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Polyadenylation Occurs at Multiple Sites in Maize Mitochondrial *cox2* mRNA and Is Independent of Editing Status

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Polyadenylation of nucleus-encoded transcripts has a well-defined role in gene expression. The extent and function of polyadenylation in organelles and prokaryotic systems, however, are less well documented. Recent reports of polyadenylation-mediated RNA destabilization in *Escherichia coli* and in vascular plant chloroplasts prompted us to look for polyadenylation in plant mitochondria. Here, we report the use of reverse transcription–polymerase chain reaction to map multiple polyadenylate addition sites in maize mitochondrial *cox2* transcripts. The lack of sequence conservation surrounding these sites suggests that polyadenylation may occur at many 3′ termini created by endoribonucleolytic and/or exoribonucleolytic activities, including those activities involved in 3′ end maturation. Endogenous transcripts could be efficiently polyadenylated in vitro by using maize mitochondrial lysates with an activity that added AMP more efficiently than GMP. Polyadenylated substrates were tested for stability in maize mitochondrial S100 extracts, and we found that, compared with nonpolyadenylated RNAs, the polyadenylated substrates were less stable. Taken together with the low abundance of polyadenylated RNAs in maize mitochondria, our results are consistent with a degradation-related process. The fact that polyadenylation does not dramatically destabilize plant mitochondrial transcripts, at least in vitro, is in agreement with results obtained for animal mitochondria but differs from those obtained for chloroplasts and *E. coli*. Because fully edited, partially edited, and unedited transcripts were found among the cloned polyadenylated *cox2* cDNAs, we conclude that RNA editing and polyadenylation are independent processes in maize mitochondria.

INTRODUCTION

Present-day mitochondria have almost certainly evolved from a prokaryotic endosymbiont (reviewed in Gray, 1992). These organelles possess their own genomes and gene expression machinery; however, during evolution, most of the genetic information of the mitochondrial ancestor was transferred to the nuclear genome. Mitochondrial genes in *Saccharomyces cerevisiae* and metazoans are transcribed by a nucleus-encoded T7-like RNA polymerase and accessory factors (reviewed in Tracy and Stern, 1995), and candidate plant nuclear genes encoding mitochondrial RNA polymerase have been identified (Cermakian et al., 1996; Hedtke et al., 1997; Young et al., 1998; Chang et al., 1999). In plants, promoter strength may play a regulatory role in gene expression (Mulligan et al., 1991), but post-transcriptional regulation also can occur by differential RNA stability (Finnegan and Brown, 1990).

The maize mitochondrial genome is typical of those found in vascular plants. It can be genetically mapped as a single circular molecule of 570 kb, with multiple repeated sequences giving rise to a variety of stably inherited subgenomic recombination products (Lonsdale et al., 1984). As in other species, maize mitochondrial primary transcripts are subject to both *cis*-splicing (e.g., Fox and Leaver, 1981) and *trans*-splicing (Pereira de Souza et al., 1991); in many cases, RNA editing, usually by conversion of cytosine to uridine, occurs widely and is often required to form an initiation or stop codon or to create a functional protein (reviewed in Maier et al., 1996).

3′ end formation and regulation of mRNA stability are poorly understood in plant mitochondria. Recent studies have shown that as in chloroplasts, 3′ untranslated region (UTR) sequences do not serve as transcription terminators but instead direct 3′ end maturation (Dombrowski et al., 1997). The role of 3′ UTR sequences and structures in RNA stability has been demonstrated in the cases of rice *cob*, in which two genes differing only in their 3′ UTRs have widely different stabilities (Kaleikau et al., 1992), and rapeseed cytoplasmic male sterility locus open reading frame 138, in which similar effects have been documented both in vivo and in vitro (Bellaoui et al., 1997).

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In this article, we report evidence for extensive polyadenylation of maize mitochondrial *cox2* transcripts. Although polyadenylation of nuclear and some bacterial and metazoan mitochondrial transcripts is well known (Gelfand and Attardi, 1981; Ojala et al., 1981; Wahle and Keller, 1992; Sarkar, 1997), data demonstrating polyadenylation of plant mitochondrial mRNAs had not been reported to our knowledge. The recent rediscovery of polyadenylation in higher plant chloroplasts and its role in RNA degradation (Kudla et al., 1996; Lisitsky et al., 1996; reviewed in Hayes et al., 1999) prompted us to look for polyadenylation in plant mitochondria. We report that polyadenylation occurs at many sites in the 3′ region of maize *cox2* mRNA, apparently at 3′ termini created by endonucleolytic and/or exonucleolytic cleavage events, including 3′ end maturation. Although polyadenylation appears not to dramatically destabilize plant mitochondrial transcripts in vitro, the low abundance of these transcripts is consistent with their association with an RNA degradation pathway. Our results emphasize that polyadenylation is a widespread process in the DNA-containing organelles of eukaryotic cells; however, its metabolic role may vary.

RESULTS

Detection of Polyadenylated mRNA in Maize Mitochondria and Identification of Poly(A) Addition Sites

We selected *cox2* for our initial studies of polyadenylation. *cox2* encodes subunit II of the cytochrome oxidase complex and was the first plant mitochondrial gene sequenced (Fox and Leaver, 1981). To look for polyadenylated *cox2* transcripts, we reverse transcribed RNA isolated from purified maize mitochondria (mtRNA) by using an oligo$(dT)_{17}$ adapter oligonucleotide, and the resulting cDNAs were polymerase chain reaction (PCR) amplified with an adapter primer and a second primer located within the *cox2* coding region (primer ZMC22; Figure 1A). Two major size classes of PCR products were purified from an agarose gel and cloned into a plasmid vector. These products were not observed using the oligo(dT) primer alone, in the absence of reverse transcriptase, or when RNA preparations were treated with oligo(dT) and RNase H, which should specifically degrade the poly(A) tails of mRNAs (Figure 1B). Positive cDNAs were detected by colony hybridization using the SnaBI-AvaI fragment of the *cox2* 3′ region (see Figure 1A), and 60 positive transformants were selected for further analysis. Most of the positive clones fell into two classes containing ~400- or 700-bp inserts.

Both strands of each positive clone were sequenced, and as expected, each contained a 3′ polyadenosine tract of variable length. A representative sequencing gel of two clones having the two most represented poly(A) sites is shown in Figure 1C. Like the vast majority of positive clones, the poly(A) tract was homopolymeric (see below). Figure 2A shows the locations of the 23 poly(A) sites identified within the *cox2* coding region and 3′ UTR. Of the 60 clones sequenced, half were polyadenylated at apparently random sites between 138 nucleotides upstream and 377 nucleotides downstream of the stop codon. The two most heavily represented sites are indicated by arrows and are located 41 (site 7) and 363 (site 20) nucleotides downstream of the stop codon, respectively, and they accounted for the remaining clones. The 322 bp between these sites correspond well to the difference between the sizes of the two major reverse transcription (RT)–PCR products shown in Figure 1B. Site 7 was found 13 times, and site 20 was found 16 times. All other sites were found once, with the exception of the sites indicated by a double-length boldface line, which were found two (sites 8 and 22) or three (sites 11, 19, and 21) times.

We examined the sequences found upstream and downstream of each polyadenylation site and the number of clones having each site. No consensus sequence was identified within the 40 nucleotides surrounding the polyadenylation sites; however, in 20 clones (33%), adenosines were found at the junction between the upstream and downstream sequences, making it impossible to determine precisely where polyadenylation occurred. There was also no obvious similarity either in sequence or in base composition between the two most heavily used sites, 7 and 20 (see also Figure 3B). This suggested that the frequency of polyadenylated RNAs might depend on the relative levels of *cox2* mRNAs having the respective 3′ termini (see below).

Figure 2B shows the nucleotide composition of the tails sequenced and how many clones had each tail type. Because the oligo(dT) primer can anneal anywhere within a poly(A) tail, these results are likely to underestimate the length of poly(A) tails, which for a few clones were apparently less than the length of the oligo(dT) primer (17 nucleotides). The randomly selected clones may also not reflect the composition of tails in vivo. Of the clones sequenced, 49 (80%) were homopolymeric for adenosines and ranged in size from 14 to 36 nucleotides, with the majority having 16 to 20 adenosines. Three clones had a single guanosine embedded in their tails, one had two guanosines separated by an adenosine, three had a single uridine at or near their 3′ ends, and several had interspersed uridines or cytosines. Overall, the average tail length was 22 nucleotides, with a range of 14 to 52 nucleotides. Taken together, these results appear to reflect an ability of the polyadenylation machinery to utilize inefficiently any of the other three nucleotides.

A Major Poly(A) Addition Site Corresponds to the Mature 3′ End of the *cox2* Transcript

The preponderance of clones with poly(A) added at either site 7 or site 20 raised the possibility that one or both of these sites might correspond to abundant termini of *cox2*

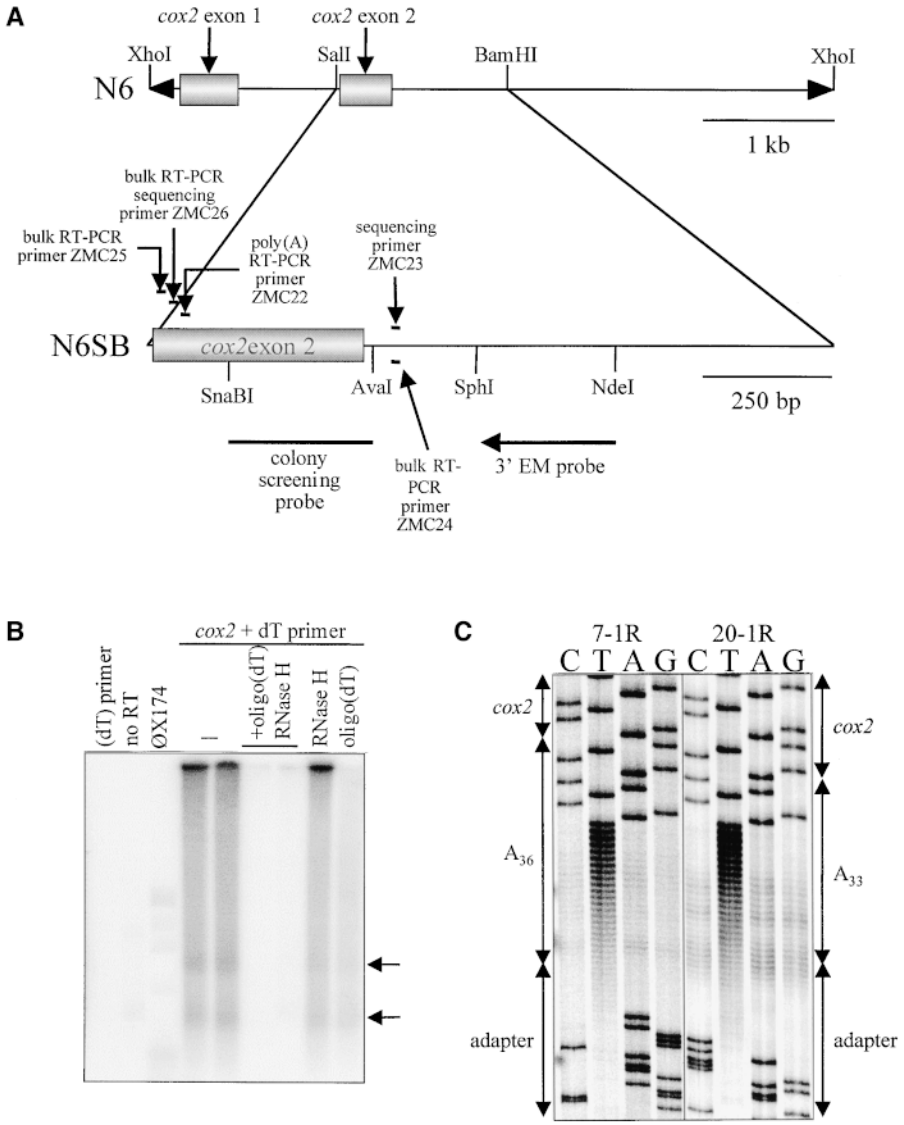

Figure 1. Isolation of cDNAs Corresponding to Polyadenylated *cox2* mRNAs.

(A) The *cox2* exon 2 and downstream region of the maize mitochondrial genome. Shown is a map of the clones, relevant restriction sites, primers (indicated by dashes), and probes (the direction of the 3′ end mapping [3′ EM] probe is indicated by an arrow) used in the experiments (see Methods). The arrow labeled N6 represents the DNA insert in the N6 clone. The other arrows connect labels with the corresponding objects.

(B) Filter hybridization analysis of RT-PCR products. Simultaneous reactions were performed with the oligo(dT) (dT) primer alone or with the oligo(dT) primer as well as the specific *cox2* primer, one of which lacked reverse transcriptase (no RT). In some cases, the mtRNA was pretreated with various combinations of oligo(dT) and RNase H; the dash column indicates reactions in which neither was added. The products were separated in an agarose gel, which was blotted and hybridized with the colony screening probe shown in **(A)**. The arrows indicate the two major size classes of PCR products obtained, and the markers were ϕX174 DNA digested with HinfI.

(C) Dideoxy sequencing reactions of two clones having the two most-represented polyadenylation sites, with A_{36} and A_{33} giving the deduced lengths of the poly(A) tails. Clones 7-1R and 20-1R were sequenced using the M13 reverse primer.

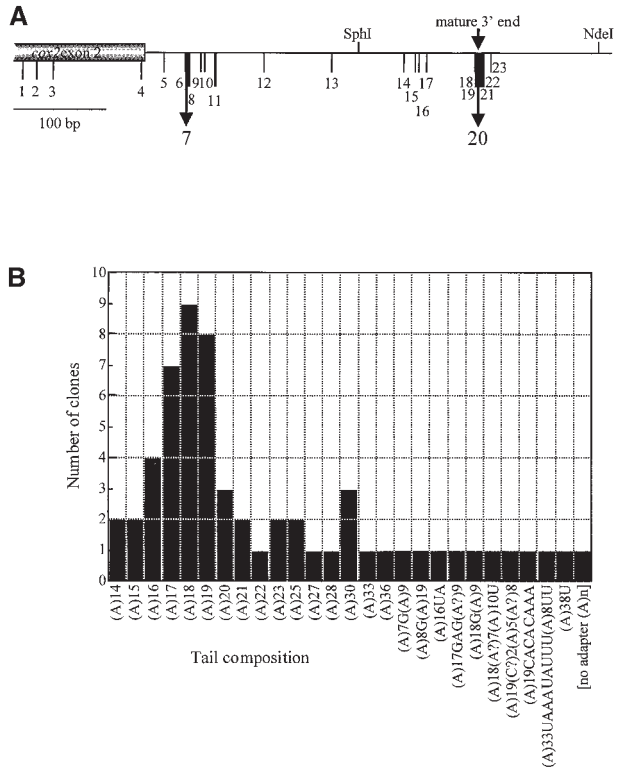

Figure 2. Polyadenylation of *cox2* Transcripts.

(A) Map showing the location and relative frequency of the 23 polyadenylation sites for *cox2* found in oligo(dT)-primed cDNAs. Exon 2 of *cox2* is indicated by a shaded box. The mature 3′ end is indicated and coincides with the region including sites 18 to 22. The sites fell into three categories: those with one representative clone, shown by a numbered vertical line; those with two or three representative clones, shown by a numbered boldface vertical line; and those with 13 to 16 representative clones, shown by a numbered boldface line ending in an arrow.

(B) A histogram showing the tail nucleotide sequences found and their frequencies. Question marks indicate the presence of ambiguous sequences. One clone lacked the adapter and polyadenosine sequences altogether.

mRNA. To map the mature 3′ ends of the *cox2* transcript, we performed S1 nuclease protection assays. The SphI-NdeI fragment shown in Figure 1A was labeled on the antisense strand and hybridized with mtRNA, or with tRNA as a control. The resulting DNA–RNA hybrids were subjected to S1 nuclease digestion and sized relative to a DNA sequence ladder and other size markers. Figure 3A shows that the major products were 139 to 144 nucleotides long, corresponding to positions 360 to 365 nucleotides downstream of the stop codon, with the predominant length being 142 nucleotides. Poly(A) addition sites 18 to 22 lie within this six-nucleotide region, accounting for polyadenylation sites in 25 (42%) of the 60 clones (Figure 3B). Thus, it appears that mature *cox2* mRNA is a common substrate for poly(A) addition. In contrast, S1 protection revealed only a minor protected product corresponding to a 3′ end at or near site 7 (D.S. Lupold and D.B. Stern, unpublished data; see Figure 3B for the sequence surrounding site 7). This suggests that polyadenylation may occur at site 7 as part of an RNA degradation pathway or that RNA processed at site 7 might be stabilized by polyadenylation.

A Maize Mitochondrial Protein Extract Possesses Polyadenylation Activity

To detect presumed poly(A) addition activity in maize mitochondria, we isolated a total soluble fraction from purified, intact mitochondria that were lysed in the presence of 0.5% Triton X-100 and 1 M KCl. Because endogenous RNA had not been removed from these lysates, we performed an experiment to see whether poly(A) addition activity could utilize these natural substrates. To do this, we incubated the lysates in the presence of 0.5 mM unlabeled ATP as well as a small amount of α-^{32}P-ATP. As shown in lanes 2 to 6 of Figure 4, a time-dependent incorporation of label was observed, yielding an increasing amount of a high molecular weight heterodisperse product as well as a discrete product of $\sim$75 nucleotides. The discrete product almost certainly corresponds to tRNA, with ATP added by a tRNA nucleotidyltransferase activity; such activities are known to be present in plant mitochondrial extracts (Hanic-Joyce and Gray, 1990). Another discrete product of $\sim$120 nucleotides may correspond to the 5S ribosomal RNA (Stern et al., 1982). To verify that the heterodisperse products consisted of RNA, we treated aliquots with RNase A or DNase I and found them to be RNase sensitive but DNase I resistant (lanes 7 and 8, respectively).

Because cDNA sequences had shown low amounts of non-adenosine nucleotides in the post-transcriptionally added tails, the mitochondrial lysates were tested for their abilities to add GMP. Lanes 9 to 12 of Figure 4 show that when 0.5 mM GTP and a small amount of ^{32}P-GTP were used, there was little incorporation. Quantification with a PhosphorImager suggested that the amount incorporated was $\sim$0.1% of the ATP/ATP level. However, when ^{32}P-GTP was provided in the presence of 0.5 mM ATP, incorporation was observed at $\sim$1% of the ATP/ATP level (lanes 12 to 14). The labeled material was sensitive to RNase T1 (Figure 4, lane 15), indicating that GMP was in fact incorporated into RNA rather than being converted into another nucleotide. Taken together, the results shown in Figure 4 strongly suggest that plant mitochondria contain a poly(A) polymerase activity that is responsible for generating the *cox2* transcripts that were amplified and sequenced. Whereas poly(A) polymerase activity is also found in plant nuclei and chloroplasts, the purification procedure used for maize mitochondria should result in undetectable levels of contamination (Moore and

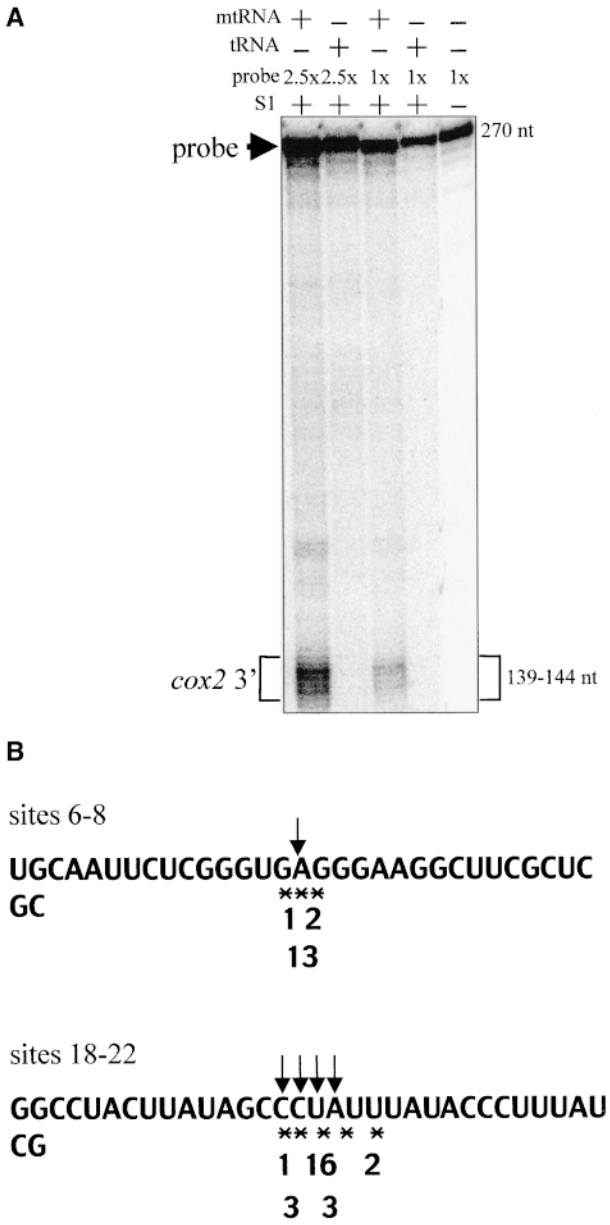

Figure 3. Major 3′ Ends and Polyadenylation Sites in the *cox2* 3′ UTR.

(A) Determination of the mature 3′ end of *cox2* transcripts by S1 nuclease protection. The probe used was the 3′ end–labeled SphI-NdeI restriction fragment of N6SB (see Figure 1A). The amount of probe used (1× or 2.5×) and the addition (+) or not (−) of mtRNA, yeast tRNA, and/or S1 nuclease are indicated. Size markers were HinfI-digested ϕX174 and an unrelated sequence ladder (data not shown). The protected fragments map from 360 to 365 nucleotides (nt) downstream of the stop codon. The self-annealed double-stranded probe is 270 bp.

(B) Sequences surrounding sites 7 and 20 in the *cox2* 3′ UTR. The heterogeneous 3′ ends are marked by arrows above the sequence, and poly(A) addition sites are marked by asterisks below the sequences, with the number indicating the number of cDNAs recovered for each site.

Proudlove, 1983). In addition, we did not observe the rapid degradation of polyadenylated transcripts in these same protein extracts that would have been expected from plastid contamination (see below). Finally, the results shown in Figure 4 suggest that although ATP is the preferred substrate for this terminal transferase activity, GTP can be incorporated at a low rate. This finding is fully consistent with the cDNA sequences shown in Figure 2.

Effect of Polyadenylation on Transcript Degradation in Maize Mitochondrial Extracts

Because polyadenylation could have a positive, neutral, or negative effect on mRNA stability, experiments were performed to compare the degradation rates of in vitro–synthesized transcripts possessing or lacking poly(A) tails. Two different substrates were used for these experiments, as shown in Figure 5A, consisting of the mRNA-like strands and ending at site 7 or site 20, which were the most frequently encountered sites of poly(A) addition. Site 7 is relatively rare as a 3′ end in the steady state *cox2* mRNA population, whereas site 20 is abundant (Figure 3). Apart from three guanosines from the T7 promoter included in one of the PCR primers used to amplify the fragments used for RNA synthesis, the substrates contained only *cox2* sequences. To test the effect of poly(A) on transcript stability, we made a second version of each substrate in which 25 adenosines were added at the 3′ end. This is similar to the average number of adenosines found in the cDNAs we sequenced (Figure 2B).

Equal counts of the ^{32}P-labeled transcripts with or without poly(A) tails were incubated alone or mixed together in maize mitochondrial S100 extracts for varying lengths of time, and the products were analyzed by gel electrophoresis. Figure 5B shows results for site 7 RNA. The nonpolyadenylated transcript decayed slowly, with 84% remaining at the end of the 90-min incubation period. The polyadenylated site 7 RNA, however, decayed faster, with only 20% remaining after 90 min. When mixed together, 61% of the nonpolyadenylated transcript was detectable after 90 min, whereas 44% of the polyadenylated transcript remained. This pattern was reproducible in several experiments and by using independent S100 preparations, with greater differences always seen when the transcripts were incubated alone rather than mixed together. This effect might be due to competition for an RNase activity and, interestingly, contrasts with what was seen during in vitro studies of chloroplast RNA polyadenylation-mediated decay, where the enhanced instability of poly(A)-containing transcripts was

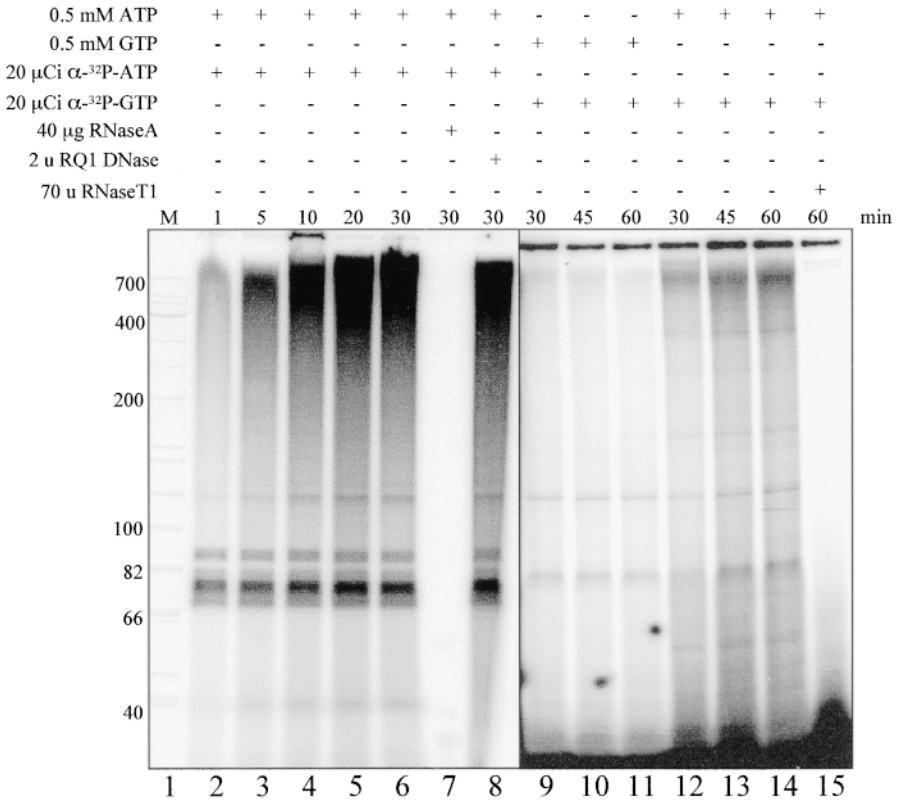

Figure 4. In Vitro Polyadenylation in a Maize S100 Extract.

Preparation of extracts and reaction conditions are described in Methods. The various additions or treatments used are indicated at the top of the gel. (+) indicates that the reagent was used, whereas (−) indicates that it was not used. Numbers at left indicate size markers (lane 1; marked M at the top) in nucleotides, whereas numbers across the top represent reaction times in minutes. Electrophoresis was in a 6% denaturing polyacrylamide gel. u, units.

most evident when mixed with their nonpolyadenylated counterpart (Lisitsky et al., 1997).

Results using site 20 RNA are shown in Figure 5C, and they resemble those obtained for site 7. Because site 20 coincides with the major 3′ end of *cox2* mRNA, we anticipated that a processing mechanism might remove the poly(A) tail, regenerating a poly(A)$^-$ molecule. Indeed, a small amount of a product of this size accumulated when the poly(A)-containing substrate was incubated alone. However, the majority of the poly(A)-containing substrate did not appear to accumulate as this product, suggesting that processing is inefficient, the product is unstable, and/or the processing machinery competes poorly with the RNA degradation machinery. Instead, many faster migrating bands are seen that could represent pauses in a putative exonucleolytic degradation mechanism. Taken together, our results suggest that in this in vitro system, transcripts terminating in 25 adenylate residues are less stable than their nonpolyadenylated counterparts.

RNA Editing and Polyadenylation Appear to Be Largely Independent Processes

Plant mitochondrial RNAs are subject to extensive post-transcriptional C-to-U editing, often resulting in codon changes required to specify functional proteins (reviewed in Maier et al., 1996). It was possible that polyadenylation was a mechanism to promote degradation of incorrectly or partially edited transcripts in maize mitochondria. The maize mitochondrial *cox2* mRNA is known to be edited (Yang and Mulligan, 1991), and six of these editing sites were present in *cox2* sequences analyzed for poly(A) addition. Therefore, the editing patterns of the polyadenylated clones were determined, and the results are shown in Figure 6A. Of the 60 cDNAs, 32 (53%) were fully edited, 13 (22%) were unedited, and 15 (25%) were partially edited. Each of the six sites was edited ~70% of the time, with the exception of site 2, which was edited 58% of the time.

To test whether polyadenylated mRNAs exhibited a biased

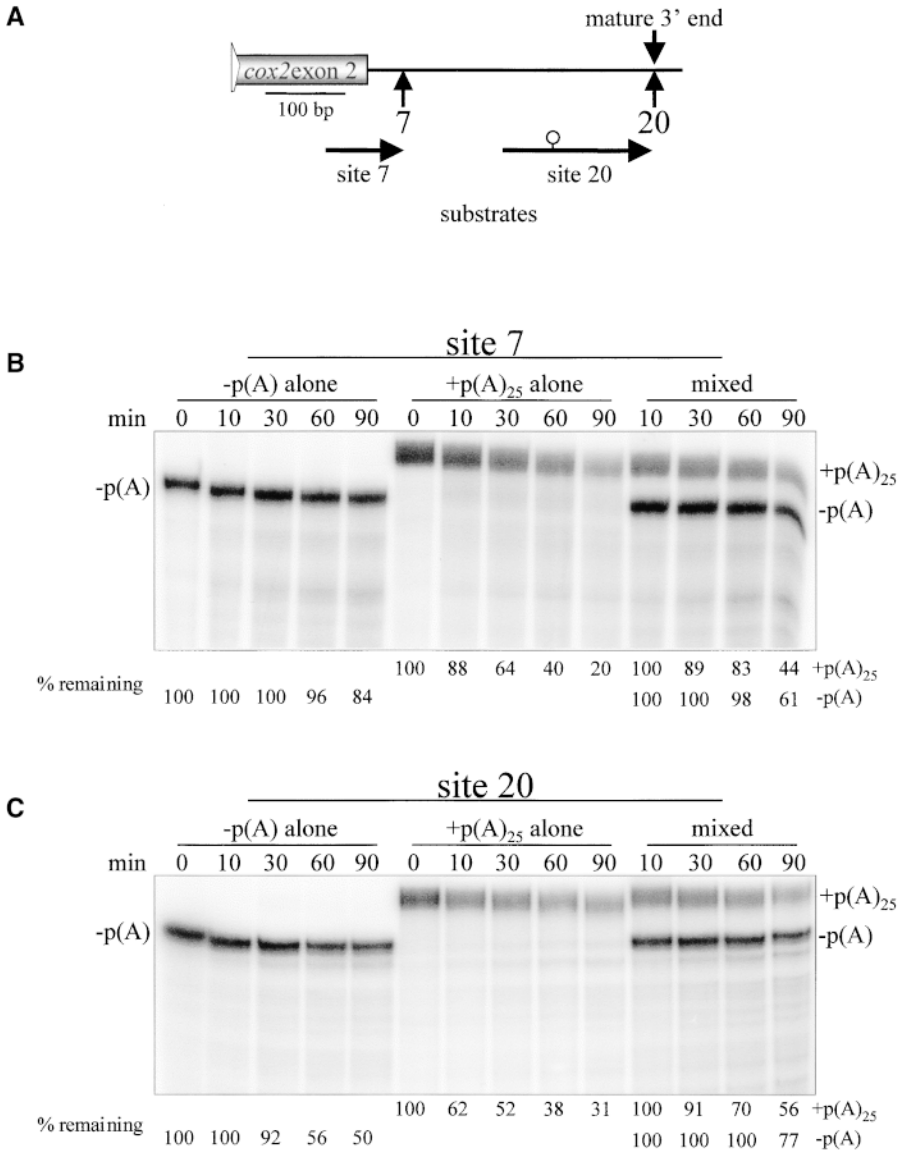

Figure 5. In Vitro RNA Degradation in a Maize Mitochondrial S100 Extract.

(A) Map of the *cox2* 3′ UTR, showing to scale the locations, polarity, and sizes of substrates. The 203-nucleotide site 7 substrate (228 nucleotides with the poly[A] tail) did not contain any known editing sites, whereas the 193-nucleotide site 20 substrate (218 nucleotides with the poly[A] tail) contains one editing site at position 1603 of the *cox2* sequence; this site was unedited in the substrate used, as indicated by the open circle.

(B) and **(C)** Six percent denaturing polyacrylamide gel analysis of reaction products at the times indicated. The numbers below the gels indicate the percentage of each RNA that remained, with the zero time point set to 100%. The values shown are for these gels; repetitions of each experiment were in good agreement. For example, for site 7 RNA, an average of 81% of the nonpolyadenylated substrate remained after 90 min when incubated alone or 67% when incubated in the presence of the poly(A)$^+$ substrate. An average of 30% of the poly(A)$^+$ substrate remained after 90 min when incubated alone or 49% when incubated in the presence of the poly(A)$^-$ substrate. Numbers across the top indicate reaction times in minutes; $+p(A)_{25}$ indicates that the substrate included a 25-nucleotide poly(A) tail; $-p(A)$ indicates that the substrate lacked a poly(A) tail. The reaction contents are indicated at the top of each gel; each substrate was added alone, or the two were added simultaneously (mixed) in approximately equal amounts. Below each gel, the percentage remaining is indicated; the top row is for the $+p(A)_{25}$ transcript, where present, and the lower row is for the $-p(A)$ transcript, where present.

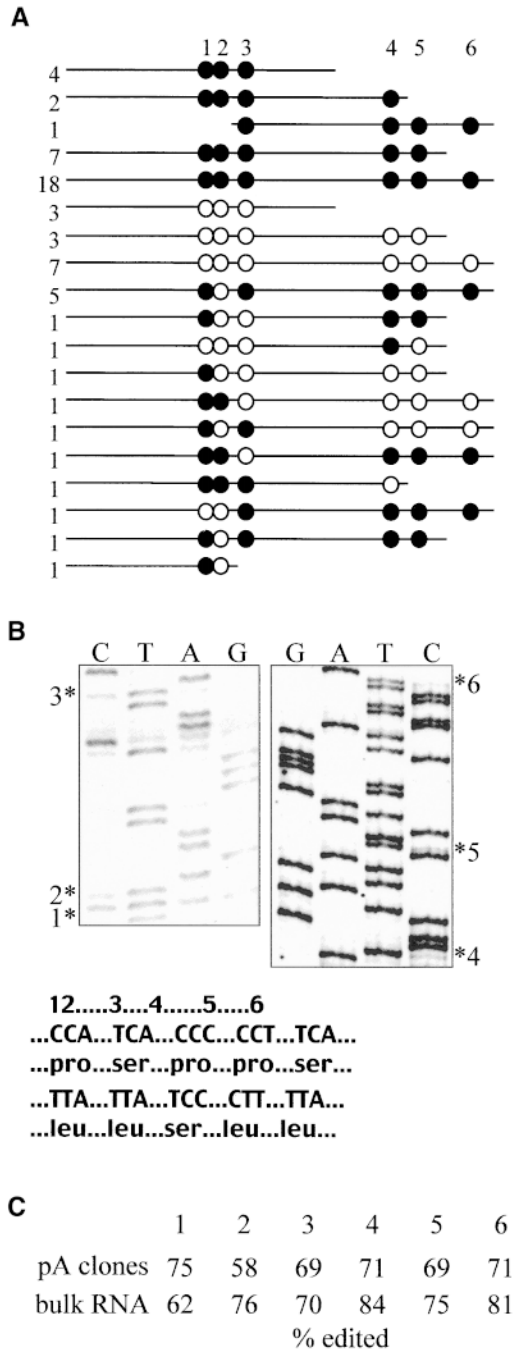

Figure 6. Editing Status of the Polyadenylated cDNAs.

(A) Filled circles represent sites that were edited in the sequenced clones, and open circles represent unedited sites. The number of polyadenylated clones having each pattern is indicated at left. Numbers at top represent editing sites.

(B) Editing status of bulk mtRNA. The sequencing gels show heterogeneity at each editing site; these sites are indicated by numbered asterisks, and the corresponding sequences and codons are indicated below the gels.

editing pattern when compared with the average status of *cox2* transcripts, we assessed the editing status of bulk mitochondrial *cox2* mRNA. RNA was isolated from intact mitochondria, reverse transcribed using the *cox2* 3′ UTR–specific primer ZMC24 (see Figure 1), and then PCR amplified using ZMC24 and the upstream *cox2* coding region–specific primer ZMC25. The product was gel purified and directly sequenced with the internal primer ZMC26. The experiment was performed in triplicate; a representative gel is shown in Figure 6B. The bands representing the edited (lanes T) and unedited (lanes C) bases for each edited position were quantified using a PhosphorImager.

Figure 6C shows that the degree of editing in the cDNA clones closely paralleled the degree of editing in bulk mitochondrial mRNA in most cases. However, site 2 was consistently edited to a higher degree in bulk mRNA than in the cDNA clones. Sites 1 and 2 are in a single codon (see Figure 6B), and when the second site is unedited, the codon specifies Pro (CCA) or Ser (UCA) instead of the correct Leu. In two-thirds of the partially edited cDNA clones, the first site was edited whereas the second was not, specifying Ser. With bulk mRNA, this editing pattern was largely reversed, leading to a high percentage of Leu (CUA or UUA) codons. Thus, at least in this instance, polyadenylation may have a slight preference for the nonfunctional transcript.

DISCUSSION

Function of Polyadenylation in Maize Mitochondria

Here, we have demonstrated polyadenylation of plant mitochondrial transcripts by mapping numerous poly(A) addition sites in maize *cox2* cDNAs. Approximately 40% of the cDNA clones possessed tails at or near the major mature 3′ end (sites 18 to 22), and ~25% had tails at an upstream site that is a minor 3′ end. Despite several attempts, we have been unable to obtain reliable data regarding the proportion of poly(A)-containing *cox2* transcripts among the steady state population; however, given the large number of PCR cycles required to amplify the cDNAs, it is unlikely to be any higher than the several percent estimated for prokaryotic systems (reviewed in Sarkar, 1997).

Regarding the function of polyadenylation in maize mitochondria, two somewhat conflicting observations were

(C) Editing frequency of bulk RNA was determined by using a PhosphorImager to quantify the edited (lanes T) and unedited (lanes C) bands in **(B)** for each edited position, using nearby unedited T and C bands as controls for variation in band intensity related to sequence length and composition. The values for bulk RNA are averages of three separate experiments. pA, poly (A).

made. The first was that two different polyadenylated synthetic transcripts were only slightly less stable when compared with their nonpolyadenylated counterparts in an in vitro system. When these same transcripts were tested in a chloroplast extract, the poly(A)$^+$ but not poly(A)$^-$ transcripts were highly unstable (data not shown), in agreement with other results published for chloroplasts (Kudla et al., 1996; Lisitsky et al., 1996). Thus, there is nothing inherently stable about the particular sequences that were chosen. The second observation is that poly(A)$^+$ transcripts are of low abundance, as determined by RNase protection, and require sensitive RT-PCR techniques to be amplified as cDNAs. This is consistent with poly(A)-mediated instability or with very inefficient poly(A) addition.

These findings suggest two main alternative interpretations. The first is that poly(A) tails do not confer relative instability to maize mitochondrial RNAs and do not have any other role in gene expression. The second possibility is that polyadenylation is indeed part of a normal RNA degradation or gene regulation pathway and that the relatively minor differential stability in the mitochondrial S100 extract reflects the particular in vitro conditions or substrates used. We believe that the available data support the second possibility for the following reasons.

First, polyadenylation is widespread in mitochondria and thus unlikely to be an evolutionary remnant in plants. For example, apart from the ample documentation in animals cited earlier, oligoadenylation of yeast (Hendler et al., 1975; Yuckenberg and Phillips, 1982; but see Groot et al., 1974) and trypanosome (Feagin et al., 1985; Bhat et al., 1992) mitochondrial transcripts has been reported. Second, whereas polyadenylation of plant mitochondrial transcripts might not confer the striking instability that it does in chloroplasts (Lisitsky et al., 1997) and *Escherichia coli* (Haugel-Nielsen et al., 1996), in other mitochondrial systems, poly(A) has still been implicated in RNA decay, for example, in rat, in which there is a correlation between poly(A) tail shortening and mRNA decay (Avadhani, 1979).

Third, whereas the half-lives of plant mitochondrial transcripts have not been directly measured, in maize, some mitochondrial transcripts that can be labeled by run-on transcription do not appear in the steady state population, implying that there is an active and selective degradation mechanism (Finnegan and Brown, 1990). Fourth, many of the poly(A) tails we found were not added at the mature 3′ end, implying that the tails were added during an RNA degradation process or after abortive transcription termination (Hajnsdorf et al., 1996).

Although we did not observe substantial differences in transcript stability using different temperatures or protein preparations (data not shown), it is possible that the S100 extract was depleted of certain ribonuclease activities. Whereas poly(A)-enhanced RNA decay occurs in chloroplast soluble protein extracts, the equivalent factors could be membrane associated in mitochondria. Although we did not test membrane protein fractions for RNase activity, Gagliardi and Leaver (1999) reported little RNase activity in matrix fractions from mitochondria of etiolated sunflower cotyledons, whereas two distinct RNase activities were found in the membrane fractions. In contrast, in vitro studies of mRNA 3′ end maturation (Dombrowski et al., 1997) and tRNA processing (Marchfelder and Brennicke, 1994) have utilized mitochondrial lysates much like ours, with lysis in the presence of nonionic detergent followed by clarification of the lysate. We also cannot rule out that tissues other than the etiolated hypocotyls used in our study might express different RNases. For example, the major cytosolic ribonucleases of Arabidopsis accumulate in a tissue-specific and developmentally regulated fashion (Yen and Green, 1991; Bariola et al., 1994).

Determination of Maize Mitochondrial Poly(A) Addition Sites

Because there was no sequence conservation surrounding the poly(A) addition sites, we concluded that polyadenylation occurs in a non-sequence-specific manner, perhaps stochastically at any available 3′ end, analogous to the tailing of most or all animal mitochondrial transcripts created by endonucleolytic processing (Hirsch and Penman, 1973). Similarly, transcripts in prokaryotes, bacteriophage T7, and chloroplasts also exhibit polyadenylation of endonuclease cleavage products without the requirement for specific sequence elements (reviewed in Sarkar, 1997), although RNA secondary structures may modulate accessibility of the poly(A) polymerase (Xu et al., 1993). This contrasts with the case in nuclei of metazoans, yeast, and plants, in which cleavage and polyadenylation are tightly coupled and the site is determined by specific sequence elements (reviewed in Wahle and Keller, 1992; Rothnie, 1996; Li and Hunt, 1997).

To determine whether other maize mitochondrial mRNAs exhibit such heterogeneity in their poly(A) addition sites, we have initiated a follow-up study focusing on *cox3*. Our preliminary data indicate that 12 of 12 maize *cox3* poly(A)-containing cDNAs have the same poly(A) site 56 nucleotides downstream of the stop codon, after UU (D. Gingerich, D.S. Lupold, and D.B. Stern, unpublished data). The identification of additional *cox3* poly(A)-containing cDNAs and those for other maize mitochondrial mRNAs is required before we can draw strong conclusions about poly(A) site selection.

Poly(A) Tail Length and Composition

The poly(A) tails of the sequenced cDNA clones ranged from 14 to 52 nucleotides; however, because the oligo(dT) primer can anneal anywhere within a poly(A) tail, these results likely underestimate the length of poly(A) tails in vivo. When unlabeled substrates consisting of endogenous mitochondrial RNAs were in vitro polyadenylated using α-^{32}P-ATP, the products were long; however, the substrates were certainly

of variable length, and thus the amount of poly(A) addition is difficult to estimate. Based on cDNA sequence data, our results are not conclusive but suggest that in maize mitochondria, poly(A) tails are relatively short, perhaps similar in size to the 57 nucleotides estimated for human mitochondria (Hirsch and Penman, 1973) and the 35 to 55 nucleotides estimated for rat mitochondria (Avadhani, 1979) but longer than those in yeast mitochondria (Yuckenberg and Phillips, 1982) and at the termini of stable *E. coli* RNAs (Li et al., 1998).

The base composition of the tails was examined during the sequencing of the cDNA clones. Eighty percent of the clones were homopolymeric for adenosine, whereas 20% had one or more other nucleotides interspersed, indicating that the poly(A) machinery can inefficiently use any of the other three nucleotides. This finding was supported by the ability of mitochondrial lysates to incorporate limited amounts of guanosine into RNA tails (Figure 4). In this regard, plant mitochondrial polyadenylation most closely resembles that of metazoans, yeast, plants, and prokaryotes and can be distinguished from that of chloroplasts and bacteriophage T7 (Wahle and Keller, 1992; Lisitsky et al., 1996; Sarkar, 1997).

The possibility of significant contamination of our mitochondrial extracts by nuclear proteins can be largely discounted for several reasons. First, our purification procedure includes both differential centrifugation and density gradient sedimentation, previously shown to yield essentially pure and intact mitochondria (Moore and Proudlove, 1983). Second, the presence of PCR-amplifiable poly(A)$^+$ cDNAs is consistent with such an activity being mitochondrially localized. Finally, such an activity must obviously be present in other mitochondrial systems where polyadenylation occurs, and indeed, poly(A) polymerase has been partially purified from rat mitochondria (Rose et al., 1975).

RNA Editing and Polyadenylation

Because fully edited, partially edited, and unedited transcripts were found among *cox2* cDNAs, we concluded that RNA editing and polyadenylation are independent processes, in accord with conclusions reached for *Trypanosoma brucei* mitochondria (Koslowsky and Yahampath, 1997). This is not surprising, because there is no defined order to post-transcriptional processes in plant mitochondria; for example, there is no 5′ to 3′ polarity of editing, and each site has a characteristic editing frequency (Wilson and Hanson, 1996). Transcript abundance and editing are linked in some cases (e.g., *atp6* in petunia; Lu and Hanson, 1992) but not in others (e.g., *atp6* in sorghum; Kempken and Howad, 1996). Examination of polyadenylation of plant mitochondrial RNAs during development, and for multiple genes in multiple species, is required to more fully elucidate its function. In this regard, newly published data suggest a role for polyadenylation in regulating the stability of a sunflower mitochondrial transcript involved in the expression of cytoplasmic male sterility (Gagliardi and Leaver, 1999).

METHODS

Seedling Growth, Mitochondrial Isolation, and RNA Extraction

Pioneer brand 3377 maize (Pioneer Hi-Bred International, Johnston, IA) was germinated and grown in the dark for 4 to 5 days. Intact mitochondria were isolated from etiolated maize seedlings as described (Rapp and Stern, 1992). RNA was extracted from mitochondria as described (Stern and Newton, 1986).

DNA Clones and Sequences

N6 has the 5.3-kb XhoI fragment from the N5G8 cosmid (kindly provided by Christiane Fauron, University of Utah, Salt Lake City; Lonsdale et al., 1984; Fauron and Havlik, 1988) inserted into pBluescript SK− (Stratagene, La Jolla, CA). N6SB has the SalI-BamHI fragment from N6 subcloned into pBluescript KS+. N6 was sequenced with oligonucleotides ZMC23 and ZMC27 extending from positions 1748 to 1762 (GenBank accession number V00712) and 541 to 559 bp downstream of the stop codon of the *cox2* gene, respectively, to provide additional *cox2* 3′ untranslated region (UTR) sequence information.

PCR Amplification and Identification of Polyadenylation Sites

Mitochondrial RNA (mtRNA; 10 μg) was used as a template for synthesis of oligo(dT)-primed cDNA with the dT-adapter primer, as described previously (Lisitsky et al., 1996). This cDNA was polymerase chain reaction (PCR) amplified, with the adapter primer and oligonucleotide ZMC22 extending from position 1338 to position 1354 of the *cox2* gene (see Figure 1A). Amplification was performed under standard conditions for 50 cycles of 1 min each at 94, 50, and 72°C, with the addition of extra enzyme (Promega) after 25 cycles. Controls were treated with 0.8 units of RNase H (Gibco BRL), 0.3 μg of oligo$(dT)_{20}$, or both, as previously described (Brewer and Ross, 1988). PCR products were size selected (two size classes, as shown in Figure 1B) in an agarose gel and ligated into pUC57/T (MBI Fermentas, Amherst, NY). Colony hybridization with the SnaBI-AvaI fragment of the *cox2* 3′ region was used to select clones for further evaluation. These were sequenced in both directions using vector-specific M13 (−20) and reverse primers to identify polyadenylation sites and check the editing status of each clone. Manganese was added to the sequencing reactions to facilitate the reading of sequences close to the primers.

Determination of the Mature 3′ End of the *cox2* Transcript

The 270-bp SphI-NdeI restriction fragment was isolated from the N6SB clone and end labeled using T4 DNA polymerase (Promega). Briefly, the T4 DNA polymerase reaction was allowed to proceed for

5 min at 16°C in the absence of deoxynucleotide triphosphates and then labeled with α-^{32}P-dCTP in the absence of dGTP for 15 min at 16°C to preferentially label the 3′ end of the antisense strand at the SphI site, chased with cold dCTP and dGTP for 5 min at 16°C, and passed over a Sephadex G-25 (Sigma) spin column to remove unincorporated deoxynucleotide triphosphates. S1 nuclease protection was performed as described (Sambrook et al., 1989) with two different amounts of probe, either 1× or 2.5×, protected by mtRNA or tRNA.

Protein Extract Preparation, in Vitro Polyadenylation, and Degradation Assays

Intact mitochondria were lysed in the presence of 1 M KCl and 0.5% Triton X-100 (Sigma) followed by centrifugation of the lysate at 100,000*g*. Endogenous RNA in the S100 extract (450 μg of protein per reaction) was polyadenylated at 30°C in the presence of poly(A) polymerase buffer (40 mM Tris-HCl, pH 7.9, 2.5 mM $MnCl_2$, 250 mM NaCl, 1 mM EDTA, and 10 mM $MgCl_2$) with the addition of labeled or unlabeled nucleotide triphosphates, as indicated in Figure 4. Reactions were stopped by incubation at 37°C for 15 min in the presence of 0.3 M NaCl, 10 mM Tris-HCl, pH 7.9, 5 mM EDTA, 0.8% SDS, and 800 μg of proteinase K. Fifty micrograms of yeast tRNA and 500 μM aurintricarboxylic acid were added before organic extractions and ethanol precipitation. Where indicated, treatment with RNase A, RNase T1, or RQI (RNA Qualified; Promega) DNase was for 15 min at 37°C.

For degradation assays, templates specifying the transcripts, as shown in Figure 5A, were amplified using PCR, and in vitro transcription was performed using T7 RNA polymerase with the inclusion of α-^{32}P-ATP. These transcripts were gel purified and polyadenylated in vitro using yeast poly(A) polymerase, under the conditions specified by the manufacturer. Under these conditions, partial polyadenylation was obtained, and the resulting mixture of polyadenylated and nonpolyadenylated transcripts was incubated at 37°C with the S100 extract (450 μg of protein per reaction) in the presence of poly(A) polymerase buffer and 1 × MTB (Rapp and Stern, 1992). Reactions were stopped as described for poly(A) addition.

PCR Amplification and Sequencing to Determine the Editing Status of Bulk mtRNA

A 30-μL reaction containing 90 ng of DNase-treated mtRNA was reverse transcribed by using 450 ng of the *cox2*-specific oligonucleotide ZMC24, extending from nucleotides 1762 to 1748 of the *cox2* sequence, and avian myeloblastosis virus reverse transcriptase (Promega) for 1 hr at 42°C. Then, 2 μL of this product was PCR amplified by using oligonucleotides ZMC24 (see above) and ZMC25, which extends from nucleotides 1293 to 1305 of the *cox2* sequence. Amplification was performed for 25 cycles of 1 min each at 94, 50, and 72°C. The product was isolated in low-melting-temperature agarose and directly sequenced as originally described (Bachmann et al., 1990) in a reaction containing manganese (to read the sequences close to the primer) using ZMC26, which extends from nucleotide 1314 to 1329 of the *cox2* sequence. The bands in Figure 6 representing the edited (lanes T) and unedited (lanes C) bases for each edited position (as well as nearby unedited T and C bands as controls for variation in band intensity related to sequence length and composition) were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The values given represent the averages of three experiments.

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