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Identification of small non-coding RNAs from mitochondria and chloroplasts

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ABSTRACT

Small non-protein-coding RNAs (ncRNAs) have been identified in a wide spectrum of organisms ranging from bacteria to humans. In eukarya, systematic searches for ncRNAs have so far been restricted to the nuclear or cytosolic compartments of cells. Whether or not small stable non-coding RNA species also exist in cell organelles, in addition to tRNAs or ribosomal RNAs, is unknown. We have thus generated cDNA libraries from size-selected mammalian mitochondrial RNA and plant chloroplast RNA and searched for small ncRNA species in these two types of DNA-containing cell organelles. In total, we have identified 18 novel candidates for organellar ncRNAs in these two cellular compartments and confirmed expression of six of them by northern blot analysis or RNase A protection assays. Most candidate ncRNA genes map to intergenic regions of the organellar genomes. As found previously in bacteria, the presumptive ancestors of present-day chloroplasts and mitochondria, we also observed examples of antisense ncRNAs that potentially could target organelle-encoded mRNAs. The structural features of the identified ncRNAs as well as their possible cellular functions are discussed. The absence from our libraries of abundant small RNA species that are not encoded by the organellar genomes suggests that the import of RNAs into cell organelles is of very limited significance or does not occur at all.

INTRODUCTION

Cells from all organisms contain two different kinds of RNAs: mRNAs, which are translated into proteins and non-protein-coding RNAs (ncRNAs). The latter ones function at the level of the RNA and are not translated into proteins (1–6). Reported sizes of ncRNAs range from very large as, for example, the ~17 kb human Xist RNA (7), to extremely small, as the 21–23 nt microRNAs (8,9). However, the sizes of the vast majority of ncRNAs known to date lie between 20 and 500 nt, well below the size of the majority of mRNAs (2). NcRNAs fulfill vital and important functions in many cellular processes, namely in transcription, translation, splicing, DNA replication or RNA processing (3).

The evolutionary origin of eukaryotic cells is marked by the endosymbiontic uptake of two eubacteria (an α-proteobacterium and a cyanobacterium) and their gradual conversion into the DNA-containing cell organelles of present-day eukaryotes, mitochondria and chloroplasts (10–12). Genetically, the evolutionary optimization of endosymbiosis was accompanied by the loss of dispensable and redundant genetic information, and the large-scale translocation of information from the genome of the endosymbiont to that of the host cell (13,14). Consequently, contemporary organellar genomes are greatly reduced and, as compared to the nuclear genome, contain relatively little information. The chloroplast genome of higher plants is a circular double-stranded molecule of 120–160 kb which harbors ~120 genes (15,16). Most of these chloroplast-encoded genes belong to either of two major gene classes: photosynthesis-related genes and genetic system genes (e.g. genes for rRNAs, tRNAs, ribosomal proteins, subunits of an RNA polymerase) (17). Plant mitochondrial genomes can be significantly larger in size (180–2400 kb), but typically contain only half as many genes as chloroplast genomes (18,19). In contrast, animal mitochondria harbor much smaller, but highly compact and gene-dense genomes (20,21). For example, the human mitochondrial genome contains 37 genes in a 16.5 kb circular genome. Of these mitochondrial genes 13 specify protein products (subunits of respiratory chain complexes), the remaining 24 encode RNA products (a complete set of ribosomal and transfer RNAs).

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An important feature of all organellar genomes is their remarkably high ploidy level, i.e. copy number per cell (22,23). A single tobacco leaf cell, for example, may contain as many as 100 chloroplasts, each harboring ~100 identical copies of the plastid genome resulting in an extraordinarily high ploidy degree of up to 10 000 plastid genomes per cell.

Clearly, the limited coding capacity of organellar genomes is far insufficient to provide all of the many components required to support its own gene expression system. The organelles are therefore highly dependent on the products of nuclear genes that are synthesized on cytoplasmic ribosomes and post-translationally imported into the organelle (24). Chloroplasts, for example, import >90% of their proteins from the cytosol. Consequently, the temporal and spatial expression of organellar and nuclear genes must be regulated in a highly coordinated fashion.

Eubacterial genomes encode a large number of ncRNAs and it seems conceivable that ncRNAs also could exist and possibly play regulatory roles in cell organelles. Indeed, the identification of a first ncRNA candidate in tobacco chloroplasts [*sprA*; (25)] points to the presence of ncRNAs in present-day cell organelles. In this study, we sought to systematically identify ncRNA species in chloroplasts and mitochondria.

Since the identification of ncRNAs solely by biocomputational approaches is severely hampered by their lack of an open reading frame, we employed an experimental approach designated as 'experimental RNomics' which is based on the generation of specialized cDNA libraries encoding potential novel ncRNA species (26,27). We thus generated cDNA libraries from fractions of small RNAs isolated from organellar RNA which was extracted from purified chloroplasts (model organism: tobacco, *Nicotiana tabacum*) and mitochondria (model organism: mouse, *Mus musculus*). In this analysis of the small RNA component of the transcriptome of chloroplasts and mitochondria, we identified a number of novel ncRNA candidates which might be involved in the regulation of organellar gene expression and thus possibly exert similar functions as ncRNAs in the bacterial ancestors of present-day organelles.

MATERIALS AND METHODS

Generation of a cDNA library from tobacco chloroplasts and mouse mitochondria

For the construction of a chloroplast library, chloroplasts were isolated from young leaves of *N.tabacum* plants grown in the light and plants grown in dark by using a Percoll gradient-based method (28). Mitochondria were isolated from mouse liver and kidney and purified by a Percoll gradient as described previously (29).

Total RNA was extracted from cell organelles by the TRIzol method (Gibco-BRL) or by directly extracting the chloroplast pellet with phenol/chloroform (1:1). Subsequently, 100 μg of total RNA was size-fractionated by denaturing 8% PAGE (7 M urea, 1× TBE buffer). RNAs in the size range between ~20 and 500 nt were excised from the gel, passively eluted and ethanol-precipitated. For the chloroplast library, RNAs were ligated to 5′- and 3′-oligonucleotide linkers by T4 RNA ligase, as described previously (26). For the mitochondrial library, RNAs were poly(C)-tailed employing poly(A) polymerase (Invitrogen). C-tailed RNAs were ligated to a 5′- oligonucleotide linker as described previously (27). RNAs from both libraries were subsequently converted into cDNAs by RT–PCR as described previously, employing complementary primers to 5′- and 3′-linkers or the poly(C) tail (27), followed by cloning into pGEM-T or pGEM-T-Easy vector (Promega).

Sequence analysis of cDNA libaries from chloroplasts and mitochondria

cDNA clones were sequenced using the M13 reverse primer and the BigDye terminator cycle sequencing reaction kit (PE Applied Biosystems). Sequencing reactions were run on an ABI Prism 3100 (Perkin Elmer) capillary sequencer. Subsequently, sequences were analyzed with the LASERGENE sequence analysis program package (DNASTAR, Madison, USA). cDNA sequences were compared with one another using the Lasergene Seqman II program package to identify identical sequences (DNASTAR). Following a BLASTN search against the GenBank database (NCBI), all RNA sequences, which were not annotated in the database were treated as potential candidates for novel ncRNAs.

Northern blot analysis

Total organellar RNA (5–10 μg) was denatured for 1 min at 95°C, separated on a 8% denaturing polyacrylamide gel (7 M urea, 1× TBE buffer) and transferred onto a nylon membrane (Quiabrane Nylon Plus, Quiagen) using the Bio-Rad semi-dry blotting apparatus (Trans-blot SD; Bio-Rad). After immobilizing of RNAs using the STRATAGENE UV crosslinker, we pre-hybridized the nylon membrane for 1 h in 1 M sodium phosphate buffer (pH 6.2) with 7% SDS. Oligonucleotides from 20 to 26 nt in size, complementary to potentially novel RNA species were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Hybridization was carried out at 58°C in 1 M sodium phosphate buffer (pH 6.2), 7% SDS for 12 h. Blots were washed twice at room temperature in 2× SSC buffer (20 mM sodium phosphate, pH 7.4; 0.3 M NaCl; 2 mM EDTA), 0.1% SDS for 15 min and subsequently at 58°C in 0.1× SSC, 0.5% SDS for 1 min. Membranes were exposed to Kodak MS-1 film from 12 h to 5 days.

RESULTS AND DISCUSSION

cDNA library containing ncRNAs from chloroplasts

Library construction and sequence analysis of cDNA clones. Tobacco plants from the species *N.tabacum* were either grown under normal light conditions (16 h light, 8 h dark) or kept under constant darkness for 3 days prior to isolation of chloroplasts. As chloroplast gene expression is strongly regulated by light (30,31), we decided to comparatively analyze small RNA species in light-grown versus dark-grown plants. From purified chloroplasts, cDNA libraries were generated encoding RNAs sized from 20 to 500 nt (Materials and Methods). Subsequently, a total of 5500 cDNA sequences were analyzed by first grouping identical cDNA clones, followed by bioinformatical analysis on their location on the chloroplast genome (Materials and Methods).

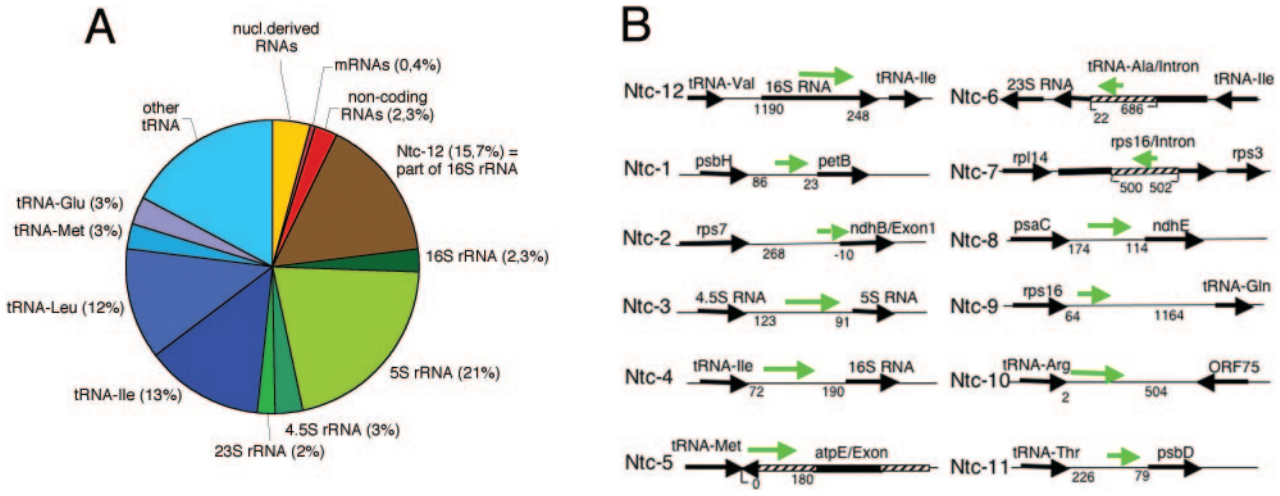

Figure 1. Sequence analysis and genomic location of ncRNA candidates from the chloroplast library of *N.tabacum*. (**A**) Sequence analysis of 5500 cDNA clones from the chloroplast library. cDNA clones representing different RNA species or categories are shown as percent of total clones. The red sector denotes candidates for novel ncRNAs in chloroplasts; note that Ntc-12 ncRNA is part of a previously assigned chloroplast gene, i.e. 16S rRNA, and thus is annotated within the number of cDNA clones from this gene. (**B**) Location of ncRNA candidates on the chloroplast genome, not drawn to scale. Novel candidates for ncRNA genes are indicated by green arrows, genes flanking the ncRNA candidate are indicated by black arrows. The distance of the ncRNA to 5′ and 3′ ends of flanking genes (in nt) is shown below. For ncRNAs mapping in sense or antisense orientation to introns/UTRs, the distance to the neighboring exon is shown (in nt); for ncRNAs that overlap with the open reading frame of a gene, the number of overlapping nucleotides is indicated by a negative number.

About 90% of the sequenced clones represented known ncRNAs from the chloroplast genome (Figure 1A). We thereby identified the full set of chloroplast-encoded tRNAs and small rRNAs (data not shown), consistent with our screen being saturating with respect to known ncRNAs. A minor fraction (0.4%) was derived from coding regions of chloroplast mRNAs, presumably representing mRNA degradation products. This low amount of putative mRNA degradation intermediates in the library indicates a high degree of intactness of the isolated organellar RNA population. About 2.7% of sequences represented novel potential candidates for chloroplast ncRNAs by two criteria. They were located mainly in intergenic regions and had not been assigned to a known chloroplast gene (Figure 1A; note that from novel ncRNAs, Ntc-12 ncRNA is part of a previously assigned chloroplast gene, i.e. 16S rRNA, and thus is annotated within the number of cDNA clones from this gene). Finally, 4% of the sequenced cDNA clones were encoded by the nuclear genome (Figure 1A). Among the nuclear genome-derived RNAs, 11 novel, previously not annotated candidates for small nucleolar RNAs (snoRNAs) were identified based on sequence and structural motifs. In addition, the nuclear-encoded 5.8S rRNA and some tRNAs were found (see below).

Novel candidate ncRNAs encoded by the chloroplast genome

Ntc-12 RNA. The most abundant cDNA clone from our chloroplast library (found in ~900 cDNA clones) was designated as Ntc-12 (*N.tabacum* chloroplast-12). It is encoded by the chloroplast genome and is derived from the chloroplast 16S ribosomal RNA (Table 1 and Figure 1B). The cDNA represents a 53 nt long portion of domain III of the 16S rRNA which folds into a stable hairpin structure (Supplementary Figure S1). Although fragments of ribosomal RNA regularly appear as contaminants in cDNA libraries encoding ncRNAs, the presence of such a highly abundant specific rRNA fragment has not been observed previously. The fact that all 900 clones are derived from one particular region of the 16S rRNA sequence and, moreover, exhibit defined 5′ and 3′ ends makes it highly unlikely that this small RNA species reflects contamination with non-specific rRNA degradation products. Consistent with this interpretation, we detect a specific signal of 53 nt in size in northern blot analyses of total RNA from light and dark grown plants (Figure 2) indicating that significant amounts of Ntc-12 accumulate in a light-independent manner. It was interesting to compare light inducibility of Ntc-12 with that of the 16S rRNA from which Ntc-12 is likely to originate by processing. Previous work has shown that a dark–light shift results in only a moderate increase in transcription from the 16S rRNA promoter [of about 30% (32)]. In contrast, no increase in the level of Ntc-12 ncRNA can be observed in northern blot experiments under light conditions (Figure 2).

The high abundance of Ntc-12 clones in our library might imply that a significant portion of the 16S ribosomal RNA is fragmented within chloroplasts. However, by a poisoned primer extension analysis (33), we demonstrated that <1% of the ribosomal 16S rRNA in chloroplasts is fragmented and thus lacking the Ntc-12 sequence (data not shown). Thus, the high abundance of Ntc-12 cDNA clones in our library is likely due to the high abundance of rRNA in general and/or to preferential cloning of the Ntc-12 RNA compared with the remaining sequences.

We cannot entirely exclude the possibility that Ntc-12 is not derived from the full-length 16S rRNA but, instead, is derived from a shorter RNA produced by internal transcription initiation. However, the rRNA operon is probably the best-characterized transcription unit in chloroplast and has

Table 1. Candidates for novel ncRNAs from a *N.tabacum* chloroplast cDNA library derived from RNAs sized 10–500 nt

Name	Nr.	Sequence	cDNA (nt)	N. blot (nt)	Remarks
Ntc-12	890	CCGGGACAAAGGGTCGCGATCCC GCGAGGGTGAGCTAACCCCAAAA ACCCGTC	53	53	Localized in 16S rDNA gene, 100% conserved in 24 different chloroplast genomes, stable stem–loop structure
Ntc-1	36	GGTAGTTCGATCGTGGAATTTC	22	22*	Localized in intergenic region, 100% conserved in 10 different chloroplast genomes
Ntc-2	20	AGTTACTAATTCATGATCTGGC	22	22	Intergenic region, 100% conserved in 960 different chloroplast genomes; homologous sequence found in *A.thaliana* chloroplast cDNA library
Ntc-3	18	TCTGCCCTCCCTCTCTATCTATCC AAGGGATGGAAGGGCAGAGG	44	45	Intergenic region, 100% conserved in 19 different chloroplast genomes, stable stem–loop structure, sncRNA in the same region identified in *A.thaliana*
Ntc-4	18	ACGTCCCCATGTTCCCCCCGTGTG GCGACATGGGGGCGAA	40	55	Intergenic region, 100% conserved in six different chloroplast genomes, stable stem–loop structure
Ntc-5	4	AAACTTATTAGATACCAGAGTCA ATGGTATCTAATAAGGTTT	42	40	Antisense to 3′-UTR of *atpE* gene, stable stem–loop structure, 100% conserved in five chloroplast genomes
Ntc-6	2	TGAGAGGCGGTGGTTTACC	19	—	Localized in tRNA-Ala intron, 100% conserved in 975 different chloroplast genomes, putative part of a miRNA precursor sequence
Ntc-7	1	GCATTCACAAGTTCCGTC	18	—	Antisense to intron in gene for ribosomal protein S16 (*rps16*), 100% conserved in nine different chloroplast genomes
Ntc-8	1	AGAAATCAAAGTATTTTGGCCCT CTCTC	28	—	100% conserved in three different *Nicotiana* chloroplast genomes
Ntc-9	1	CAACCAATGACTATTCATGATTC	23	—	Intergenic region/promoter region of gene for ribosomal protein S16,100% conserved in 19 different chloroplast genomes
Ntc-10	1	AACCGGCCCAAAAGGGAAGTACC TTTCCCTCTGGGGGTAGGA	42	—	Intergenic region, 100% conserved in 10 different chloroplast genomes
Ntc-11	1	ATCCATTCGAAAGGTTAGA	19	—	Intergenic region, 100% conserved in three different chloroplast genomes

Nr., number of independent cDNA clones identified from each RNA species; Sequence, sequence of cDNA; cDNA (nt), length of cDNA encoding a ncRNA candidate as assessed by sequencing; N. blot (nt), length of RNAs as assessed by northern blot analysis or by an RNase protection assay (indicated by asterisk).

been extensively used to map and dissect promoter elements [for a review see (34)]. No evidence for the presence of an internal promoter has been found, making it less likely that Ntc-12 is produced by internal transcription initiation.

Ntc-1 RNA

The next most abundant ncRNA candidate identified in our screen (designated as Ntc-1) was present in 36 identical cDNA clones. Ntc-1 is located in an intergenic region of the chloroplast genome, and maps between the *psbH* (encoding a small subunit of the photosystem II complex) and *petB* (encoding the cytochrome b subunit of the chloroplast cytochrome b_6f complex) genes (Table 1 and Figure 1B). As assessed by its cDNA sequence, the Ntc-1 RNA has a size of 24 nt and thus is in the same size range as miRNAs present in the nucleocytosolic compartment of plant cells (8). Although being the second most abundant novel ncRNA candidate in our library, we were unable to confirm expression of Ntc-1 by northern blot analysis. This might be due to the design of the oligonucleotide probe used for detection of Ntc-1 RNA, an explanation that would be consistent with previous results from analysis of a mouse brain cDNA library (35). Owing to the small size of Ntc-1 (24 nt), it was not possible to design a probe to a different region of the RNA. However, we were able to confirm expression of Ntc-1 RNA by an alternative method, employing an RNase A protection assay and using a radiolabeled RNA antisense probe directed against the Ntc-1 RNA (data not shown).

Ntc-2 RNA

Ntc-2 RNA is represented by 20 cDNA clones and exhibits a predominant size of 22 nt (Table 1 and Figure 1B). Expression of the RNA can be confirmed by northern blot analysis (Figure 2) and the size of the northern blot signal is in good

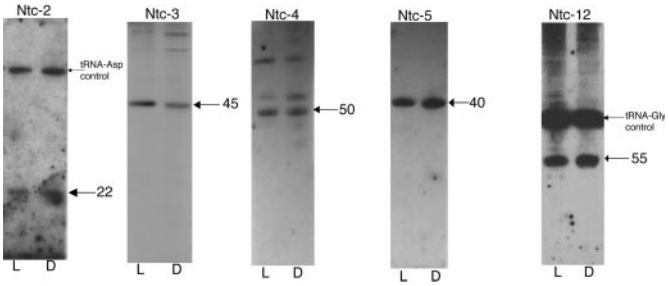

Figure 2. Northern blot analysis of selected ncRNAs from the chloroplast library. Clone names for each ncRNA are indicated above each lane, sizes of RNAs, as estimated by comparison with an internal RNA marker, are indicated by arrows on the right. RNAs isolated from light or dark grown plants (Materials and Methods) are indicated below by L or D, respectively.

agreement with the length of the cloned cDNAs. The DNA sequence specifying the Ntc-2 RNA is located 3′ of a ribosomal protein gene (*rps7*) and overlaps with the initiation codon of the *ndhB* gene following immediately downstream (Figure 1B). Interestingly, the *ndhB* gene encoding a subunit of the chloroplast NAD(P)H dehydrogenase complex is not an essential gene in chloroplasts, but seems to play a functional role under certain stress conditions (36–40). A similar sequence to Ntc-2 was detected recently in a cDNA library encoding small RNAs (sized between 20 and 30 nt) from *N.tabacum* generated from total cellular RNA (41). In this case, the ncRNA was found to be 2 nt longer at the 3′ end. However, among the 20 cDNA clones from our library, only 3 contained this 2 nt extension while the remaining 18 clones were 22 nt in size.

Homologs to Ntc-2 can been found in the chloroplast genomes from >1000 different plant species indicating that the Ntc-2 sequence is evolutionarily highly conserved. Since Ntc-2 overlaps with the open reading frame of the *ndhB* gene, two scenarios can be envisioned, how this RNA is generated: (i) alternative transcription producing distinct transcripts for Ntc-2 and *ndhB* gene, respectively, or (ii) alternative processing of a primary transcript spanning Ntc-2 and *ndhB* RNAs.

Ntc-3 RNA

Next abundant in our library, represented by 18 independently identified cDNA clones, is Ntc-3, a 44 nt long ncRNA candidate mapping to the intergenic region between two ribosomal RNA genes, the 4.5S rRNA and the 5S rRNA (Table 1 and Figure 1B). The ncRNA candidate can be detected as a distinct band in a northern blot analysis pointing to a rather high expression level (Figure 2). A homolog of Ntc-3 RNA has been identified previously in *Arabidopsis thaliana*, designated as Ath-243. Ntc-3 and Ath-243 share 70% sequence identity and can both be folded into a similar stable stem–loop structure by employing the M-fold program (for secondary structures of selected ncRNAs from chloroplasts see Supplementary Figure S2).

Interestingly, the *A.thaliana* homolog Ath-243, which exhibits a similar size as RNA (as assessed by northern blotting), was shown to be expressed tissue-specifically and detected at significant levels only in roots (42). Ntc-3, however, was identified in a cDNA library produced from leaf chloroplasts suggesting that, at least in tobacco, the occurrence of this small RNA is not strictly confined to roots.

Ntc-4 RNA

As for Ntc-3, Ntc-4 also maps to an intergenic region of the chloroplast genome, flanked by two known ncRNA genes, 16S rRNA and $tRNA^{Ile}$ (Table 1 and Figure 1B). We have isolated 18 independent cDNA clones from our library that contained this RNA, all of them exhibiting a size of 40 nt. As for Ntc-12 and Ntc-3, Ntc-4 folds into a very stable stem–loop structure (Supplementary Figure S2). Expression of Ntc-4 can be verified by northern blot analysis resulting in a signal of the expected size (i.e. at ~40 nt) as well as two larger bands, which might represent precursors of Ntc-4 RNA (Figure 2).

Ntc-5 RNA

Ntc-5 is represented by four independent cDNA clones. The sequence is located immediately adjacent to the $tRNA^{Met}$ gene with no spacer region present between the two ncRNAs (Table 1 and Figure 1B). Previously, it has been shown that a novel endonuclease, termed tRNase Z, is able to process the mature 3′ ends of tRNAs (43). 5′-Processing of Ntc-5 as well as 3′-processing of $tRNA^{Met}$ thus might be simultaneously exerted by the same tRNase Z enzyme. In fact, by an *in vitro* assay, employing recombinant tRNase Z, we could show that Ntc-5 is processed by tRNase Z from a longer precursor RNA including $tRNA^{Met}$ (A. Hüttenhofer and A. Marchfelder, unpublished data).

Ntc-5 can fold into a stable stem–loop structure (Supplementary Figure S2) and is transcribed in opposite orientation to the 3′-untranslated region (3′-UTR) of the *atpE* gene located on the complementary strand. A possible function of Ntc-5 could be the regulation of gene expression of the *atpE* gene, in analogy to miRNAs, which target 3′-UTRs in eukaryal mRNAs. Thereby, Ntc-5 RNA and the 3′-UTR of the atpE gene form stem–loop structures with complementary loop sequences, prerequisite for the formation of a so-called 'kissing complex' (Supplementary Figure S3). This spatial arrangement has been shown previously to be a hallmark of bonafide regulatory sense/antisense interactions in bacteria (44). However, so far no miRNA-like gene regulation mechanism has been described in chloroplasts which would resemble the one observed for cytoplasmatic mRNAs of eukaryotic cells (8).

Alternatively, Ntc-5 might function in analogy to an antisense RNA identified previously in bacteria, designated as *GadY*, which is transcribed in opposite orientation to the 3′-UTR of the *GadX* gene. It was shown that expression of GadY resulted in an increase in *GadX* mRNA levels and that this increase is dependent on the complementarity to the 3′-UTR of the *GadX* gene (45). Thus, Ntc-5 RNA might have a similar, positive effect on *aptE* expression. Since Ntc-5 is presumably co-transcribed with the $tRNA^{Met}$ gene (see above) it is tempting to speculate, that by that mechanism protein synthesis (by $tRNA^{Met}$ transcription) could be coupled to ATP synthesis (by *atpE* transcription) in chloroplasts.

Alternatively, Ntc-5 RNA might have a destabilizing effect on *atpE* mRNA, since in bacteria, antisense RNAs to 3′-UTRs of mRNAs have been reported, which trigger mRNA decay by an RNase III-dependent mechanism (46). Based on the structure of identified cleavage sites in chloroplast RNAs, the existence of an RNase III-like enzyme activity in chloroplasts has been suggested (25,47). However, no such enzyme has been unambiguously identified to date.

The expression of the Ntc-5 ncRNA could be verified by northern blot analysis and represents the strongest expressed ncRNA candidate from our screen (Figure 2).

Ntc-6 RNA

Two independent cDNA clones have been identified for the Ntc-6 RNA, which is located in the intron of the $tRNA^{Ala}$ gene and exhibits a size of 19 nt. A northern blot signal with a band at the expected size could not be observed. The tRNA intron can be folded in a hairpin precursor structure (somewhat reminiscent of a miRNA precursor, from which the Ntc-6 RNA could be processed; Supplementary Figure S4).

Ntc-7, Ntc-8, Ntc-9, Ntc-10 and Ntc-11 RNAs

From each of the ncRNAs candidates Ntc-7, Ntc-8, Ntc-9, Ntc-10 and Ntc-11, only a single cDNA clone was isolated. In addition, we could not verify their expression by northern blot analysis, pointing to a very low expression level, as compared with most of the ncRNA candidates discussed above. All of these low-abundant ncRNAs map to intergenic regions of the chloroplast genome (Table 1 and Figure 1B). Except for Ntc-10, they do not fold into extended stem–loop structures. Owing to their limited abundance (inferred from missing northern blot signals and single cDNA clone occurrence), these candidates might less likely represent functional ncRNAs in chloroplasts. However, we cannot rule out the possibility that at least some of them might still be functional despite their low expression level.

Nuclear-encoded ncRNAs from the chloroplast cDNA library

We also identified cDNA clones from the chloroplast library encoding RNA transcripts from the nuclear genome of *N.tabacum*. From these, 94 cDNA clones were assigned to various nuclear tRNAs, 38 cDNA clones to nuclear 5.8S rRNA and 25 cDNA clones represented 11 novel candidates for C/D box snoRNAs. (Supplementary Table S1).

To address the possibility that these nuclear-encoded ncRNAs are post-transcriptionally imported into the chloroplast compartment, northern blot analyses were conducted to compare signal intensities in purified chloroplast RNA versus total cellular RNA. However, for none of the selected ncRNAs of nuclear origin, including snoRNAs and nuclear-encoded tRNAs, did these experiments provide evidence for import into chloroplasts at significant levels (data not shown). However, we cannot exclude the possibility that these RNAs are imported into chloroplasts at low levels. To date, the import of RNAs into chloroplasts has not been directly demonstrated, although indirect evidence may suggest that at least tRNAs can be imported into chloroplasts (48–50).

Nonetheless, in the absence of expression data demonstrating enrichment of nuclear-encoded ncRNAs inside chloroplasts, we tentatively explain the presence of these sequences in our chloroplast library as RNA contaminations, which might stick to the outer membrane of chloroplasts during organelle purification and thus were co-isolated with the endogenous chloroplast RNA.

We have considered pre-treatment of isolated chloroplasts with RNases as a possibility to eliminate cytosolic RNAs associated with the outer membrane of the chloroplast. However, nuclease treatment of chloroplasts is known to be problematic and often results in complete degradation of the chloroplast nucleic acids (51). This is consistent with earlier findings that isolated chloroplasts are not impermeable to exogenously added enzymes, including restriction endonucleases (52).

cDNA libary containing ncRNAs from mouse mitochondria

Library construction and sequence analysis of cDNA clones. A mouse cDNA library encoding mitochondrial ncRNAs was generated as described for the chloroplast library (see above Materials and Methods). About 1700 cDNA clones were analyzed from this library. Thereby, the majority of clones were identified as mitochondrial-encoded rRNAs (i.e. 16S or 12S rRNAs) or mitochondrial tRNAs (Figure 3A). As for the chloroplast library, we could identify the full set of known mitochondrial ncRNAs, e.g. mitochondrial tRNAs and ribosomal RNAs. Only 1.4% of the cDNA clones are derived from mitochondrial mRNA fragments which is consistent with an extremely low abundance of mRNA degradation intermediates in the RNA population. About 1% of the total cDNA clones could be assigned as putative novel ncRNA candidates, since they did not contain any sequence or structural motifs which allowed classification as any of the known mitochondrial ncRNAs (Table 2). In addition, 6.6% of cDNA clones were nuclear-encoded RNAs, such as rRNAs, mRNA fragments as well as miRNAs (see below).

Novel candidates for ncRNAs encoded by the mitochondrial genome

Mt-1, Mt-2, Mt-3 and Mt-4 RNAs. We cloned several RNA species derived from the D-loop region of the mitochondrial genome. The mitochondrial D-loop region contains the major transcription initiation sites of the genome as well as the origin of heavy strand (H-strand) DNA replication, (53,54). Synthesis of an RNA primer required for DNA replication of the H-strand and transcription of the entire L-strand polycistronic transcript both originate at the same initiation site on the L-strand (Figure 3B) (55). Before replication, the RNA primer for H-strand replication is thought to undergo processing by cleavage with RNase MRP (Figure 3B).

We have identified altogether 15 cDNA clones that encode the ncRNA candidate Mt-1 (Table 2 and Figure 3B). The Mt-1 RNA exhibits an identical 5′ end to the predicted RNA primer for DNA replication. The 3′ end of the Mt-1 RNA is heterogeneous by 4 nt and coincides with the CBS III motif, a conserved sequence motif that, together with CBS I and II, is found in mitochondrial D-loop regions

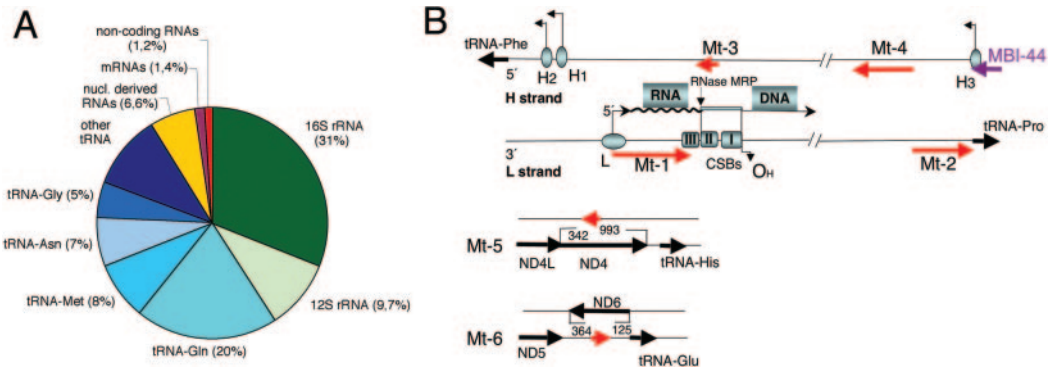

Figure 3. Sequence analysis and genomic location of ncRNA candidates from the mitochondrial library of *M.musculus*. (**A**) Sequence analysis of 1700 cDNA clones from the mitochondrial library. cDNA clones representing different RNA species or categories are shown as percent of total clones. The red sector denotes candidates for novel ncRNAs in mitochondria. (**B**) Location of ncRNA candidates on the mitochondrial genome, not drawn to scale. Respective novel candidates for ncRNA genes are indicated by red arrows, genes flanking the ncRNA candidate are indicated by black arrows. Upper panel: Mitochondrial D-loop region involved in genome replication: Locations of Mt-1, Mt-2, Mt-3 and Mt-4 RNAs, respectively, are indicated by red arrows, the location of MBI-44 (see text) is shown by a purple arrow. Conserved sequence boxes CSB I, II and III, which are characteristic of mitochondrial origins of replication are also shown. L, light-strand transcription initiation site; H1, H2 and H3 represent three heavy-strand transcription initiation sites, respectively. O_H indicates the origin of replication of the H-strand. RNase MRP, the arrow points to potential RNase MRP cleavage site involved in RNA primer processing. Lower panel: Location of Mt-5, Mt-6 ncRNAs on the mitochondrial genome. Distance of RNAs to 5′ or 3′ ends of the reading frames of genes ND-4 and ND-6, respectively, (which are located on the opposite strand) is indicated in nt.

Table 2. Candidates for novel ncRNAs from a *M.musculus* mitochondrial cDNA library derived from RNAs sized 10–500 nt

Name	Nr.	Sequence	cDNA (nt)	Remarks
Mt-1	15	GAATTGATCAGGACATAGGGTTTGATAG TTAATATTATATGTCTTTCAAGTTCTTAG TGTTTTTGGGG (A)4–37	68	Maps to D-loop L-strand encoded, Four sequences are partly polyadenylated
Mt-2	1	ATAGTTTAATGTACGATATACATAAATG TACTGTTGTACTATGTAAATTTATGTACT	57	Maps to D-loop L-strand encoded
Mt-3	1	CACCCCCTCCTCTTAATGCCAAA	23	Maps to D-loop H-strand encoded
Mt-4	1	CATTTGGTCTATTAATCTACCATCCTCCG TGAAACCAACAACCCGCCCACCAATG	55	Maps to D-loop H-strand encoded
Mt-5	1	TTGGGATTAAGGTTGCTTCAAATAAAAT ATAAAATATAATTAG	43	Antisense to ND-4 mRNA
Mt-6	1	CAACATCGTCAACCTCATATATCAA TCAAT	30	Antisense to ND-6 mRNA three mismatches to mitochondrial sequence, two mismatches to a nuclear-encoded pseudogene

Nr., number of independent cDNA clones identified from each RNA species; Sequence, sequence of cDNA; cDNA (nt), length of cDNA encoding a ncRNA candidate as assessed by sequencing.

(56). The Mt-1 RNA sequences, however, terminate shortly before the putative cleavage site by RNase MRP within CBS III. Thus it seems feasible that premature transcription termination of the RNA primer for DNA replication, which in turn prevents cleavage by RNase MRP regulates replication of the mitochondrial genome. This would be consistent with the previous hypothesis that regulation of replication of the mitochondrial genome is exerted at the level of RNA priming, based on the observation that the rate of transcription initiation at the D-loop exceeds that of mitochondrial DNA replication (57).

Remarkably, 4 out of the 15 Mt-1 sequences identified in our library were polyadenylated with polyadenylation ranging from 4 to 37 adenine residues. It has been demonstrated recently that polyadenylation in animal mitochondria serves a dual role in that it can either produce stable mRNAs or mark transcripts for rapid degradation (58). Thus, whether Mt-1 is stabilized or destabilized by poly(A) tail addition remains to be determined.

A second RNA species from the D-loop region is represented by a single cDNA clone, designated as Mt-2 RNA (Table 2 and Figure 3B). The 3′ end of Mt-2 RNA is spaced by 1 nt to the 5′ end of $tRNA^{Pro}$ and thus might reflect a processing product of mitochondrial RNase P cleaving the $tRNA^{Pro}$ precursor.

An interesting aspect of Mt-3, a short, 23 nt long RNA from the mitochondrial D-loop, is that it is encoded by the H-strand and transcribed in antisense orientation to the RNA primer for DNA replication and spans the predicted RNase MRP cleavage site (Table 2 and Figure 3B) (59). Potentially, the Mt-3 RNA could regulate the activity of the catalytic RNA component of RNase MRP by an antisense mechanism and thereby influence the rate of transcription and/or replication of the H-strand. Lastly, the RNA Mt-4 with a size of 55 nt also locates to the D-loop region of the mitochondrial genome and is presumably transcribed from the H3 promotor of the H-strand (Figure 3B).

In a cDNA library generated from total cellular RNA of mouse brain, we have identified previously a clone

(MBI-44) that maps close to the mitochondrial D-loop and exhibits a size of 97 nt (35). The Mt-1, Mt-2, Mt-3 and Mt-4 RNAs, however, map to a different region than MBI-44, with the latter RNA species being in antisense orientation to $tRNA^{Pro}$ (Figure 3B).

Mt-5 and Mt-6 RNAs

We also have identified two antisense RNA species transcribed in opposite orientation to two different mitochondrial mRNAs encoding NADH dehydrogenase subunits. While Mt-5 RNA is transcribed in antisense orientation to the NADH dehydrogenase subunit 4 gene (ND-4), Mt-6 RNA is transcribed in antisense orientation to the NADH dehydrogenase subunit 6 gene (ND-6; Table 2 and Figure 3B). Mt-5 and Mt-6 cDNA clones display sizes of 43 and 30 nt, respectively. Interestingly, the Mt-6 RNA exhibits three mismatches to the published mitochondrial genome sequence (60), which might be due to polymerization errors of the reverse transcriptase in the process of library construction and/or sequencing errors. Remarkably, the Mt-6 RNA exhibits only two mismatches to a nuclear-encoded pseudogene of ND-6. The Mt-5 and Mt-6 ncRNA species could potentially be involved in regulation of gene expression of the ND-4 and ND-6 genes by an antisense mechanism, as observed previously for numerous bacterial antisense RNAs (44). This would be compatible with the eubacterial origin of mitochondria and the evolutionary conservation of the mechanisms regulating gene expression in both systems.

For none of the above ncRNA candidates from mouse mitochondria, we were able to detect a clear and unambiguous northern blot signal, presumably pointing to their low accumulation level. This is consistent with the notion that from all but one ncRNA (Mt-1), only a single cDNA clone has been isolated. The low abundance of ncRNA candidates in mouse mitochondria is not necessarily indicative of their lack of functional significance. It is well established that ncRNAs can be functional even at very low levels, as observed for some miRNAs which also cannot be detected by northern blot analysis (8,9).

Nuclear-encoded ncRNAs identified in a cDNA libary from mitochondria

As observed for the chloroplast cDNA library, we also found clones derived from the nuclear genome (112 sequenced cDNAs; Figure 3A). Among them, about half of the clones (i.e. 2.5% of all cDNA clones sequenced) represent 5.8S rRNA sequences (with a size of 160 nt) while about another half encodes RNA fragments of the 28S rRNA (varying in size between 25 and 132 nt). The remaining sequences represent clones of 5S rRNA and fragments of the 18S rRNA. In addition, we identified 12 cDNA clones representing nuclear-encoded mRNA fragments as well as four nuclear-encoded miRNAs, namely let7f-1, let-7g, miR-122a,b and miR-101b (8). Only one cDNA clone was identified from each miRNA species.

The predominant occurrence of 5.8S rRNA clones in our library, but not of the similar sized and equally abundant 5S rRNA, could hint towards a mitochondrial import of this RNA species, as observed for 5S rRNA in human mitochondria (61). However, in a previous study on ncRNAs from mouse, analyzing total cellular ncRNAs (35), we noted a preferential occurence of 5.8S rRNA cDNA clones compared to 5S rRNA clones in our library (ratio ~4:1); a similar ratio was observed in the mitochondrial library indicative that the predominant occurrence of 5.8S rRNA might be due to preferential cloning of this RNA species compared to 5S rRNA.

Interestingly, we could not detect two nuclear-encoded RNAs, MRP RNA and RNase P, in our library, which have been predicted to be imported. However, the import of these two RNA species remains still to be proven and their presence within mitochondria, especially of MRP RNA, is still a subject of debate. Thus, the lack of clones from these species in our mitochondrial library, while identifying all other mitochondrial-encoded ncRNAs (see above), might shed some new light on this debate.

CONCLUSION

By an experimental RNomics approach, we have investigated the small transcriptome representing RNAs, sized 20–500 nt, from the two DNA-containing cell organelles, mitochondria (from *M.musculus*) and chloroplasts (from *N.tabacum*). Although mouse mitochondria exhibit a small-sized genome of 16.6 kb, chloroplast from *N.tabacum* exhibit a considerably larger genome size of 156 kb (62,63). For eukaryal nuclear genomes, it has been speculated previously that a considerable portion of the genome (up to 50%) might code for novel regulatory RNA transcripts, amounting to many thousands of regulatory ncRNAs in the nucleus or cytoplasm (3,5,6). In our analysis of the small transcriptome of the two cell organelles we do not find evidence for a similarly large number of small ncRNA candidates within these cellular compartments. It might be argued that a considerable number of ncRNAs might have escaped detection due to the experimental strategy used in our screen; e.g. some RNAs might not be reverse transcribed into cDNAs because of their structure and/or modification and thus could not be identified. Although we cannot completely exclude this possibility, we would like to point out that in both libraries we could detect the full set of all known organellar ncRNAs, including mitochondrial tRNAs that are modified as well as highly structured. Thus, in our RNomics screen we are confident to have detected the majority of small stable RNA transcripts in cell organelles ranging from 20 to 500 nt.

In the chloroplast library from the plant *N.tabacum*, we have identified 12 candidates for ncRNAs. For six of these ncRNAs, we could confirm expression by northern blot analyses while the remaining five appear to be expressed at low levels. Unlike animal mitochondrial genomes, chloroplast genomes possess many promoters of widely different strength and, even for one and the same gene or operon, often multiple transcription initiation sites are found (64–67). Another level of complexity is added by the presence of two different types of RNA polymerases in chloroplasts: a chloroplast-encoded eubacterial-type RNA polymerase depending on sigma factors and a nuclear-encoded bacteriophage-type RNA polymerase (68,69). The presence of a large number of promoters and alternative transcription initiation sites as well as the interplay of the two different RNA polymerase

activity result in a highly complex transcript pattern in chloroplasts with different transcripts displaying widely different abundances and stabilities (70–72). Thus, the strong differences observed in the accumulation levels of the identified candidate ncRNAs are unsurprising.

The majority of ncRNAs from the chloroplast genome map to intergenic regions (nine ncRNAs), two to intronic sequences and one is transcribed in antisense orientation to the 3′-UTR of a chloroplast gene (*atpE*). Most tobacco ncRNA candidates exhibit sequence homology to other chloroplast genomes including the genomes from rather distantly related species which may hint to a conserved function. However, at present we cannot exclude the possibility that some of the identified RNA species are by-products of chloroplast RNA processing, which may or may not be functionally relevant.

From the mouse mitochondrial library, six ncRNA candidates could be identified. Four of these map to the mitochondrial D-loop region involved in genome replication, while two others are transcribed in antisense orientation to known mitochondrial mRNAs (ND-4 and ND-6, respectively). For none of the candidates, expression could be confirmed by northern blot analysis probably pointing to their low expression levels. Since transcription for both strands of the mitochondrial genome starts from the D-loop region and results in the production of large polycistronic transcripts, the identified ncRNA candidates very likely reflect processing products of these primary polycistronic transcripts. The strong bias of expressed RNA sequences towards the mitochondrial D-loop region may reflect an increased transcriptional activity in this region of the genome.

The larger genome size of chloroplasts compared to animal mitochondria may in part account for the larger number of novel ncRNA candidates found in this organelle. Nonetheless, the generally rather low total number of novel ncRNA candidates in cell organelles seems a bit surprising, considering their eubacterial descent. In bacteria, regulatory ncRNAs are widespread. For example, to date, well over 60 regulatory ncRNA have been identified in *E.coli* (4,73). Bacteria have to react fast to steadily changing environments and this might require numerous ncRNAs as fast genetic switches which do not have to be translated into proteins before exerting their functions.

This is demonstrated by independent studies which identified numerous ncRNAs in various bacterial genomes (4,74,75) as well as a cyanobacterial genome (76), which is commonly considered as an ancestor to chloroplasts and their genomes. Owing to the evolutionary distance between bacteria, cyanobacteria on the one side and chloroplasts and mitochondria on the other, no sequence homologs could be identified between those species. The general location of ncRNA genes in cell organelles is thereby similar to those found in bacterial species, which are mainly found to be intergenic with the exception of a considerable number of antisense RNAs transcribed *in cis* from the opposite strand of protein coding or ncRNA genes (75).

Although one could argue that the environment of cell organelles within eukaryotic cells might be more stable and thus the demand for regulatory ncRNAs might be lower—compared to bacteria and cyanobacteria; the main functions of chloroplasts and mitochondria also require very fast adaptation responses: Most chloroplast-encoded genes are involved in photosynthesis, a key bioenergetic pathway that, when not adjusted properly to changing light conditions, can result in massive photooxidative stress and the concomitant release of highly cytotoxic free radicals and reactive oxygen species (77,78). Similar considerations hold true for mitochondria with most mitochondrial gene products functioning in the respiratory electron transport chain. Thus, at least in theory, post-transcriptional regulation via ncRNAs would provide a fast and efficient mechanism for the rapid adjustment of organellar gene expression to changing environmental conditions and/or metabolic demands of the cell.

At present, our study provides no experimental evidence for a pathway that would promote import of nuclear-encoded RNAs into cell organelles to a significant extent. Most of the nuclear-derived RNAs found in our cDNA libraries from chloroplast and mitochondria were highly abundant RNAs, such as rRNAs or snoRNAs, and moreover, were not enriched in purified organellar RNAs compared to total cellular RNA preparations. Thus, these RNA species might represent nuclear contaminations, which were co-isolated and cloned during library construction. We cannot exclude, however, that at least some of the identified nuclear-encoded ncRNAs are imported into mitochondria or chloroplasts at low levels.

In summary, we present here the first comprehensive analysis of the small RNA component of the transcriptome from mitochondria and chloroplasts. We could identify a total of 18 novel candidates for ncRNAs in these cellular compartments. Functional studies on these organellar ncRNA candidate genes will be required to assign biological functions to the presumptive novel ncRNA species.

Unfortunately, the production of mutants or the targeted inactivation of ncRNA genes in chloroplast and mitochondria is far from being trivial. No transgenic technologies suitable to generate gene knockouts are currently available for animal mitochondria making it difficult to directly test the identified ncRNA candidates for their biological functions. In contrast, recent progress with the genetic transformation of chloroplasts has facilitated reverse genetics approaches in chloroplast genomes (79–82). Although the procedures involved in the genetic transformation of higher plant chloroplasts are demanding and time consuming, the targeted knockout of the candidate ncRNA genes identified here certainly represents the most promising approach towards determining ncRNA functions in chloroplasts.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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