±3) nmol·min^{-1}·mg^{-1}, calculated per 1 mg of the Dss1p subunit) is significantly higher (about tenfold) than that of the Dss1p alone (6(±0.6) nmol·min^{-1}·mg^{-1}), and the difference is significant at the 95% confidence level. These values correspond to a k_{cat} of about 6 min^{-1} and 0.6 min^{-1}, respectively. By the way of comparison, bacterial RNase R has a k_{cat} between about 1.2 min^{-1} and 0.6 min^{-1}, depending on the substrate.[36]

When the RNase activity of the reconstituted mtEXO complex was compared with that of the Dss1 protein alone, the K_m for the entire complex appeared to be higher than for Dss1 protein alone, the activity of Dss1p with low substrate concentrations was, however, too weak to allow reliable estimation of K_m.

The significant decrease in RNase activity of the Dss1 protein in the absence of the Suv3p helicase, observed for the polynucleotide substrates (Figure 2 (a)), could be attributed to the inhibitory properties of secondary structure elements in the degraded RNA. Similar dependence of the RNase activity on the presence of the Suv3p helicase was, however, observed in Figure 3 using a shorter ssRNA oligonucleotide substrate. Using the RNAfold program, we predicted that the 30 nt RNA oligonucleotide 5W

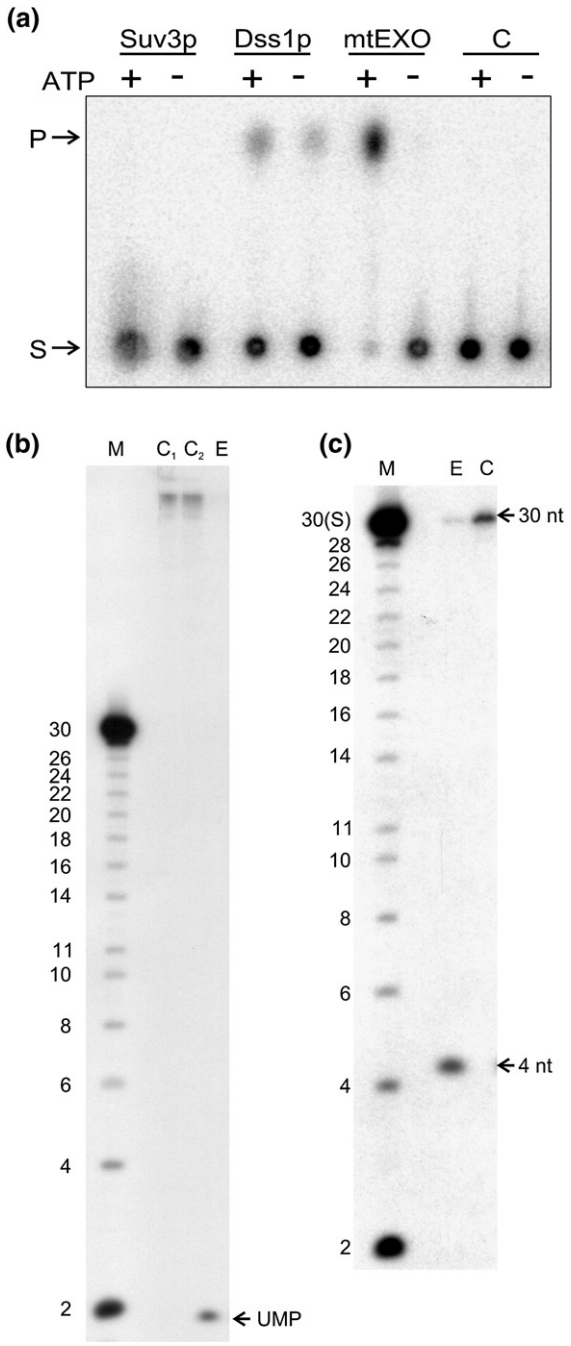


Figure 2. Ribonuclease activity of the mtEXO complex and its components. (a) The single-stranded polynucleotide RNA substrate (heterologous 341 nt RNA) labeled with [α-^{32}P]UTP (S) was incubated for 30 min with 0.1 μg of Suv3p, Dss1p or the reconstituted mtEXO complex, or with the reaction buffer without protein as control (C) and the mixtures were separated by thin-layer PEI-cellulose chromatography and visualized by autoradiography as described in Materials and Methods. Reaction products (P), corresponding to nucleoside monophosphates, are indicated. (b) The single-stranded polynucleotide substrate (a fragment of the yeast *CYTB* transcript) was incubated with the mtEXO (E) complex, or with water (C1), or with reaction buffer (C2) as controls. Reaction conditions were the same as in (a). Reaction products were separated on a denaturing 20% polyacrylamide gel. M is the RNA size marker resulting from limited RNase T_1 digestion of the 5W oligonucleotide labeled at the 5′ end. Fragment sizes are indicated at the left. (c) The 5W RNA oligonucleotide (S, 30 nt) labeled on the 5′ end was incubated with the mtEXO complex (E) or with the reaction buffer with no protein added (C). Reaction conditions were the same as in (a) and (b). Reaction products were separated on a denaturing 20% polyacrylamide gel with the RNA size marker (M) obtained by limited digestion of the 5W oligonucleotide with RNase T_1 as in (b). Positions of the marker bands and the residual 4 nt core left by the mtEXO complex are indicated.

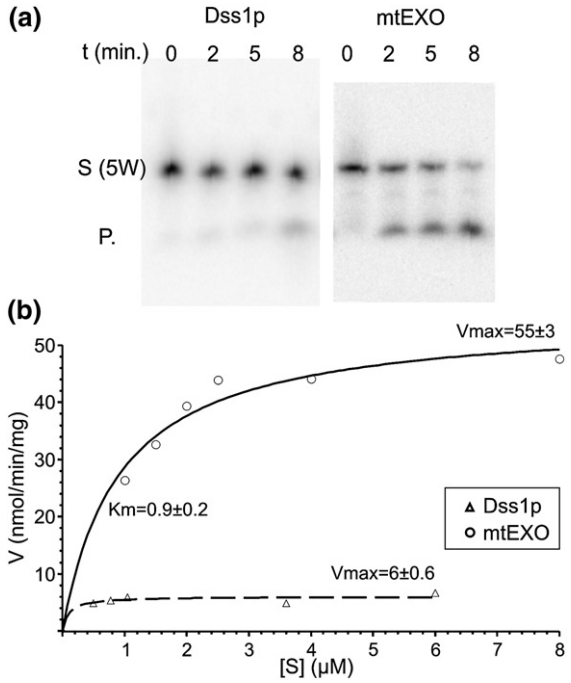


Figure 3. Kinetics of the exoribonuclease activity of Dss1p and the reconstituted mtEXO complex. The 5W RNA oligonucleotide labeled on the 5′ end was used as substrate, with 0.1 μg of each protein/reaction. (a) Typical time-course results obtained with Dss1p and mtEXO. Reaction mixtures were separated on denaturing 10% polyacrylamide gels, the positions of substrate (S) and product (P) are indicated. (b) Michaelis–Menten reaction kinetics obtained using various concentrations of substrate. The theoretical curve was fit by non-linear regression, estimated values of K_m and V_{max} are shown together with the standard error of estimation derived from the curve fit. The activity of Dss1p with low concentrations of substrate is too weak to allow reliable estimation of K_m.

used as substrate in those assays does not fold into any secondary structure stable at 30 °C.[53]

Both subunits of the mtEXO complex have similar RNA-binding capability

Our results suggest that the presence of Suv3p in the mitochondrial degradosome greatly enhances its ribonuclease activity, even when the single-stranded substrate is devoid of any secondary structure. In order to verify whether the Suv3p subunit is required for efficient binding of the RNA substrate by the reconstituted mtEXO complex, we performed RNA-binding assays for the Suv3 and Dss1 proteins alone or in complex using the filter-binding method, with the single-stranded RNA oligonucleotide 5W as substrate. The results, shown in Figure 4 and Table 1, indicate that both Suv3p and Dss1p can bind this RNA substrate with similar affinities, corresponding to a K_d value of about 200 nM. The binding experiments were performed in the absence of divalent metal cations and ATP to avoid RNA hydrolysis by mtEXO and Dss1p. The addition of 0.5 mM ATP did not influence the binding affinities of either protein (data not shown). The RNA-binding affinity of the reconstituted mtEXO complex is comparable to that of the single subunits with a K_d of about 125 nM (assuming monomeric binding). The lower apparent K_d value of the complex may suggest that both subunits contribute to the binding of RNA under the experimental conditions. These results indicate that the Dss1p RNase subunit is capable of binding RNA on its own, and that the contribution of Suv3p to the increased activity of the entire complex is not related directly to stronger RNA binding.

Similar binding affinity analysis for Suv3p, Dss1p and the mtEXO complex was also performed using a longer (831 nt) RNA polynucleotide corresponding to the 3′ portion of the mature yeast mitochondrial *CYTB* mRNA. All three protein preparations bound this RNA substrate with similar affinities, with 50% saturation achieved at ~20–30 nM protein (data not shown). The lower concentration of protein required for 50% saturation of binding suggests that the longer RNA presents multiple binding sites for the proteins, the quality of the data is, however, not sufficient for detailed quantitative analysis. Results obtained with the long polynucleotide RNA still support the conclusion that the Suv3p and Dss1p subunits do not differ significantly in their RNA-binding capability.

RNA-dependence of the ATP hydrolysis reaction by the Suv3p helicase is increased in the presence of the exoribonuclease

The Suv3 protein belongs to the DExH/D superfamily of RNA helicases,[29,34,47] which possess the

Table 1. Summary of the main properties of the mitochondrial degradosome components Suv3p and Dss1p and of the entire complex reconstituted *in vitro*

Activity	Dss1p	Suv3p	mtEXO
ATPase[a]	–	3.2(±0.16) μmol·min^{-1}·mg^{-1} [b]	3(±0.12) μmol·min^{-1}·mg^{-1} [c]
RNase	6(±0.6) nmol·min^{-1}·mg^{-1}	–	55(±3) nmol·min^{-1}·mg^{-1}
Helicase	–	–	++
RNA binding[d]	K_d=209(±47) nM	K_d=203(±43) nM	K_d=125(±30) nM

[a] In the presence of RNA.
[b] Significant background activity in the absence of RNA (~50%).
[c] Low background activity in the absence of RNA (~16%).
[d] Oligonucleotide substrate, assuming monomeric binding.

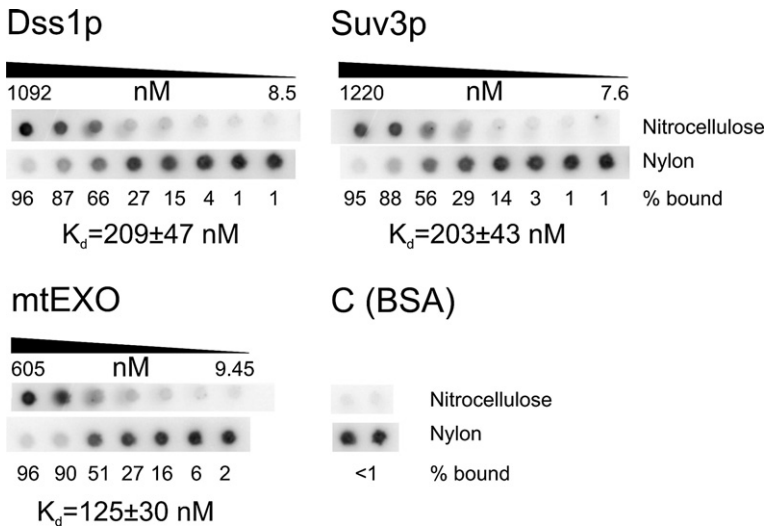
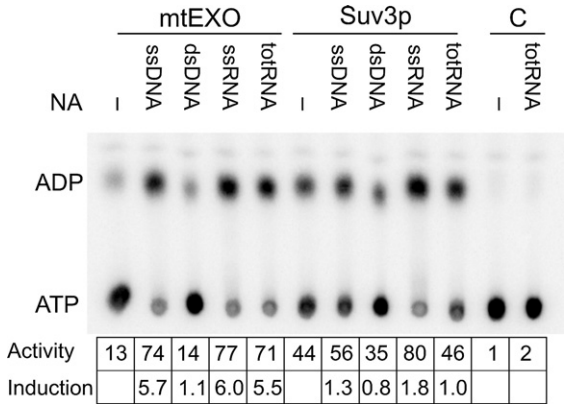


Figure 4. RNA binding by Suv3p, Dss1p and the reconstituted mtEXO complex. The 5W RNA oligonucleotide labeled on the 5′ end was used in a double-filter assay at a concentration of 87.5 pM (1.75 fmol/20 μl reaction) with a series of twofold dilutions of each protein, with starting and ending values shown for each series. BSA was used as a negative control (C). Protein-bound RNA is retained on the nitrocellulose membrane, the remaining unbound RNA binds to the nylon membrane. Results of a typical experiment are shown, the percentage of bound RNA was calculated as the mean of four independent assays. K_d was estimated by a non-linear regression fit of the data to the formula $B=[P]/(K_d+[P])$, where B is the bound RNA fraction and [P] is the concentration of protein. The standard error of estimation was derived from the curve fit.

Walker A and Walker B ATPase motifs. We analyzed the ATPase activity of the Suv3 protein alone and in complex with the RNase subunit Dss1p using radiolabeled ATP as substrate. The results shown in Figure 5 demonstrate that the Suv3 protein and the reconstituted mtEXO complex can hydrolyze ATP *in vitro*, and that this ATPase activity is, as expected, stimulated by the presence of RNA, both short oligoribonucleotides and total yeast RNA. Short single-stranded DNA oligonucleotides also stimulated the ATPase activity of Suv3p, while the longer double-stranded DNA molecules had no effect on either Suv3p alone or the mtEXO complex.



Activity	13	74	14	77	71	44	56	35	80	46	1	2
Induction		5.7	1.1	6.0	5.5		1.3	0.8	1.8	1.0		

Figure 5. ATPase activity of Suv3p and the reconstituted mtEXO complex. The Suv3 protein (0.4 μg) and the reconstituted mtEXO complex (0.8 μg) were incubated with [α-^{32}P]ATP as substrate in the presence or in the absence of the indicated nucleic acid (NA): 20 nt single-stranded DNA oligonucleotide (ssDNA), plasmid DNA (dsDNA), single-stranded RNA oligonucleotide 5W (ssRNA) or total yeast RNA (totRNA) as indicated (1 μg of NA each time). The control reaction (C) contained no protein. Reaction mixtures were separated on PEI-cellulose thin-layer chromatography plates as described in Materials and Methods, the positions of substrate (ATP) and product (ADP) are indicated. Relative activity is expressed as the activity of product divided by the sum of activities of substrate and product (100·ADP/(ADP+ATP)). Induction is given as the fold change in activity relative to the reaction with no nucleic acid added for a given protein. The values are means of two independent experiments.

The ATPase activity of the reconstituted degradosome in the presence of RNA was comparable to the activity of the Suv3 protein alone. The activity of the reconstituted complex in the absence of RNA or single-stranded DNA was, however, significantly lower than for the Suv3 protein alone. The background activity (without any stimulating nucleic acid) of the Suv3 protein was more than threefold higher than the background activity of the reconstituted complex (Figure 5). Thus, RNA or ssDNA increased the activity of the mtEXO complex about sixfold, while for the Suv3 protein alone, the difference was no greater than twofold. By way of comparison, the ATPase activity of Mss116p is increased fourfold in the presence of RNA.[54] Therefore, the presence of the RNase subunit did not increase the RNA-dependent ATPase activity of Suv3p significantly, but it increased its inducibility by decreasing the non-specific activity in the absence of the nucleic acid substrate.

We measured the Michaelis–Menten kinetics of ATP hydrolysis by Suv3p and the reconstituted mitochondrial degradosome complex in the presence of RNA. Non-linear regression was used to fit theoretical curves to the obtained data. The results are shown in Figure 6 and Table 1. The K_m value for the Suv3 protein alone (~200 μM) appears to be higher than for the complex (~100 μM), but the difference is not significant at the 95% confidence level. The V_{max} for Suv3p and for the entire complex is very similar (about 3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) and corresponds to a k_{cat} of about 250 min^{-1}. This value is similar to that of the yeast Ded1p helicase (210–680 min^{-1})[55,56] and appears to be higher than that of the yeast Mss116p protein (56.7 min^{-1})[5]. Measured

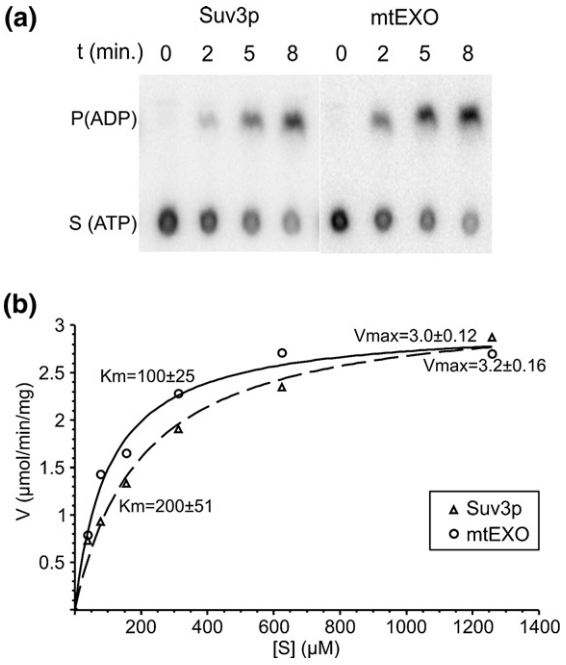


Figure 6. Kinetics of the ATPase activity of Suv3p and the reconstituted mtEXO complex. [α-^{32}P]ATP was used as substrate, with 0.1 μg of each protein per reaction. (a) Typical time-course results obtained with Suv3p and mtEXO. Reaction mixtures were separated on PEI-cellulose thin-layer chromatography plates as described in Materials and Methods, the positions of substrate (ATP) and product (ADP) are indicated. (b) Michaelis–Menten reaction kinetics obtained using varying substrate concentrations. The theoretical curve was fit by non-linear regression, estimated values of K_m and V_{max} are shown together with the standard error of estimation derived from the curve fit.

K_m and k_{cat} values for Suv3p are, in fact, in the range reported for several other DExH/D family RNA helicases.[20]

The helicase activity is directional and depends on the presence of the exoribonuclease subunit

The Suv3 protein contains sequence motifs typical for the DExH/D family of RNA helicases, and the native yeast mitochondrial degradosome complex was shown to unwind double-stranded RNA (dsRNA) substrates.[29] We analyzed the helicase activity of heterologously expressed Suv3p and the reconstituted heterologously expressed mitochondrial degradosome complex towards different dsRNA duplex oligonucleotide substrates. The substrates were either blunt-ended, or had protruding 5′ or 3′ termini (Figure 7(a), substrates C, A and B, respectively). The Suv3 protein alone was not capable of unwinding any of the provided substrates (Figure 7(b)). The reconstituted mtEXO complex exhibits RNase activity (see the previous section) and degraded the provided substrates, although with varying efficiency (Figure 7(b)). The substrate with the protruding 3′ terminus (substrate B) was degraded very rapidly, while the blunt-

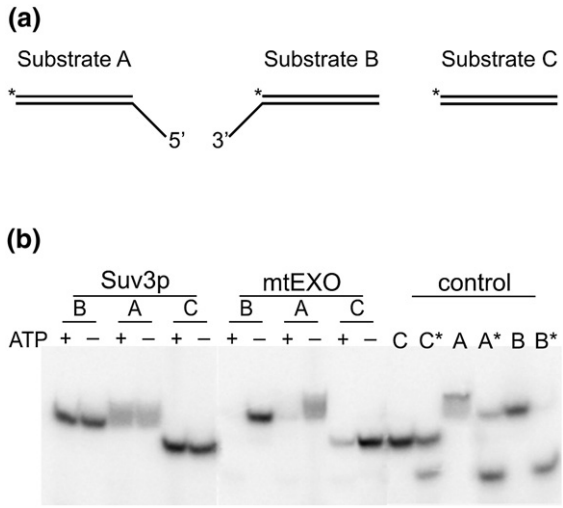


Figure 7. Helicase/ribonuclease activity of Suv3p and the entire mtEXO complex. (a) Schematic structure of the dsRNA substrates used in the assay. Sequences can be found in Materials and Methods. The asterisk (*) indicates the 5′-^{32}P label. (b) The substrates were incubated with the indicated proteins in the presence or in the absence of ATP, or in the reaction buffer without any protein (control). Asterisks in control samples indicate substrates denatured by heating to 65 °C to obtain single-stranded products. After stopping the reaction, samples were digested with proteinase K, analyzed on a native 15% polyacrylamide gel, and visualized by autoradiography.

ended substrate (substrate C) and the substrate with the protruding 5′ terminus (substrate A) were degraded with a much lower efficiency.

We performed time-course analysis of the concerted activity of RNase and helicase subunits of the mtEXO complex using the same substrates. The results, shown in Figure 8, demonstrate that the substrate with the protruding 3′ terminus (substrate B) was unwound and then degraded rapidly, so that the unwound intermediate was visible only at the

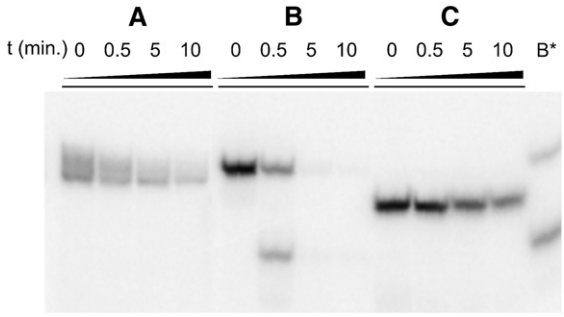


Figure 8. Time-course analysis of the helicase/exoribonuclease activity of the reconstituted mitochondrial degradosome using dsRNA oligonucleotide substrates shown in Figure 7(a). The substrates were incubated with the mtEXO complex in the presence of ATP for the indicated time. After stopping the reaction, samples were digested with proteinase K, analyzed on a native 15% polyacrylamide gel, and visualized by autoradiography. B* is the substrate B denatured by heating to 65 °C.

first time-point (0.5 min.). The blunt-ended substrate (substrate C) and the substrate with the protruding 5′ terminus (substrate A) were degraded very slowly and no unwound intermediate was apparent. These results suggest that the helicase activity is directional and requires a free 3′ single-stranded substrate.

In order to analyze the timing of the duplex unwinding and RNA degradation steps in the reaction catalyzed the mtEXO complex we performed the time-course analysis using the substrate with the protruding 3′ terminus with either the loading strand or the complementary strand labeled at the 5′ terminus (Figure 9). Unwound intermediates were observed only when the complementary strand was labeled, when the label was on the loading strand no unwound intermediates were apparent. This suggests that digestion of the loading strand by RNase quickly follows unwinding by the helicase.

In order to test the substrate specificity of the helicase activity residing in the reconstituted mtEXO complex we performed the helicase assays using RNA/DNA and DNA/DNA substrates. The substrates used in this assay corresponded to the substrate with the protruding 3′ terminus (substrate B) from the previous assays. In the case of the RNA/DNA heteroduplex, the loading strand was RNA. The strand complementary to the loading strand was labeled. The results shown in Figure 10 demonstrate that the helicase activity of the mitochondrial degradosome complex is not entirely substrate-specific. The RNA/DNA heteroduplex was efficiently unwound, the DNA strand was, however, not digested. This suggests that while, as demonstrated by previous assays (Figure 7; Table 1), the presence of the exoribonuclease subunit is essential for the helicase activity, the actual digestion of the substrate is not a requirement. This

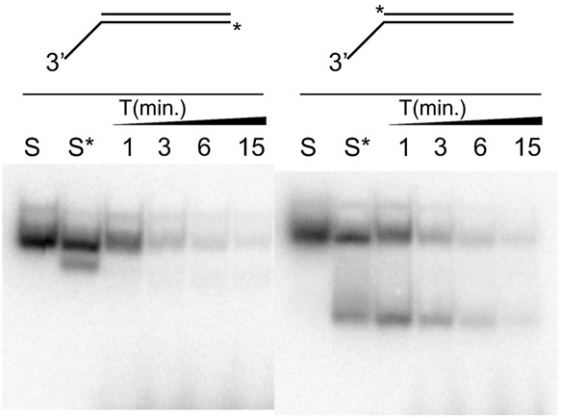


Figure 9. Time-course analysis of the helicase/exoribonuclease activity of the reconstituted mitochondrial degradosome using dsRNA oligonucleotide with the protruding 3′ end (substrate B of Figure 7(a)) with either the loading strand (left) or the complementary strand (right) labeled at the 5′ end with ^{32}P. S is the dsRNA substrate, S* is the substrate denatured by heating to 65 °C. After stopping the reaction, samples were digested with proteinase K, analyzed on a native 15% polyacrylamide gel, and visualized by autoradiography.

agrees with published results demonstrating that an RNase-deficient mutant allele of the Dss1 protein is capable of supporting the helicase activity of the degradosome.[29]

Surprisingly, the DNA/DNA substrate was unwound by the helicase activity of the mitochondrial degradosome in an ATP-dependent manner, albeit with a lower level of efficiency.

Discussion

The 3′ to 5′ exoribonuclease complexes have a key role in RNA metabolism in many genetic systems. The yeast mitochondrial degradosome (mtEXO) represents a very basic form of such a complex, as it is composed of only two subunits: an exoribonuclease (Dss1p) and an RNA helicase (Suv3p). It could therefore serve as a good model for analyzing functional and structural relationships between the components of exoribonucleolytic complexes.

We expressed both subunits of the yeast mitochondrial degradosome in *E. coli* and reconstituted the complex either by co-purifying the two proteins or by mixing the separately purified subunits. In both cases, the functional complex is easily formed, suggesting that the interaction between Dss1p and Suv3p is strong and does not require additional assembly factors, at least *in vitro*. The apparent molecular mass of the reconstituted complex, determined by size-exclusion chromatography, equals the sum of the molecular masses of both components, which suggests that the complex has a 1:1 heterodimer stoichiometry and does not form higher-order aggregates upon purification.

The RNase activity of the reconstituted mtEXO complex is broadly similar to that of other RNases belonging to the RNR family. It is a progressive 3′ to 5′ exoribonuclease that releases nucleoside monophosphates and leaves a short residual core of four nucleotides. This makes mtEXO more similar to bacterial RNase II, which leaves residual cores of 3–5 nt,[50–52] than to bacterial RNase R,[52] or yeast exosome subunit Dis3p,[26] which leave shorter cores of about 2–3 nt.

Various single-stranded RNA substrates are degraded efficiently by the mtEXO complex *in vitro* regardless of their sequence. At this point it is not entirely clear whether the mtEXO complex *in vivo* is capable of discriminating between mature transcripts and improperly terminated RNAs or unprocessed precursors, or what could be the mechanism underlying such specificity. Genetic evidence gathered from observations that a missense mutant in the *SUV3* gene suppresses a defect related to improper processing of the *VAR1* transcript,[57] and that strains deficient in degradosome function accumulate unprocessed RNA precursors and intronic sequences,[15,29,32,34,44,46] suggest that the mtEXO complex may function in RNA surveillance in yeast mitochondria. Most likely, functional mature RNA transcripts are protected by bound protein factors, while unprocessed

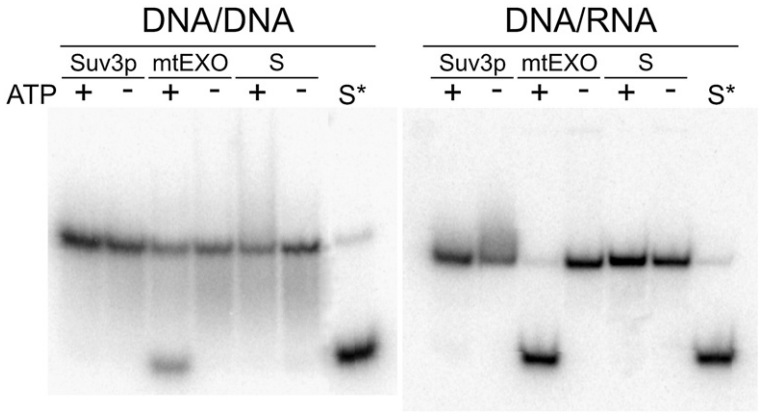


Figure 10. Helicase activity of Suv3p and the entire mtEXO complex towards DNA/DNA and DNA/RNA substrates, corresponding to the substrate with the protruding 3′ terminus (substrate B from Figure 7(a)). In the case of the RNA/DNA heteroduplex substrate, the loading strand was RNA. The strand complementary to the loading strand (DNA in both cases) was labeled at the 5′ end with ^{32}P. Suv3p or the reconstituted mtEXO complex was incubated with the substrate in the presence or in the absence of ATP, as indicated. After stopping the reaction, samples were digested with proteinase K, analyzed on a native 15% polyacrylamide gel, and visualized by autoradiography. S is the double-stranded substrate, S* is the substrate denatured by heating to 65 °C.

precursors and other "junk RNAs" lack this protection and are degraded quickly by mtEXO. This hypothesis is, however, at this point purely speculative and requires further research.

Analysis of the *in vitro* activities of the protein components of the mitochondrial degradosome and of the reconstituted mtEXO complex reveals a very strong functional interdependence. For every enzymatic activity associated with the proteins forming the mitochondrial degradosome, we observed significant changes when the relevant protein is present in the complex, compared to the activity measured for the protein alone (Table 1).

The Suv3p helicase is capable of hydrolyzing ATP in the presence of RNA alone as well as in the complex, and neither the reaction rate, nor the ATP substrate binding (as determined by K_m) seem to be changed significantly upon forming a complex with Dss1p. The ATPase properties of Suv3p are similar to those established for many other proteins belonging to the DExH/D superfamily.[20] While the Suv3 protein alone displays noticeable ATP hydrolysis in the absence of RNA, this background activity is reduced greatly when the protein is in the complex with Dss1p. This suggests that, while the presence of Dss1p does not enhance the catalytic ATPase activity of Suv3p, it probably changes its conformation in a way that restricts the background, non-RNA-dependent activity.

The effect of the complex formation on the helicase activity is, on the other hand, remarkable. The Suv3 protein alone does not display any detectable unwinding activity towards any tested substrate. The helicase activity becomes apparent only when Suv3p is in complex with Dss1p. Only the substrate with the protruding 3′ end is efficiently unwound by the helicase activity of mtEXO; Suv3p is therefore a directional helicase requiring a free 3′ single-stranded substrate. Given the tight association between Suv3p and Dss1p, it is not surprising that in the mitochondrial degradosome, as demonstrated by the time-course experiments, the digestion by RNase immediately follows duplex unwinding.

Unwinding activity has been demonstrated only for a small subset of known proteins containing sequence motifs indicative of the RNA helicase function.[20,21] As most such proteins were not tested in the context of an assembled complex,[20] it is possible that the dependence of the helicase activity on protein–protein interactions within native complexes is a more general feature of this class of enzymes. An increase of the helicase and ATPase activity was observed for the eIF4A protein in the presence of eIF4B or eIF4F.[58–61] The case of the Suv3p helicase and the mitochondrial degradosome is, however, unique in the absolute character of the functional interdependence.

While the presence of the Dss1p subunit is essential for the helicase activity of Suv3p, the actual hydrolysis reaction is not required. RNase-deficient mutant alleles of Dss1p are capable of supporting the helicase activity of Suv3p,[29] and in our experiments RNA/DNA and even DNA/DNA substrates were unwound in an ATP-dependent manner.

Probably the most interesting example of functional interdependence relates to the RNA hydrolysis activity of the mitochondrial degradosome, which is its main function *in vivo*. While the Dss1 protein alone does have some basal exoribonuclease activity, which is not ATP-dependent, the activity of the entire complex is at least an order of magnitude higher and is entirely ATP-dependent, with no residual activity observed in the absence of ATP. Such absolute ATP-dependence is unique among the known exoribonuclease complexes. The Suv3p helicase therefore plays a critical role in RNA degradation by the degradosome.

RNA helicases have been implicated in the degradation of RNA, for example in the eubacterial degradosome,[16,62,63] and in the eukaryotic exosome.[64,65] In the bacterial degradosome, the RNA helicase encoded by the RhlB gene is required to unwind RNA secondary structures that impede the progress of both PNPase and RNase II.[62,63] In the case of the mitochondrial degradosome, however, we observed that the presence of the Suv3p helicase was required for efficient RNA degradation even for

the single-stranded oligonucleotide substrate that does not fold into any secondary structure stable at 30 °C, as predicted using the RNAfold program.[53] It is therefore unlikely that unwinding of the secondary structure elements of the RNA substrate by Suv3p is the main factor contributing to the observed activity increase of the mtEXO ribonuclease in our experiments.

The results of RNA-binding assays demonstrate that both Dss1p and Suv3p are capable of binding RNA with similar affinities, corresponding to a K_d value of about 200 nM for the short (30 nt) RNA oligonucleotide and about 20–30 nM (approximate) for longer RNA polynucleotides. A binding affinity value similar to that of Suv3p was reported recently for another yeast mitochondrial DExH/D family protein Mss116p.[54] The binding of the reconstituted mtEXO complex (and Dss1p alone) to the single-stranded RNA oligonucleotide substrate is significantly weaker than in the case of bacterial RNase R,[36] where K_d values ranged from ~1 nM to ~20 nM, depending on the composition of the substrate. Similar RNA-binding affinities of Suv3p and Dss1p suggest that the dependence of the Dss1p RNase on the presence of the Suv3p helicase subunit cannot be explained by differences in their RNA-binding capabilities.

Recent results obtained for another yeast mitochondrial DExH/D family protein, Mss116p, indicate that its function in splicing may not be related directly to the helicase activity, and that the protein could instead act as an RNA chaperone by assisting in the assembly of RNA tertiary structures.[54] Interestingly, over-expression of the *MSS116* gene can compensate for some defects related to the inactivation of *SUV3*.[66] However, the function of Suv3p in the mitochondrial degradosome complex is probably different, as it is manifested also with short oligonucleotide substrates where RNA structure is essentially absent.

An alternative explanation for the involvement of the DExH/D helicases in RNA degradation was proposed for proteins interacting with the eukaryotic exosome, the yeast Ski2p helicase[64] and the human RHAU protein.[65] In these models, the RNA helicase acts as a "molecular motor", or adapter that binds RNA and feeds the substrate to the catalytic core of the RNase. This model could be easily reconciled with the recently published structure of RNase II.[67,68] The catalytic center of the enzyme is located at the bottom of a long, funnel-shaped channel. The substrate has to be threaded through this channel to reach the catalytic site. In the mitochondrial degradosome, the Suv3p helicase could be located near the entrance to the RNase channel and, in an ATP-dependent manner, feed the substrate towards the catalytic site. The Dss1p protein alone is capable of RNA binding, but the overall level of activity is very low, perhaps due to the inefficient passage of the substrate to the catalytic site. The presence of Suv3p in the complex provides an active mechanism of threading the substrate to the active site. Even though the detailed structure of Suv3p is not known, homology modeling (data not shown) indicates that its structure is typical of the DExH/D family of proteins, with two globular domains joined by a short linker. The proposed role of Suv3p in the mitochondrial degradosome complex is consistent with the "inchworm" model of helicase activity,[20–22] in which the cleft between the two domains opens and closes, allowing the protein to track along the RNA strand. Such movement, which depends on ATP hydrolysis, would enable the helicase subunit to move the RNA strand into the opening of the RNase subunit. In the absence of ATP, RNA would still bind, as it was observed in our *in vitro* binding assays, but no movement would be possible, the complex and substrate would remain locked and thus no RNA degradation activity would be observed. This model explains why the presence of the Suv3p helicase greatly enhances the ATP-dependent RNA hydrolysis activity of the mitochondrial degradosome, but completely abolishes the RNase activity in the absence of ATP. Obviously this model is, in the absence of structural information, entirely speculative.

One of the particular characteristics of the mitochondrial genetic system is the close physical association between different components of the gene expression machinery. RNA processing and translation have been shown to be physically coupled to transcription through a network of protein–protein and protein–RNA interactions.[69–71] As the mitochondrial degradosome is associated with the ribosome,[29] it should also be considered a part of this network. In such complex systems, tight regulation of enzymatic activities is absolutely essential for maintaining their function. One can therefore speculate that the absolute dependence of the helicase activity of Suv3p on the presence of Dss1p, and particularly the absolute ATP-dependence of the exoribonuclease activity of the complex, have an important biological function in controlling these potentially destructive activities. The levels of mitochondrial ATP were recently shown to be a key factor in controlling mtDNA transcription,[7,72] the absolute dependence of the main mitochondrial exoribonuclease complex on ATP could be another element of this regulatory network.

Functional interdependence between different subunits of the exoribonuclease complexes, such as revealed in our studies of the yeast mitochondrial degradosome, has a very important part in shaping their activity. While the actual mechanisms of mitochondrial gene expression, including RNA degradation, show great divergence in different eukaryotic lineages,[3] exoribonucleolytic complexes play an important role in all the known systems, from yeast to man. Results obtained with the yeast mtEXO suggest that interactions with other subunits should always be taken into consideration when making assumptions about function of proteins forming such complexes.

Materials and Methods

Expression plasmids

Construction of His_6MBP Fusion protein vectors for both Suv3p and Dss1p was performed using Gateway Recombinational Cloning (Invitrogen) according to the procedure described by Tropea *et al.*[48] DNA encoding the entire Suv3 protein without the 26 amino acid residue N-terminal presequence was amplified using the following primers:

5′-GAGAACCTGTACTTCCAGGGTTACCACAGC-GAGCCGCATAG (N1-27-737ySUV3);
5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTAT-TATGTACGCAATCTTCTTCTCGA (C-ySUV3) and
5′-GGGGACAAGTTTGTACAAAAAAAG-CAGGCTCGGAGAACCTGTACTTCCAGGGT (N2).

During PCR, the initial ratio of primers was 1:20:20, N127737ySUV3/C-ySUV3/N2, respectively.

The PCR product was cloned into pDONR201 (Invitrogen) by recombinational cloning to generate the entry plasmid pDON-SUV3-27-737. Finally, recombination of the encoding fragment from entry plasmid to pDEST-His_6MBP[48] produced destination vector His_6MBP-ySUV3-27-737. The same procedure was used to obtain His_6MBP-DSS1p-27-969 over-expressing fragment 27–969 of DSS1p. The following primers were used:

5′ GAGAACCTGTACTTCCAGGGTACCAGAGG-CAAACGACAGCGA (N1-27-969);
5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTAT-TATAGCTTTTCCAACTCTAACATTC (C-DSS1p), N2.

Protein expression and purification

E. coli BL21(DE3) CodonPlus-RIL cells (Stratagene) containing the His_6MBP-ySUV3-27-737 or His_6MBP-DSS1p-27-969 were grown overnight at 37 °C in Luria broth supplemented with 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol. Overnight cultures were diluted 1:100 (v/v) of the same medium and grown to an A_{600} value between 0.6 and 0.8. At this point, flasks were cooled rapidly to ~20 °C and IPTG was added to a final concentration of 0.3 mM. The culture was grown for an additional 4–5 h followed by centrifugation. The cell pellet was suspended in buffer A (50 mM Tris, 50 mM K_2PO_4, 0.3 M KCl, 10% (v/v) glycerol, 25 mM imidazole, 5 mM β-mercaptoethanol (β-ME), pH adjusted to 8.0) and benzamidine–HCL was added to a final concentration of 3 mM. Suspended cells were stored at −80 °C.

All of the subsequent purification steps were performed at 4 °C. Suspended cells were thawed and lysed by sonication. Crude extract was centrifuged at 40,000*g* for 40 min and supernatant was loaded onto a Ni-NTA (Qiagen) column equilibrated with buffer A. Unbound proteins were eluted with ten volumes of buffer A adjusted to 2 M KCl followed by ten volumes of buffer A. Bound proteins were eluted with buffer A supplemented with 200 mM imidazole. Protein fractions were pooled, adjusted to 1 mM DTT and 1 mM EDTA. TEV protease (autoinactivation-resistant mutant S219V expressed from the pRK793 plasmid,[49] courtesy of Dr David Waugh) was added at a ratio of 1 mg:100 mg of TEV to total protein. After overnight digestion at 4 °C, the protein solution was precipitated with 0.39 g/ml ammonium sulfate and centrifuged at 10,000*g* for 20 min. The pellet was suspended in buffer A lacking imidazole and applied to a NiNTA column. Proteins washed out with buffer A containing 25 mM imidazole were precipitated again with ammonium sulfate (0.39 g/ml) and centrifuged. The pellet was resuspended in 2 ml of buffer C (0.2 M NaCl, 10% glycerol, 10 mM Hepes, 1 mM TCEP, 0.02% NaN3, pH adjusted to 7.5) and applied onto a Superdex 200 size-exclusion chromatography column equilibrated with the same buffer. Fractions containing purified proteins were collected, concentrated to 10 mg/ml, frozen in liquid nitrogen and stored at −80 °C.

Oligonucleotide substrates

The following RNA nucleotides were used to prepare substrates for RNase and helicase activity assays.

T (CAAACUCUCUCUCUCAAC),
5W (AGAGAGAGAGGUUGAGAGAGAGAGA-GUUUG),
3W (GUUGAGAGAGAGAGUUUGAGAGAGA-GAG),
B (GUUGAGAGAGAGAGUUUG).

The dsRNA substrate with the protruding 5′ terminus (substrate A) was prepared by annealing oligonucleotides 5W and T; the substrate with the protruding 3′ terminus (substrate B) was prepared by annealing oligonucleotides 3W and T; and the blunt-ended substrate (substrate C) was prepared by annealing oligonucleotides B and T. DNA equivalents of oligonucleotides 3W and T were used to prepare the double-stranded DNA (dsDNA) substrate, and the DNA equivalent of oligonucleotide T together with the 3W RNA oligonucleotide was used to prepare the RNA/DNA heteroduplex substrate. Oligonucleotides were radiolabeled at the 5′ terminus with ^{32}P using the phage T4 polynucleotide kinase (NEB) when indicated.

Polynucleotide substrates

The ssRNA substrates were generated by *in vitro* transcription using the T7 Transcription Kit (Fermentas), according to the manufacturer's instructions. [α-^{32}P]UTP was added to the reaction to produce radiolabeled substrate. The templates were either the PCR product corresponding to 831 bp of the 3′ sequence of the mature yeast mitochondrial *CYTB* gene obtained using primers Lp; TAATACGACTCACTATAGGGAGATATTACTAATTTA-TTCTCAG, containing the T7 promoter as a 5′ overhang, and Rp; TTAAGAATATTATTAAAGTA or the heterologous 341 nt control sequence from the T7 Transcription Kit.

Helicase activity assay

RNA/DNA helicase activity was assayed by the strand displacement method. The double-stranded RNA, RNA/DNA or DNA/DNA substrates described above were prepared by radiolabeling one strand as described above, and annealing the cold complementary strand in tenfold molar excess. Substrates after annealing were purified by electrophoresis through a native 15% polyacrylamide gel. Helicase assay was performed in a 20 μl reaction volume containing 10 mM Tris–HCl (pH 8.0), 25 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 0.1 pmol of labeled duplex RNA at 30 °C. In each case, reaction was supplemented by 1 pmol of cold RNA trap to avoid reannealing of the reaction products. The reaction was terminated by adding solution containing 10 mM Tris–HCl

(pH 7.6), 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol FF, 60% (v/v) glycerol, 60 mM EDTA. After the reaction, each sample was incubated for 10 min with 10 μg of proteinase K at room temperature. Samples were analyzed on native 15% polyacrylamide gel and visualized by autoradiography.

ATPase activity assay

ATPase assays were carried out in a 20 μl reaction volume containing Tris–HCl (pH 8.0), 25 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP and 10 fmol of [α-^{32}P]ATP. The reactions were supplemented with 1 μg of either total yeast RNA, 5W RNA oligonucleotide, a 20 nt DNA oligonucleotide or plasmid DNA, when indicated. Assays were performed at 30 °C for 30 min. Reactions were terminated by adding 2 μl of 0.5 M EDTA, and 1 μl of each reaction mixture was spotted onto PEI-cellulose thin-layer chromatography plates and developed in 2 M formic acid, 0.5 M LiCl. Chromatograms were quantified by autoradiography using the PhosphorImager with the Image Quant software (Molecular Dynamics).

Exoribonuclease activity assays

Assays were carried out at 30 °C for 30 min in the buffer described for the ATPase assay using as substrates the 341 nt ssRNA substrate or the yeast *CYTB* mRNA (generated by *in vitro* transcription) or the 5W oligonucleotide labeled on the 5′ end. Reaction products were analyzed by thin-layer PEI-cellulose chromatography as described,[45] or by denaturing electrophoresis in 7 M urea/20% polyacrylamide gels. Limited digestion of the 5W oligonucleotide using ribonuclease T_1 (Roche) was used to generate an RNA size marker for the electrophoresis.

Filter-binding assays

The double-filter RNA-binding assay[73,74] was performed essentially as described.[36] Nitrocellulose filters were presoaked in 0.5 M KOH for 10 min and washed in water until the pH was neutral. Nylon filters were washed once in 0.1 M EDTA (pH 8.0) and three times for 10 min in 1.0 M KCl , followed by rinsing with 0.5 M KOH and washing with water until the pH was neutral. Nitrocellulose and nylon filters were equilibrated in binding buffer (10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 25 mM KCl, 1 mM DTT, 10% glycerol) at 4 °C for at least 1 h before use. Each well of the dot-blot apparatus was washed with 100 μl of the binding buffer before and immediately after applying the sample. Reaction mixtures containing 1.75 fmol of RNA in 20 μl (87.5 fM) and various amounts of protein were preincubated for 20 min at room temperature before the application to the blot. Single-stranded RNA oligonucleotide 5W or polynucleotide corresponding to the terminal 831 nt of the yeast *CYTB* mRNA (generated by *in vitro* transcription) were used as binding substrates.

Enzyme kinetics

For measurement of exonuclease reaction kinetics, the 5W oligonucleotide labeled on the 5′ end was used as a substrate. Reactions were supplemented with appropriate amounts of cold oligonucleotide to obtain the indicated total concentrations of substrate. Reactions were terminated by adding stop buffer containing 20 mM EDTA. Time-course samples from each reaction were separated on denaturing 10% polyacrylamide gels. Reaction progress was quantified by autoradiography using the PhosphorImager with the Image Quant software (Molecular Dynamics).

V_0 values for the ATPase reaction were measured as described above using [α-^{32}P]ATP supplemented with appropriate amounts of cold ATP as a substrate. Each reaction was supplemented with 1 μg of total yeast RNA. Time-course samples from each reaction were separated on thin-layer chromatography. Reaction progress was quantified by autoradiography using the PhosphorImager with the Image Quant software (Molecular Dynamics).

The data points were fitted to the Michaelis–Menten equation by non-linear regression using the Levenberg–Marquardt algorithm (QtiPlot software†) and the values of K_m and V_{max} were estimated.

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† http://soft.proindependent.com/qtiplot.html

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