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Genome-Based Analysis of *Chlamydomonas reinhardtii* Exoribonucleases and Poly(A) Polymerases Predicts Unexpected Organellar and Exosomal Features

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ABSTRACT

Enzymes from several gene families modify RNA molecules at their extremities. These reactions occur in several cellular compartments and affect every class of RNA. To assess the diversity of a subclass of these enzymes, we searched Chlamydomonas for open reading frames (ORFs) potentially encoding exoribonucleases, poly(A) polymerases, and proteins known to associate with and/or regulate them. The ORFs were further analyzed for indications of protein localization to the nucleus, cytosol, mitochondrion, and/or chloroplast. By comparing predicted proteins with homologs in Arabidopsis and yeast, we derived several tentative conclusions regarding RNA 5′- and 3′-end metabolism in Chlamydomonas. First, the alga possesses only one each of the following likely organellar enzymes: polynucleotide phosphorylase, hydrolytic exoribonuclease, poly(A) polymerase, and CCA transferase, a surprisingly small complement. Second, although the core of the nuclear/cytosolic exosome decay complex is well conserved, neither nucleus-specific activators nor the cytosolic exosome activators are present. Finally, our discovery of nine non-canonical poly(A) polymerases, a divergent family retaining the catalytic domains of conventional poly(A) polymerases, leads to the hypothesis that polyadenylation may play an especially important regulatory role throughout the Chlamydomonas cell, stabilizing some transcripts and targeting degradation machinery to others.

OUR initial aim was to define and categorize Chlamydomonas enzymes involved in either elongating or trimming the 3′-ends of RNAs encoded in the nuclear, mitochondrial, and chloroplast genomes. Indeed, RNA metabolism is one of the few processes occurring in all three organelles as well as the cytosol, which together represent three different endosymbiotic origins. The release of Chlamydomonas genome version 3 (Merchant *et al.* 2007)—now supplemented by version 4—gave us an opportunity to perform this analysis in a unicellular photosynthetic eukaryote, where we have focused on comparing its suite of enzymes and regulators to those found in a nongreen unicellular organism, yeast, and the multicellular Arabidopsis. A related analysis focused on endoribonucleases, such as those of the Dicer family, is published elsewhere (Casas-Mollano *et al.* 2008).

For this work, we initially reviewed all known and predicted exoribonucleases (reviewed in Carpousis *et al.* 1999; Bollenbach *et al.* 2004; Meyer *et al.* 2004). Cells contain both 3′ → 5′ and 5′ → 3′ exoribonucleases (Figure 1), of which only the former class has been found in organelles and prokaryotes. 3′ → 5′ decay overcomes either secondary structure or stabilizing proteins or acts on molecules that lack these features. Transcripts particularly vulnerable to exoribonuclease digestion include incomplete or aberrant RNAs such as premature termination products, endonucleolytic cleavage products (intermediates of RNA decay), and mRNAs that have lost their protective cap and long poly(A) tail. Conversely, 3′ → 5′ activity contributes to maturation of tRNAs, chloroplast mRNAs, and organellar rRNAs. A summary of most nonorganellar pathways involving 3′ → 5′ degradation, which involve, among others, rRNAs, snRNAs, and small nucleolar RNAs, can be found in Houseley *et al.* (2006). 5′ → 3′ exoribonucleases are encoded by small gene families and are involved in nuclear and cytosolic RNA decay (Newbury 2006). In plants, the family size is slightly larger, and members play a major role in RNA-silencing mechanisms (Gy *et al.* 2007). With respect to organelles, at least Chlamydomonas chloroplasts have a net 5′ → 3′ pathway (Drager *et al.* 1999; Hicks *et al.* 2002), but the enzymes involved are unknown. Exoribonucleases are often found in complexes, such as the bacterial degradosome and the nuclear/cytosolic exosome, which are discussed in more detail in the results. These complexes include accessory factors, including

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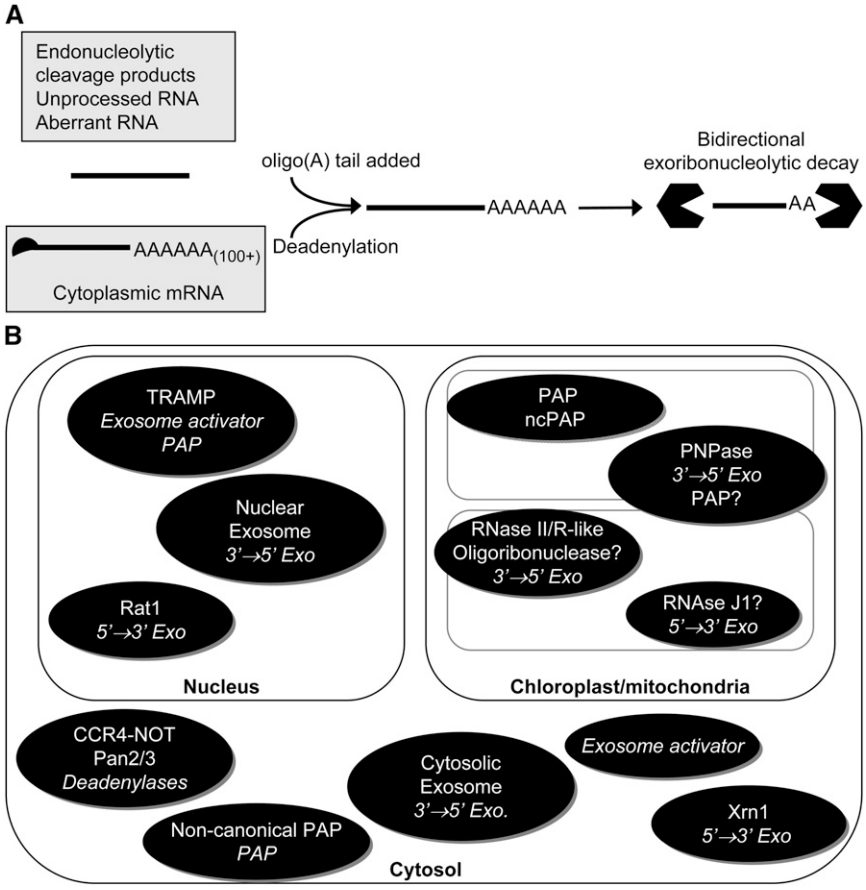

FIGURE 1.—Synopsis of prokaryotic/organellar and eukaryotic exoribonucleolytic decay. (A) Two pathways for RNA decay. (Top) Stimulation of decay by addition of a poly(A) tail. (Bottom) Removal of long stabilizing poly(A) tails. In both cases, decay is completed by exoribonuclease activities acting from both ends of the molecule. (B) Known or suspected subcellular localization of the RNA metabolism components discussed in this article. For nuclear and cytosolic proteins, yeast nomenclature is used. Protein names are in non-italic type, and their catalytic activities are in italic type. For the organellar proteins, names of bacterial homologs are given.

helicases and RNA-binding proteins, which were also investigated for this article.

Certain RNA-specific nucleotidyltransferase (NTR) family members were also studied, in particular polyadenylate polymerases (PAPs) and CCA transferases (CCAtrs), the latter of which add -CC, -A, or -CCA to the 3′-ends of tRNAs (MARTIN and KELLER 2007). Of the PAPs, the classical nuclear version adds long tails to mRNAs, promoting nuclear export and translation. The PAP in *Escherichia coli* is of a different NTR class and, like its chloroplast and mitochondrial counterparts, is responsible for transient polyadenylation, which can increase the affinity of mRNAs or RNA fragments for the exoribonuclease polynucleotide phosphorylase (PNPase) (LISITSKY *et al.* 1997; LISITSKY and SCHUSTER 1999). PNPase is also a polynucleotide polymerase, although not of the NTR family. The organellar poly(A)-stimulated degradation pathway is partly related to the metabolism of nucleus-encoded mRNAs, where deadenylation of a long poly(A) tail ultimately results in a short poly(A) tail that can serve as a foothold for the exoribonuclease decay machinery, known as the exosome (MEYER *et al.* 2004). Finally, there is a distinct family of noncanonical PAPs (ncPAPs), whose nuclear and cytosolic members have been implicated in decay-inducing, as well as stabilizing, polyadenylation (MARTIN and KELLER 2007), while a human mitochondrial member is responsible for addition of mRNA tails (TOMECKI *et al.* 2004).

Taking together the protein classes described above, one can infer that the structure of the major exoribonucleolytic machinery seems to be conserved in most systems. This encompasses PNPase multimers in prokaryotes and organelles and the exosome in Archaea, the nucleus, and the cytosol. In prokaryotic and many or perhaps all organellar systems, PNPase is arranged in a ring-shaped trimer (SYMMONS *et al.* 2000). The same structure is maintained in the exosome, except that the domains reside on different proteins and come together to form the core exosome (SYMMONS *et al.* 2002; HOUSELEY *et al.* 2006). We describe below how our findings for Chlamydomonas fit into these previously established paradigms.

MATERIALS AND METHODS

Data resources: *Chlamydomonas reinhardtii* genome sequences, protein sequences (version 3.1), and EST unigene sequences were downloaded from the Joint Genome Institute (http://genome.jgi-psf.org/Chlre3/). A query interface for genome versions 3.1 and 4 was implemented at the Boyce Thompson Institute (http://codequest.bti.cornell.edu/chlre). Arabidopsis (version 7) and *Saccharomyces cerevisiae* protein sequences (updated 09/06/2007) were downloaded from The Arabidopsis Information Resource (http://www.arabidopsis.

TABLE 1

Chlamydomonas nucleotidyltransferases, exoribonucleases, and similar proteins for which sequence analysis suggests a role in RNA metabolism

Protein	Gene name	GenBank accession no.[a]	Domain	Subcellular location[b]	cDNA support
		RNAi			
MUT68	*MUT68*	ABI79451	PAP/25A core	x	Yes
		Organellar RNA decay			
PNPase	*PNP1*	ABF57675	RNase PH, KH, SI	cp, mt	Yes
RNase II/R	*RNB2*	ABO20871	RNase II	cp	Yes
Oligoribonuclease	*ORN1*	EDP06341	DEDD exonuclease	NP	No
RNase J1	*RNJ1*	EU518648–EU518649[c]	RNA β-lactamase	cp	Yes
PAP4	*PAP4*	EU311729	PAP/25A core	cp	Yes
Suv3	*SUV3*	*142113*	DExDC helicase	mt	No
		Class II NTR			
	PAP3	EDP00507[d] *206563*	PAP head domain	cp (weak)	Yes
		PAP (other noncanonical)			
	PAP2	EDP08764[d] *206516*	PAP/25A core	cp (strong)	Yes
	PAP5	EDP08040[d] *206568*	PAP_associated	No target	No
	PAP6	EDP01690[d] *206567*	PAP/25A core	mt (weak)	Yes
	PAP7	EDP09686	PAP/25A core	n	No
	PAP8	EDP02954	PAP/25A core	cp	Yes
	PAP9	EDP01828	PAP/25A core	mt	No
	PAP10	EDO99083	PAP/25A core	No target	Yes

[a] Where a gene model has not been deposited in GenBank, only the Chlamydomonas version 3.1 protein ID is listed (in italics).
[b] x, cytosolic; cp, chloroplast; mt, mitochondrial; n, nucleus; NP, available sequence does not allow prediction to be made. Chlamydomonas locations that are underlined have experimental support. All others are predictions based on the software described in MATERIALS AND METHODS.
[c] The two accession numbers represent alternative splicing variants.
[d] EST-based corrected versions of these protein predictions were constructed for targeting analysis. The corresponding Chlamydomonas version 3.1 protein ID is listed in italics.

org) and the *Saccharomyces* Genome Database (http://www.yeastgenome.org/), respectively.

Methods: Chlamydomonas, Arabidopsis, and yeast protein sequences and Chlamydomonas EST unigene sequences were used to perform HMM searches against the Pfam domain database (BATEMAN *et al.* 2004) with a TimeLogic Decypher system (http://codequest.bti.cornell.edu/chlre). The same sequences were also searched against the InterPro database (MULDER *et al.* 2002) with a parallel InterProScan on the Cornell Computational Biology Service Unit MS Windows computer cluster (http://cbsuapps.tc.cornell.edu/piprscan.aspx). Predicted proteins involved in some aspect of trimming or extending the ends of RNA were identified on the basis of their Pfam and InterPro domains. Pfam domains utilized were 01138, 00773, 03725, 01909, 04857, 01743, and 03828. Interpro domains utilized were 003029, 001900, 011082, 011545, 001650, 001201, 002058, 002934, 004087, 001878, and 002646. To identify any genes missed by prediction programs in Chlamydomonas version 3.1 proteins, a subset of Pfam hidden Markov models was compared to the translated Chlamydomonas whole-genome sequences (versions 3.1 and 4.0). For all the sequence database searches, a cutoff value of $1e^{-5}$ was used.

To predict potential mitochondrial or plastid targeting, Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html; SMALL *et al.* 2004) and TargetP (http://www.cbs.dtu.dk/services/TargetP/; EMANUELSSON *et al.* 2000) were used. This was done only if the gene model appeared complete and began with a Met codon. For nuclear localization signals, we used PredictNLS (http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl; COKOL *et al.* 2000), and for proteins displaying ambiguous results, LOCTree was used (http://cubic.bioc.columbia.edu/cgi/var/nair/loctree/query; NAIR and ROST 2005). Multiple sequence alignments were performed using ClustalW (CHENNA *et al.* 2003). Phylogenetic trees were constructed on the basis of amino acid differences (*p*-distance) by the neighbor-joining (NJ) method using the MEGA3 program (KUMAR *et al.* 2004). Robustness of the NJ trees was tested using the bootstrap method with 1000 replicates.

RESULTS

Summary of relevant Chlamydomonas proteins

Tables 1 and 2 contain the complete collection of Chlamydomonas proteins likely to play a role in trimming or extending the ends of RNA molecules. Defining domains of each protein are listed, along with potential subcellular localization, whether there is EST or cDNA support and whether the cDNA support indicates an error in the predicted protein.

Known or predicted organellar enzymes and those with no obvious yeast homologs are listed in Table 1. Of the 15, 10 are known or likely to be organellar on the basis of published data, targeting experiments, or prediction software. Of these, 6 are from prokaryotic gene families and 4 are ncPAPs. Only 5 lack EST or cDNA support. However, only one of the protein predictions can be verified as correct with cDNA support; all others

TABLE 2

Putative Chlamydomonas orthologs of *S. cerevisiae* exoribonucleases and associated proteins

		Chlamydomonas			Location[b]		
	Sc	Gene ID	GenBank[a]	Domain	*Sc*	*Cr*	cDNA
			Exosome complex				
			Subunits with domains also found in PNPase[c]				
Archaeal Rrp41-like	Rrp41p	RNPH1	EDP01605	RNase PH	n/x		Yes
	Rrp46p	RRP46	EDP03843[d] *206570*	RNase PH	n/x		Yes
	Mtr3p		EDP04906	RNase PH	n/x		No
Archaeal Rrp42-like	Rrp42p	RNPH2	*205556*[d] *206571*	RNase PH	n/x		Yes
	Rrp43p	RRP43	EDP05793	RNase PH	n/x		No
	Rrp45p	RNPH3	EDP06132	RNase PH	n/x		No
RNA binding	Rrp4p	RRP4	EDO97018	S1/KH	n/x		No
	Rrp40p	RRP40	EDP07280	S1/KH	n/x		Yes
	Csl4p	CSL4	EDP07209	S1/zinc ribbon	n/x		Yes
			Other subunit				
	Rrp44p	RNB1	EDP03330	RNase II/R	n/x	**x or n**	Yes
			Location-specific subunits[e]				
	Rrp6p		Many exos	RNase T/D	n		
	Rrp47p		None	RNA binding?	n		
	Ski7p		None	GTPase?	x		
			TRAMP complex				
	Mtr4p	MTR4	EDO96924[d] *206573*	DExH-box helicase	n/x	n	Yes
		HEN2	EDP01779	DExH-box helicase	n/x	—	Yes
or	Trf4p	TRF4	EDP00455	PAP/25A core, PAP_associated	n	n	Yes
	Trf5p		None	PAP/25A core, PAP_associated	n		
or	Air1p	CGLD20	EDP02869	RING Zn finger	n	—	
	Air2p	RSZ22	EDP00877	RING Zn finger	n		
			Nuclear PAP and cytosolic deadenylases				
	PAP1	PAP1	EDP06435[d] *206508*	PAP_central	n	n	Yes
	Pan2p	PAN2	EDP05128	RNase D family	x		No
	Pan3p		None	RNase D family	x		
or	Caf1p	CAF1	EDP00109	RNase CAF1	n/x		Yes
	Caf1p	CAF2	EDP06748	RNase CAF1	n/x		No
or	Ccr4p	CCR4	EDO97283	Exonuclease III and leucine rich	x		Yes
	Ccr4p	CCR5	EDP04957	Exonuclease III and leucine rich	x		No
	PARN (human)		None	DEDD 3′ exonuclease	x		
			5′ → 3′ exoribonucleases				
	Xrn1p	XRN1	*149305*	5′ → 3′ exonuclease	x	m	Yes
	Rat1p	XRN2	*206539*	5′ → 3′ exonuclease	n	—	Yes
	Xrn1p	XRN3	*149248*	5′ → 3′ exonuclease	x	—	Yes

[a] Where a gene model has not been deposited in GenBank, only the Chlamydomonas version 3.1 protein ID is listed (in italics).
[b] x, cytosol; n, nucleus; m, mitochondria; —, no subcellular location was predicted. Boldface type indicates that a location has experimental support; all others are predictions.
[c] Underlining indicates groups of proteins; there may be no 1:1 association.
[d] EST-based corrected versions of these protein predictions were constructed for targeting analysis. The corresponding Chlamydomonas version 3.1 protein ID is listed in italics.
[e] Underlining indicates protein models with similar domains, not necessarily homologous to the proteins listed.

were corrected before submission to GenBank, had to be amended before analysis, or are correct but only within a limited region of cDNA support. One amended model, the putative NTR PAP3, is unusual in that it contains a long intron, which encodes, in the opposite orientation, a ubiquitin-specific protease.

Table 2 includes those enzymes that have been extensively studied in yeast. This comprises 15 exosome or exosome-associated enzymes, three 5′ → 3′ exoribonucleases, one nuclear PAP, and five deadenylases. One of the 5′ → 3′ exoribonucleases was predicted to be mitochondrially localized, but was included with its gene family in Table 2 because such a localization has no biological precedent. Of the 15 predicted proteins with cDNA support, 3 were proven correct, 5 were incorrect and had to be amended, and partial cDNA support shows

no errors in the remaining 7. The sections below discuss each of these protein classes in further detail.

Organellar RNA end trimming and extension

Chloroplast exoribonucleolytic decay: Following endonucleolytic cleavage, 3′ → 5′ decay can occur from the newly-formed 3′ end, a process which can be accelerated by transient polyadenylation. The enzymes and mechanisms involved are discussed in Bollenbach *et al.* (2008), and are similar to what is found in bacteria.

Poly(A) polymerases and CCA transferases: In *E. coli*, PAP1 generates the transient poly(A) tail, although PNPase, being a readily reversible enzyme, also generates A-rich tails and can substitute for PAP1 (Mohanty and Kushner 2000). It is still unknown whether a dedicated PAP is necessary to generate the transient poly(A) or A-rich tails observed in Chlamydomonas, Arabidopsis, spinach, and maize chloroplasts (Haff and Bogorad 1976; Kudla *et al.* 1996; Lisitsky *et al.* 1996; Komine *et al.* 2000; Walter *et al.* 2002), or whether this role can be entirely fulfilled by PNPase, as appears to be the case in the cyanobacterium Synechocystis PCC6803 (Rott *et al.* 2003).

E. coli PAP1 is a class II NTR, as are eukaryotic and bacterial CCAtrs. Class II NTRs contain the same active domain fold as class I NTRs, such as nuclear PAPs and archaeal CCAtrs, although the classes are otherwise divergent in sequence. Although it is difficult to utilize primary sequence to distinguish activities of members within each class, a particular Glu residue appears to be essential for the incorporation of cytosine during tRNA modification (Martin and Keller 2004). We expected to find at least three class II NTRs in Chlamydomonas to provide CCAtrs for both the chloroplast and mitochondria and PAP for the chloroplast, but found only two, one of which possesses the distinguishing Glu. The *PAP4* gene encodes the NTR lacking the Glu, whereas the *PAP3* gene product contains the Glu and shares very high homology with the putative Arabidopsis NTR At1g22660. Both Chlamydomonas proteins possess predicted chloroplast transit peptides. Although prediction software is not as well trained on Chlamydomonas proteins as on those of higher plants, if both *PAP3* and *PAP4* were chloroplast localized, they would likely encode tRNA maturation and polyadenylation enzymes, respectively. However, this would raise the question of the source of CCA-adding activity in the mitochondria. Because in some organisms, including plants, CCAtrs can be dual targeted (von Braun *et al.* 2007), it is not unreasonable to suppose that this might also be the case in Chlamydomonas.

Helicases: The DEAD-box helicase RhlB is present in the bacterial degradosome and stimulates the RNases therein (Coburn *et al.* 1999; Khemici *et al.* 2005). If chloroplast exoribonucleases operated in this context, we would anticipate finding an RhlB homolog in Chlamydomonas, but no such gene was located. On the other hand, tobacco VDL, a plastid protein, does have similarity to RhlB, and its absence has severe defects on chloroplast differentiation (Wang *et al.* 2000).

3′ → 5′ exoribonucleolytic decay: E. coli utilizes several enzymes to perform 3′ → 5′ decay, namely PNPase and the hydrolytic RNases II and R. PNPase is associated with the degradosome complex that includes RhlB, the endonuclease RNase E, and enolase, although several forms of the complex exist with alternative levels of activity (reviewed in Carpousis 2007). In Arabidopsis and Chlamydomonas chloroplasts, PNPase depletion affects RNA stability and decay of polyadenylated transcripts and in Chlamydomonas also the ability to withstand phosphorus deprivation (Walter *et al.* 2002; Nishimura *et al.* 2004; Yehudai-Resheff *et al.* 2007). Arabidopsis RNR1, a homolog of RNase R, is required for chloroplast rRNA maturation (Bollenbach *et al.* 2005), but also appears to be targeted to mitochondria (Perrin *et al.* 2004).

In Chlamydomonas, three potential organellar 3′ → 5′ exoribonucleases were identified. One is the chloroplast PNPase mentioned above, and the other two are hydrolytic enzymes of the RNase II/R family, RNB1 and RNB2. To provide an experimental test of their possible organellar localization, transient expression of putative transit peptide-GFP fusions was performed. The 115 N-terminal amino acids of RNB2 localized GFP to the chloroplast, but results with RNB1 were ambiguous due to low GFP expression (Yehudai-Resheff *et al.* 2007). Since then, higher-expressing transformants indicate that the RNB1 N-terminal 189 amino acids cannot target GFP to an organelle (data not shown). We therefore conclude that RNB1 is likely to be an ortholog of the yeast exosome subunit Rrp44, which is discussed in the exosome section.

5′ → 3′ exoribonucleolytic decay: As discussed in the Introduction, there is evidence of 5′ → 3′ exoribonucleolytic decay in Chlamydomonas chloroplasts. Prokaryotes do not encode canonical 5′ → 3′ exonucleases, although a net 5′ → 3′ maturation pathway is catalyzed by RNase J1 in *Bacillus subtilis* (Mathy *et al.* 2007). In Chlamydomonas chloroplasts, the enzyme responsible for 5′ → 3′ decay could be related to RNase J1 or might be a paralog of a cytosolic or nuclear Xrn-type enzyme or a dually localized protein. As discussed in the section on 5′ → 3′ exonucleases, Chlamydomonas encodes three homologs of yeast Xrn1/Rat1 5′ → 3′ exoribonucleases, as well as a putative RNase J1.

Mitochondrial exoribonucleolytic decay: Mitochondrial polyadenylation, which would be expected to participate in a 3′ → 5′ decay pathway, is not confined to this role. While destabilizing polyadenylation is part of mitochondrial RNA decay in Arabidopsis, in other organisms such as humans, polyadenylation is required to complete translation termination codons and may stabilize the transcripts, as well as act in the decay pathway. *S. cerevisiae*, in contrast, appears to have no mitochondrial

polyadenylation whatsoever (reviewed in Gagliardi *et al.* 2004; Slomovic *et al.* 2005). Assuming that Chlamydomonas mtRNA does undergo polyadenylation, what enzyme is most likely to be responsible for this activity? In human mitochondria, the ncPAP hmtPAP fulfills this role (Tomecki *et al.* 2004), and members of this family are encoded in Chlamydomonas, as described in the section on PAPs. Because the only two putative organellar NTRs (PAP3 and PAP4) are both predicted to be chloroplast proteins, they are not optimal candidates for either mitochondrial polyadenylation or CCAtr activity.

Yeast mtRNA decay is postulated to initiate with endonucleolytic cleavage, followed by processing by mtEXO, a complex that includes the helicase Suv3 and the exonuclease Dss1, which has several short regions of similarity to RNase II (Dmochowska *et al.* 1995). AtSUV3, the Arabidopsis Suv3 homolog, has helicase activity and is located in the mitochondria (Gagliardi *et al.* 1999); likewise, we found a Chlamydomonas Suv3 homolog. While a close homolog of Dss1 has been found in trypanosomes (Penschow *et al.* 2004), the less- or unrelated proteins RNR1 and PNPase are known to have roles in RNA processing and/or decay in Arabidopsis mitochondria (Perrin *et al.* 2004; Holec *et al.* 2006). Chlamydomonas differs from Arabidopsis, however, since it appears to encode only one PNPase, already implicated in chloroplast RNA decay. Furthermore, initial experiments suggest that neither of the RNase II/R proteins, RNB1 or RNB2, is predominantly mitochondrial.

One remaining candidate for a mitochondrial exoribonuclease in Chlamydomonas is a predicted homolog of oligoribonuclease, ORN1, which in *E. coli* is responsible for converting oligoribonucleotides to monoribonucleotides (Ghosh and Deutscher 1999) and is also found throughout the eukaryotes (reviewed in Zuo and Deutscher 2001). The potential localization of the Chlamydomonas protein is ambiguous because the gene model is clearly incomplete, as the predicted ORF does not begin with a Met. Thus, the correctly annotated protein could include an N-terminal transit peptide.

Exoribonuclease digestion of the nuclear transcriptome: the exosome

The exosome is the basic enzymatic unit carrying out decay of nucleus-encoded and archaeal RNA, best studied in *S. cerevisiae,* Archaea, kinetoplasts, and humans, where the exosome has been reconstituted and/or crystallized (Buttner *et al.* 2005; Liu *et al.* 2006; Lorentzen *et al.* 2007; Cristodero *et al.* 2008). It has also been recently purified by tandem affinity purification (TAP) tagging in Arabidopsis (Chekanova *et al.* 2007). Each component of the 9- or 10-subunit core exosome is necessary for cell viability in yeast and humans, and this core is common to the nuclear and cytosolic complexes, where it contains additional location-specific components. The core consists of six exonucleolytic RNase PH domain-type polypeptides, three unrelated RNA-binding proteins, and in yeast but apparently not humans, the RNase II/R family protein Rrp44 (Mitchell *et al.* 1997; Allmang *et al.* 1999; Liu *et al.* 2006). The PH domain proteins fall into two groups: one more related to Rrp41 (Rrp41/Rrp46/Mtr3) and the other more related to Rrp42 (Rrp42/Rrp43/Rrp45). Certain members of both groups in plants and Archaea have shown $3' \rightarrow 5'$ exonuclease activity using *in vitro* assays (Chekanova *et al.* 2002; Lorentzen *et al.* 2005; Hooker *et al.* 2007), but the yeast and human orthologs do not (Liu *et al.* 2006). The RNA-binding proteins have S1 and in some cases KH domains and are represented in yeast by Rrp4, Rrp40, and Csl4. Curiously, the Arabidopsis Csl4 mutant has no growth phenotype (Chekanova *et al.* 2007).

The situation in Chlamydomonas appears to mostly parallel that in yeast. When protein predictions from Chlamydomonas and Arabidopsis were searched for RNase PH domains, nine Arabidopsis and seven Chlamydomonas predicted proteins were found, although one Chlamydomonas homolog had only weak sequence similarity with the RNase PH domain consensus and was not analyzed further. The Arabidopsis and Chlamydomonas proteins were compared to the six PH-domain core exosome components from yeast, and the resulting relationships are shown in Figure 2. A separate clade is formed by the Chlamydomonas and Arabidopsis PNPase proteins, which also have PH domains. Chlamydomonas PNP1 is more closely related to the chloroplast than the mitochondrial isoform from Arabidopsis, consistent with the published data cited earlier.

With respect to the exosome subunits, two Chlamydomonas proteins fall into the Rrp41 group and three into the Rrp42 group. The corresponding numbers in Arabidopsis are four and four. This reflects, in the former case, a possible duplication of Rrp41 and the presence of an Mtr3 homolog, which is not found in Chlamydomonas. In terms of the Rrp42 group, Arabidopsis has a clear and probably recent duplication of Rrp45, two of which exhibit some functional redundancy (Hooker *et al.* 2007). While neither organism appears to encode a protein in the Rrp42/Rrp43 clade, bootstrap values suggest that this may be artifactual. As presented in Figure 2 and Table 2, Chlamydomonas has one fewer catalytic core component than yeast (and two fewer than Arabidopsis). Whether an Mtr3 homolog is encoded in as-yet-unsequenced DNA, or whether CrRNPH1 and CrRRP46 together fulfill the function of the Rrp41 group, remains to be established. On the basis of inferred active site residues (Lorentzen *et al.* 2005), CrRNPH1 (Rrp41) is predicted to exhibit exonuclease activity, whereas Rrp46 is not. Chlamydomonas would thus resemble plants/Archaea more than yeast/humans in this respect.

As shown in Table 2, the RNA-binding components Rrp4, Rrp40, and Csl4 have a one-to-one relationship with predicted Chlamydomonas proteins. Furthermore, the Rrp44 subunit could be represented by Chlamydomonas

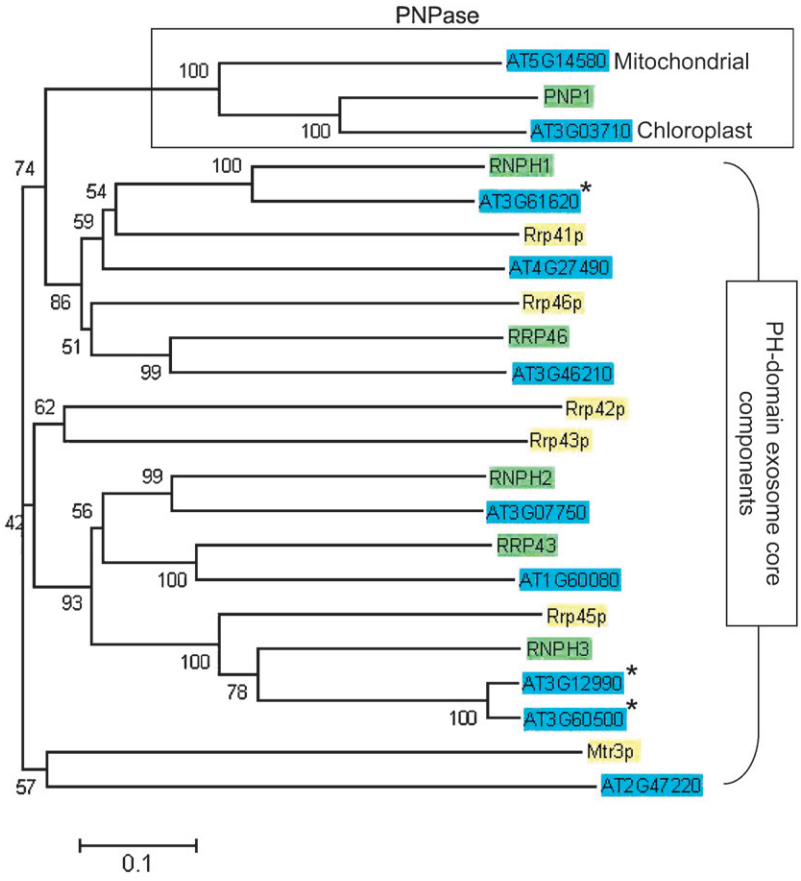

FIGURE 2.—Phylogenetic analysis of Arabidopsis (blue), Chlamydomonas (green), and yeast (yellow) PH exoribonuclease domain-containing proteins. Asterisks indicate cases where ribonuclease activity of the protein has been experimentally verified. Regarding the Arabidopsis gene models, CHEKANOVA *et al.* (2007) list At4g27490 as the Mtr3 homolog and do not identify At2g47220. They further note that At3g60500, but not At3g12990, copurify with the TAP-tagged exosome.

RNB1, which, as discussed above, has the requisite RNase II motifs and is apparently not organellar. Overall, the core exosome components are largely conserved, represented at present by nine Chlamydomonas gene models.

Other exosome and exosome-related proteins

Nuclear exosome components: In yeast, the exonuclease Rrp6 and putative RNA-binding protein Rrp47 join the core to complete the nuclear exosome (ALLMANG *et al.* 1999; MITCHELL *et al.* 2003). Chlamydomonas homologs were not found for Rrp47 or Rrp6, a D/T family exonuclease, although several putative proteins (EDP00977, EDP01505, EDP06690, EDP08895, and EDO96190) share domains with RNase D and thus are members of the exonuclease superfamily (ZUO and DEUTSCHER 2001). In contrast, Arabidopsis possesses three Rrp6 homologs, one of which has been implicated in degradation of nuclear polyadenylated RNA (LANGE *et al.* 2008).

TRAMP: The yeast nuclear exosome requires activation by the TRAMP complex (LACAVA *et al.* 2005). TRAMP has the dual roles of adding the short poly(A) tail to the target transcript and activating the exosome (HOUSELEY *et al.* 2006). It contains an ncPAP, either Trf4 or Trf5 (HOUSELEY and TOLLERVEY 2006), the helicase Mtr4p (DE LA CRUZ *et al.* 1998), and either Air1p or Air2p (WYERS *et al.* 2005), homologous proteins that contain a zinc-knuckle domain thought to bind RNA. The likely Chlamydomonas homolog of the Trf4/Trf5 proteins, CrTRF4, is discussed below along with other ncPAPs. Since yeast Mtr4 has a paralog, Ski2, which acts in cytosolic exosome activation (ANDERSON and PARKER 1998), phylogenetic analysis was performed on Chlamydomonas and Arabidopsis proteins with helicase domains related to those of Mtr4 and Ski2 (Figure 3). High bootstrap values lend confidence to the placement of two putative Chlamydomonas and Arabidopsis proteins in the Mtr4 clade. One of the Chlamydomonas proteins appears to be the true ortholog, with the second most closely related to the putative helicase HEN2, a plant regulator of floral homeotic genes (WESTERN *et al.* 2002).

Concerning Ski2, this protein appears to have an Arabidopsis ortholog; however, such a predicted protein is lacking in Chlamydomonas. Finally, a BLASTp performed with yeast Air1 and Air2 produced no hits on the translated Chlamydomonas genome, and no Pfam or Interpro consensus domains for the Air1 zinc-knuckle subfamily were available for domain searches. However, we did perform a search for the less specific CCHC-type zinc-finger Interpro consensus sequence and found 10 predicted Chlamydomonas proteins, 2 of which are robust protein models with EST support (Table 2). One of these, CGLD20, belongs to the green lineage of Chlamydomonas genes (MERCHANT *et al.* 2007). To summarize exosome accessory components, it seems likely

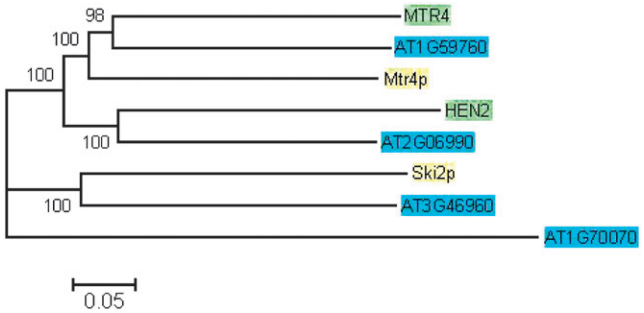

Figure 3.—Phylogenetic analysis of Arabidopsis (blue), Chlamydomonas (green), and yeast (yellow) exosome-associated helicase proteins.

that transient polyadenylation by a TRAMP-like complex occurs in Chlamydomonas nuclei, but activation of the nuclear exosome appears not to occur as it does in yeast.

Cytosolic exosome components: In addition to the core, the yeast cytosolic exosome possesses Ski7 (Araki *et al.* 2001), but this protein is apparently lacking in Chlamydomonas. Ski7 contains only general domains and predicted functions, so domain-based searches of the Chlamydomonas databases were not performed. Furthermore, no homologs of the yeast exosome-activating complex Ski2-3-8 were found (Ski2 was discussed above).

Stabilizing poly(A) polymerase and cytosolic deadenylases: When the initial substrate for decay is cytosolic mRNA, the process begins with exoribonucleolytic digestion to remove most of the long, stabilizing poly(A) tail. Yeast possesses several deadenylation complexes, the major deadenylase being CCR4-Not and the minor deadenylase Pan2/Pan3. Deadenylation initiates decapping and, following this, 5′ → 3′ exonucleolytic decay is possible (reviewed in Meyer *et al.* 2004). At the same time, the shortened poly(A) tail minus its binding proteins is an ideal substrate for the cytosolic exosome, which begins degrading from the opposite (3′-) end. The class I NTR PAP responsible for adding stabilizing tails to mRNA is likely encoded in Chlamydomonas by *PAP1*. Although the corresponding protein sequence lacks the expected nuclear localization signal, it also lacks EST support in the region where the NLS should be present, according to alignments with other eukaryotic PAPs (Martin and Keller 1996). Additionally, LOCTree (Nair and Rost 2005) predicts nuclear localization on the basis of other factors.

In terms of deadenylation components, the deadenylase domain from Caf1, one of the CCR4-Not complex deadenylases, was found in two predicted proteins, although these Caf1 homologs lack the N-terminal 100 or more amino acids found in the yeast protein. Ccr4, the other CCR4-Not deadenylase, contains both a nuclease domain and a leucine-rich repeat, but no Chlamydomonas predicted proteins were found with both of these features, even though two models did possess the nuclease domain. Some Ccr4-containing organisms possess additional proteins of unknown function containing the same nuclease domain but similarly lacking this repeat. Other components of the CCR4-NOT complex in yeast have unknown functions and were not investigated. One predicted protein similar to Pan2 of the minor deadenylase Pan2/Pan3 was found. The deadenylase PARN, which has been found in mammals, Xenopus, and Arabidopsis (Korner and Wahle 1997; Copeland and Wormington 2001; Chiba *et al.* 2004), was not found in Chlamydomonas, strengthening the view that this is an enzyme specific for regulating development and stress responses in multicellular organisms.

Noncanonical poly(A) polymerases

In our search for predicted proteins containing PAP domains, we identified nine putative proteins that were not part of the traditional NTR family, but contained similar PAP catalytic domains. These ncPAPs lack RNA recognition motifs found in the NTR family members (Keller and Martin 2002; Wang *et al.* 2002) and have only recently begun to be characterized, initially with *Schizosaccharomyces pombe* Cid1 and Cid13 (Read *et al.* 2002; Saitoh *et al.* 2002) and *Caenorhabditis elegans* GLD2 (Wang *et al.* 2002). The family also includes Trf4 and Trf5 of the TRAMP complex, which was mentioned above.

The Chlamydomonas family of 9 ncPAPs is large—not as many as the 12 in *C. elegans* (Martin and Keller 2007), but slightly exceeding the 6 in *S. pombe* (Stevenson and Norbury 2006) and the 8 in Arabidopsis, and sharply contrasting with the 2 in *S. cerevisiae* (Trf4 and Trf5). In Tables 1 and 2, the Chlamydomonas ncPAPs are MUT68, TRF4, and the seven homologs listed under the heading "PAP (other noncanonical)." To attempt to establish possible orthologous relationships, we constructed several phylogenetic trees either on the basis of global sequence alignments or by using, individually or in combination, the two domains found in this family, PAP/25A_core and PAP_associated. These analyses all showed that with the exception of a few closely related proteins, there is great diversity among the ncPAPs of Arabidopsis and Chlamydomonas, whether looking within or between organisms. Bootstrap values indicated that most of the relationships are not robust and are thus not informative (data not shown). None of the Chlamydomonas ncPAPs appear to have arisen through relatively recent gene duplications, and in only one case is there a clear relationship between Chlamydomonas and Arabidopsis proteins—those being PAP5 and AT2G45620, neither of which has been characterized.

5′ → 3′ exoribonucleases

S. cerevisiae contains a nuclear 5′ → 3′ exonuclease, Rat1 (Xrn2), and a related cytosolic homolog, Xrn1

(Johnson 1997). Chlamydomonas encodes three similar proteins (Table 2), equal to the number previously identified in Arabidopsis (Kastenmayer and Green 2000). All three have some level of EST support, and *XRN1* mRNA (equivalent to Arabidopsis *XRN2*) is alternatively spliced near its 5′-end (D. Higgs, personal communication), although there is no evidence that this is the case in Arabidopsis. None of the Chlamydomonas proteins contain predicted nuclear localization signals, but both splice variants of *XRN1* encode putatively mitochondrial proteins. It is possible that the mitochondrion, or perhaps the chloroplast, is now utilizing one of these proteins for decay of its own RNA, despite its absence in the presymbiotic organelle. A second possibility is that 5′ → 3′ exonuclease activity in chloroplasts is catalyzed by RNase J1, for which the Chlamydomonas homolog is predicted to be chloroplast targeted, whether encoded by either of two mRNA splice variants (Table 1). Although RNase J1 and a related protein RNase J2 were originally characterized as endoribonucleases in *B. subtilis* (Even *et al.* 2005), RNase J1 has been newly associated with 5′ → 3′ exonuclease activity (Mathy *et al.* 2007) and ultimately accommodates both (de la Sierra-Gallay *et al.* 2008).

DISCUSSION

Given the increasing recognition of RNA-based regulatory mechanisms, we felt it was important to identify the spectrum of RNA metabolic enzymes encoded in Chlamydomonas, where chloroplast RNA stability is subject to gene-specific regulation (Monde *et al.* 2000) and where some of the earliest results related to RNA-based gene silencing were obtained (Cerutti *et al.* 1997). Because a great variety of enzymes can modify RNA through polymerization, degradation, or covalent modification, we focused on subgroups responsible for 5′- and 3′-end metabolism. Even with this restriction, the resultant protein complement is active in four subcellular compartments in photosynthetic eukaryotes, as well as in prokaryotes. The catalytic members are targeted to substrates by inherent preferences or through regulatory subunits embedded in multisubunit complexes, and some of these accessory moieties were also studied here.

Several key concepts emerged from our analysis, one being an apparent shortage with respect to the number of exoribonucleases and NTRs that we believed to be necessary for RNA end metabolism in Chlamydomonas organelles. There are several possible reasons for this. First, there is a chance that the proteins of interest are encoded in the relatively small, unsequenced portion of the genome. Second, we may have identified the full complement of proteins, but perhaps a number of them are dual targeted, for example, PNPase, RNB2, the putative poly(A) polymerase PAP4, and/or the putative CCA-transferase PAP3. Dual-organelle targeting is difficult to predict computationally, although perhaps surprisingly common (reviewed in Millar *et al.* 2006). A third possibility is that proteins acting on the nuclear transcriptome, or their paralogs, have been recruited to the organelles to perform these functions. This may be the case with the 5′ → 3′ exoribonucleases, as one example. In addition, a number of the noncanonical PAPs have predicted organellar transit peptides (see below), and there is precedent for a mitochondrial PAP of this family in humans and *S. pombe* (Stevenson and Norbury 2006). We also raised the possibility that Chlamydomonas homologs of oligoribonuclease and RNase J1 fulfill some of the "missing" functions.

A second general outcome of our study is that core exosome components are largely conserved in Chlamydomonas. Five of six PH-domain-containing subunits appear to have a Chlamydomonas homolog, with Arabidopsis evincing instances of gene duplication, for example, in the case of Rrp41. Concerning the other components of the core exosome, it was relatively easy to assign Chlamydomonas genes to the RNA-binding subunits Rrp4, Rrp40, and Csl4. Finally, targeting experiments revealed that the RNase II/R protein RNB1 is likely to be the Rrp44 homolog, which may or may not be part of the exosome in Chlamydomonas.

A surprising feature of the Chlamydomonas exosome is that, unlike *S. cerevisiae*, it appears to have no cell-compartment-specific components. Although putative orthologs of the Mtr4 and Trf4 enzymes of the nuclear TRAMP complex were present, the proteins utilized in yeast to activate the cytosolic exosome are apparently absent in Chlamydomonas. Although Ski2, the cytosolic Mtr4 paralog, has a putative Arabidopsis ortholog, there does not appear to be one in Chlamydomonas. Additionally, the other proteins composing the Ski2-Ski7-Ski8 complex are missing. Our conclusion is that Chlamydomonas either does not need to activate its cytosolic exosome or has another mechanism for doing so.

A third major finding was the large family of noncanonical PAPs encoded in Chlamydomonas. To see if this was typical of green (chlorophyte) algae, we searched the genomes of *Ostreococcus tauri* (Derelle *et al.* 2006) and *Volvox carteri* (http://www.jgi.doe.gov/Volvox), the latter being a close relative of Chlamydomonas that is nonetheless multicellular. We found evidence for only three ncPAPs in Ostreococcus but eight in Volvox, suggesting that some degree of expansion may have occurred in the order Volvocales.

The ncPAPs are found throughout the eukaryotes and contain a typical PAP catalytic domain, but otherwise lack similarity to PAPs, as well as consensus RNA-binding domains (Stevenson and Norbury 2006). On the basis of our knowledge of the current pathways utilizing ncPAPs, we may speculate that Chlamydomonas ncPAPs play roles in both cytosolic and nuclear-exosome-catalyzed decay, targeting the exosome to specific substrates. In this way, Chlamydomonas could also enhance the specificity of its cytosolic exosome,

which as mentioned above does not appear to contain the accessory subunits found elsewhere.

An ncPAP studied in Chlamydomonas is MUT68, which is proposed to function in RNA interference (RNAi) by adding a short poly(A) tail to DICER cleavage products, thus creating exosome substrates (Ibrahim *et al.* 2006). One or more of the other ncPAPs could function like Cid1 and Cid13, polyadenylating a set of mRNAs to stabilize them, or could be involved in newly described modification pathways involving polyuridylation (Wilusz and Wilusz 2008). One potentially significant observation is that MUT68 and PAP6 are linked in the nuclear genome, being in the same orientation and separated by ~72 kb in version 4. They also are found in the same clade in various phylogenetic trees built for Chlamydomonas ncPAPs, with bootstrap values of 73–80 (data not shown). These facts together could reflect an ancient gene duplication and, possibly, functional conservation. None of the putative Chlamydomonas ncPAPs, including MUT68, belong to the "green cut" proteins present in only plant lineages (Merchant *et al.* 2007), which could have implicated them in the chloroplast RNA decay pathways described earlier.

In summary, the ancient roots of RNA metabolism are consistent with conservation of mechanisms and individual enzymes, as we have found here in a number of cases. As is often found in genomewide analyses, however, each organism has its peculiarities even within an otherwise conserved context. In the case of Chlamydomonas, this points to ncPAPs and the cytosolic exosome as particularly fruitful areas for future research, along with potential dual targeting of one or several organellar ribonucleases.

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