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Evidence for a novel mitochondrial promoter preceding the *cox2* gene of perennial teosintes

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We have characterized two promoters of the cytochrome oxidase subunit 2 (*cox2*) gene in *Zea perennis* mitochondria present in maize lines. Initiation at a site 907 bases upstream of the start codon results in the major ~1900 nt *cox2* transcript. A sequence just upstream of this site conforms to the consensus described for maize mitochondrial promoters and its transcription is correctly initiated in a maize mitochondrial *in vitro* transcription extract. A second transcription initiation site (−347) is used only when the dominant allele of a nuclear gene, *Mct*, is present and its use results in an additional, shorter major transcript. Sequences flanking the *Mct*-dependent transcription initiation site, which we have termed the conditional promoter of *cox2* (*cpc*), do not fit the maize mitochondrial promoter consensus and do not function in the maize *in vitro* transcription extract. The *cpc* region does not hybridize with mitochondrial, chloroplast or nuclear DNAs from most maize or teosinte lines. However, the *cpc* sequence is found in the same position upstream of the *cox2* gene in *Zea diploperennis* mtDNA and it has striking similarity to the previously reported 'ORF of unknown origin' fused to the ATPase subunit 6 gene in maize CMS-C mitochondria. *cpc* appears to represent a new type of mitochondrial promoter. Further analysis of both conditional and constitutive promoters should help us to better understand the control of transcription in plant mitochondria.

Key words: *cox2*/maize/mitochondrial gene/promoter/transcription

Introduction

Relatively little is known about how promoter sites are selected and transcription is initiated in the mitochondria of higher plants (reviewed in Gray *et al.*, 1992). As a result of extensive rearrangements, mitochondrial genes in closely related higher plants can occur in different genomic environments (reviewed by Hanson and Folkerts, 1992). Therefore, different sequences can act as promoter elements for the same gene. The molecular analyses of plant mitochondrial promoters that have been published to date suggest that they deviate significantly from the well-characterized yeast and mammalian mitochondrial promoters (see Gray *et al.*, 1992; Rapp *et al.*, 1993; Stern and Rapp, 1993). Indeed, plant mitochondrial promoters have very limited primary sequence identity. Furthermore, in plants such as maize, there are usually multiple promoters for each mitochondrial gene and, often, more than one transcription initiation site within the same promoter (see Stern and Rapp, 1993).

In contrast, within the small mammalian mitochondrial genomes there are only two mitochondrial promoters, each of which contains a polymerase recognition site and an upstream binding site for a specificity factor (reviewed by Clayton, 1992). In yeast, there are ~20 copies of a 9 nt promoter consensus sequence in the whole mitochondrial genome. This sequence has been shown to be both necessary and sufficient to direct transcript initiation, which occurs at the final adenine (reviewed by Constanzo and Fox, 1990).

Transcript initiation sites for several plant mitochondrial genes have been identified by *in vitro* 'G-capping' (labeling of the 5′ ends of primary transcripts with [α-^{32}P]GTP) in combination with direct RNA sequencing or primer extension analyses (Mulligan *et al.*, 1988a,b; Brown *et al.*, 1991; Covello and Gray, 1991). After comparing sequences immediately upstream of the transcript initiation sites for a number of maize mitochondrial genes, Mulligan *et al.* (1988a,b, 1991) were able to propose an 11 nt consensus sequence for maize mitochondrial promoters which included CRTA as an invariant core.

With the development of *in vitro* transcription systems, the requirements for plant mitochondrial promoter function could be dissected (Hanic-Joyce and Gray, 1991; Rapp and Stern, 1992; Rapp *et al.*, 1993). In a detailed analysis of the maize *atp1* promoter, Rapp and Stern (1992) found that the 11 nt consensus sequence proposed by Mulligan *et al.* (1991) was necessary, but not sufficient, to direct transcript initiation *in vitro*, i.e. when the consensus sequence was inserted in front of other sequences, most were not transcribed *in vitro*. Further analysis led to the proposal that there are three elements required for efficient *in vitro* transcription of maize *atp1*, one of which is a core region of 5 nt bearing the sequence YRTAT (Rapp *et al.*, 1993), which corresponds to the conserved CRTA of Mulligan *et al.* (1991).

At present, virtually nothing is known about the proteins that function in plant mitochondrial transcription. The mitochondrial RNA polymerases are probably encoded by nuclear genes, but the composition of the polymerase(s) has yet to be determined. Furthermore, the potential role of nuclear coded protein factors in the selection of transcription initiation sites in plant mitochondria has not been previously examined.

One possible approach to identify nuclear and mitochondrial components that cooperate to express mitochondrial

genes exploits existing evolutionary divergence. When nuclear and cytoplasmic genomes that have evolved under different selective pressures are combined, abnormal phenotypes and alterations in mitochondrial gene expression can result. Maize and its relatives offer a defined system for such an analysis of nuclear–cytoplasmic interactions. The teosintes most distantly related to maize (*Zea mays mays*) are found in Section Luxuriantes, comprising three species: *Z.perennis*, *Z.diploperennis* and *Z.luxurians* (Doebley and Iltis, 1980). These teosinte cytoplasms have been introduced into different maize inbred lines using the maize line as the male parent in a program of recurrent back-crossing to the teosinte-derived female line (Allen *et al.*, 1989).

Several phenotypes resulting from nuclear–cytoplasmic interactions have been reported in the maize lines carrying these teosinte cytoplasms, including cytoplasmic male sterility (CMS; Gracen and Grogan, 1974; Laughnan and Gabay-Laughnan, 1983) and small kernel/plant phenotypes (Kermicle and Lonnquist, 1973; Allen *et al.*, 1989). In addition, allelic products of maize nuclear genes have been shown to interact with the different teosinte mitochondria to affect mitochondrial gene expression. These include the synthesis of a novel polypeptide in *Z.luxurians* mitochondria (Cooper and Newton, 1989) and the presence of an additional major cytochrome c oxidase subunit 2 (*cox2*) transcript in *Z.perennis* and *Z.diploperennis* mitochondria (Cooper *et al.*, 1990). Thus, nuclear genes with specific effects on mitochondrial gene expression can be identified using these maize–teosinte nuclear–cytoplasmic combinations.

A dominant allele of a single nuclear gene, *Mct* (modifier of *cox2* transcripts), has been shown to be responsible for the difference in the major *cox2* transcripts in the perennial teosinte mitochondria (Cooper *et al.*, 1990). In the W23 (*mct/mct*) nuclear background, the most abundant transcript is 1.9 kb, whereas in the A619 (*Mct/Mct*) background, there is a novel abundant 1.5 kb transcript. No effect of nuclear background on *cox2* transcripts was observed for A619 and W23 plants possessing either a *Z.luxurians* or a normal maize cytoplasm. Immunoblot analysis suggested that the 1.5 kb *cox2* transcripts in the mitochondria of the perennial teosintes may be translated relatively poorly or that the corresponding protein products are less stable (Cooper *et al.*, 1990). We now report that the *cox2* transcript difference is due to an unusual *cox2* promoter in the mitochondrial DNA of the perennial teosintes. The use of this novel mitochondrial transcription start site requires the presence of the dominant *Mct* allele in the nucleus.

Results

Sequence analysis and expression of the Zp-cox2 gene

The *Z.perennis cox2* (*Zp-cox2*) transcript difference in the presence of homozygous *mct/mct* (−) and *Mct/Mct* (+) alleles is illustrated in Figure 1A (see also Cooper *et al.*, 1990). The most abundant mature transcript is 1.9 kb in the absence of the dominant *Mct* allele (−) and 1.5 kb in the presence of *Mct* (+). The 1.7 and 1.3 kb transcripts seen in Figure 1A are thought to have the same 5′ ends as the 1.9 and 1.5 kb transcripts respectively, but to have shorter 3′ ends (Cooper *et al.*, 1990). It should be noted that the nuclear genomes of the perennial teosintes probably carry a recessive *mct* allele: no *cox2* 1.5 kb transcript has been identified in mtRNAs from *Z.perennis* or *Z.diploperennis* (unpublished data).

In order to identify the mitochondrial sequences that could potentially be involved in specifying the difference in transcript initiation or processing, the *Zp-cox2* locus was cloned and sequenced. In order to have sufficient 5′ and 3′ regions for transcript analysis, the *Zp-cox2* gene was originally cloned as a large 10.9 kb *Pvu*II fragment (ZpA49). Several subclones were generated from this original clone, including those shown in Figure 1B, and were completely sequenced on both strands. Currently, over 6 kb in the vicinity of the *Zp-cox2* gene have been sequenced.

In Figure 1C, the sequence of the *Zp-cox2* gene from 1120 nt upstream to 80 nt downstream of the translation start site is shown. The transcription initiation sites we have identified in this study are designated by flags. Unusual features in the region preceding the translational start are highlighted. Sequences identical to the maize *atp6* coding region are fused immediately 5′ to the *cox2* coding sequence (shown by the double underlining). A short stretch of DNA upstream of the boxed region (underlined) shares homology with the 2D9 cosmid clone from maize CMS-T mtDNA. This region is near a site that is involved in a recombination event leading to a case of fertility reversion in CMS-T (Rottman *et al.*, 1987). The protein coding region of the *Zp-cox2* gene itself was found to be identical to that of maize (Fox and Leaver, 1981), with the exception of a single nucleotide at position 1421 downstream from the start codon (A in maize, G in *Zp-cox2*). This nucleotide substitution does not result in a change of the corresponding amino acid. The 794 bp intron is identical in sequence between maize (*Zm-cox2*) and *Zp-cox2*. However, 2 bp upstream of the start codon, the *Zp-cox2* gene has a 6 bp deletion relative to the *Zm-cox2* gene. Similarity between the *Zm-cox2* and *Zp-cox2* 5′ regions then resumes for nearly 100 bp.

These results are very similar to those found for the *Z.diploperennis* sequence previously reported (Gwynn *et al.*, 1987). The coding regions of the *Zp-cox2* and the *Zd-cox2* genes are identical and this sequence identity continues 5′ to the protein coding region. Only 99 nt upstream of the translational start site have been reported for the *Z.diploperennis* gene (Gwynn *et al.*, 1987), however, the *Z.perennis–Z.diploperennis* homology appears to continue much further upstream, based upon restriction site analysis of mtDNA (data not shown). As expected, the *Zp-cox2* transcripts are edited and nearly all of the editing sites are similar to those reported in maize (Table I; Covello and Gray, 1990; Yang and Mulligan, 1991). Edit sites are also indicated in Figure 1C (individually boxed nucleotides).

Identification of a constitutive and an alternative transcription initiation site for the Zp-cox2 gene

Our data show that both the sequence of the *Zp-cox2* gene and its RNA editing patterns are similar to those of maize. However, 100 bp upstream of the start codon, the sequences diverge radically. RNA gel blot analysis suggested that the site that determined the difference in

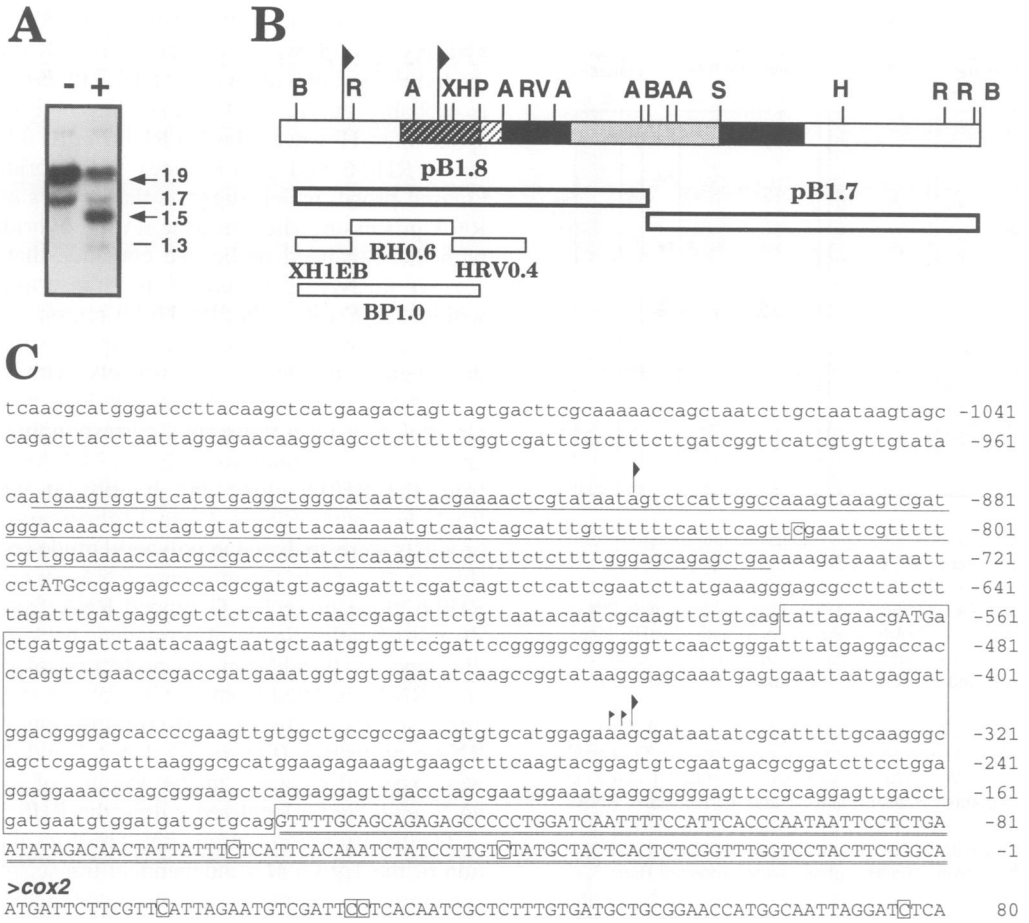

Fig. 1. The *Zp-cox2* gene. (**A**) *cox2* transcripts from *Z.perennis* mitochondria in the absence (−) or presence (+) of the dominant *Mct* nuclear gene. (**B**) Diagram of 3700 bp surrounding the *Zp-cox2* gene, indicating six of the subclones used for sequencing and transcript analysis. The protein coding regions are indicated by black boxes and the intron by a stippled box. The light striping upstream of the ATG translational start codon (+1) indicates *atp6* homology and the heavy striping indicates the region containing the conditional transcription initiation site (flag). The flag just upstream of the *Eco*RI site represents the upstream constitutive initiation site. Restriction enzyme sites are indicated: B, *Bam*HI; R, *Eco*RI; A, *Ava*II; X, *Xho*I; H, *Hin*dIII; P, *Pst*I; RV, *Eco*RV; S, *Sal*I. (**C**) DNA sequence of the region upstream of the *Zp-cox2* ORF. Protein coding sequences are shown in upper case. The *cpc* region is boxed (corresponds to heavy striping in B), the homology to the *atp6* coding region is double underlined and capitalized (see light striped area in B). Two possible upstream translation start sites (ATG) are shown as upper case. A region with homology to the 2D9 cosmid clone of cms-T maize is underlined (prior to the boxed region). Nucleotides that are edited in the RNA sequence are individually boxed. The flags indicate the transcription start sites determined by primer extension.

Table I. Edited sites in *cox2* genes

	Position																								
Zp	−61	−38	−22	14	30	31	77	144	148	162	167	169	259	284	385	449	466	467	482	550	563	587	620	638	704
Zm	−67	−44	−28																						
Zp	P	U	−	U	P	P	U	U	P	P	U	U	U	U	P	U	U	P	U	U	U	U	U	P	U
ZmB	−	U	−	P	−	P	P	−	−	−	P	P	P	P	U	U	P	U	P	P	P	P	P	P	P
ZmG	−	U	P	U	P	U	U	U	−	P	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U

Zea perennis (Zp) mRNA was reverse transcribed and amplified by PCR. The PCR product was sequenced and compared with previously published maize *cox2* RNA sequences. ZmB, from *Z.mays* B37N genotype (Yang and Mulligan, 1991); individual cDNA clones were sequenced. ZmG, *Z.mays* Golden Bantam Early (Covello and Gray, 1990); the mRNA was directly sequenced. Position 1 corresponds to the first nucleotide of the protein coding sequence in mRNA. Sites that appear to be fully edited (U) or partially edited (P) are indicated. Sites that were not reported as edited for one of the genotypes relative to the others are indicated (−).

the *cox2* transcripts from the perennial teosinte mitochondria was located 5′ to this site of divergence (data not shown). Our initial hypothesis was that a factor coded by the dominant *Mct* allele influenced the processing of the *cox2* transcripts. However, efforts to detect processing in this region, using *in vitro*-transcribed *Zp-cox2* RNAs and S100 extracts of mitochondria, yielded only negative results (data not shown).

An alternative hypothesis was that the 1.5 kb transcript (Figure 1A) resulted from MCT-mediated activation of a cryptic transcription initiation site. Methods to distinguish between mitochondrial transcript ends resulting from

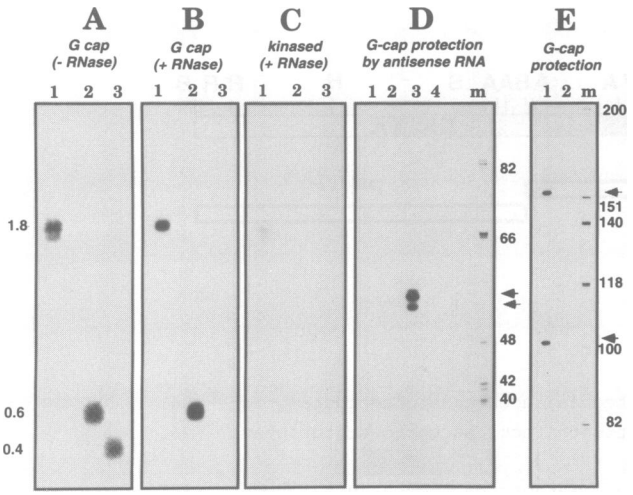

Fig. 2. RNase protection assays of *Zp-cox2* transcripts initiated from the *cpc* and *upc* promoters. (**A**–**C**) DNA gel blot analyses. Hybridization of G-capped RNA from Zp-A619 plants (*Mct/Mct*) to DNA gel blots before (A) and after (B) washing the blots with RNase A and RNase T1. Lanes 1, ZpA49 clone digested with *Bam*HI; lanes 2, RH0.6 subclone digested with *Eco*RI and *Hin*dIII; lanes 3, HRV0.4 digested with *Hin*dIII and *Eco*RV. (C) Hybridization with kinased RNA from Zp-A619 plants (*Mct/Mct*) to a duplicate DNA gel blot after RNase washes. (**D**–**E**) Protection of *in vitro* G-capped RNAs with antisense RNAs. (D) The *cpc* transcript initiation site. G-capped mtRNAs were from Zp-W23/Mo17 plants (*mct/mct*, lanes 1 and 2) or from Zp-A619/B73 plants (*Mct/Mct*, lanes 3 and 4). Antisense RNAs transcribed *in vitro* from RH0.6 were used to protect the G-capped RNAs from digestion with RNase A and RNase T1 (lanes 1 and 3). Only G-capped RNAs from *Mct/Mct* plants were protected (lane 3, arrow). A shorter protected band (lower arrow) appears to be a partial degradation product, because its intensity increases with the exposure time to RNase. As controls, sense transcripts from RH0.6 were used in the assays (lanes 2 and 4). (E) The *upc* transcript initiation site. G-capped mtRNAs were from Zp-W23/Mo17 plants (*mct/mct*) and were protected with either antisense (lane 1) or sense (lane 2) *in vitro* transcripts from the clone XH1EB. Two protected bands were seen (arrows). Markers (m) were end-labeled *Hin*fI digestion fragments of ϕX174 DNA.

initiation or processing were first published by Christianson *et al.* (1983) and were adapted for the analysis of maize mitochondrial transcripts by Mulligan *et al.* (1988a,b). Duplicate gel blots of cloned DNAs digested with appropriate restriction enzymes were hybridized with *in vitro* G-capped RNAs (representing primary transcripts) or with RNAs whose ends had been radiolabeled using polynucleotide kinase (processed transcripts). G-capped RNAs from plants that had both Zp mitochondria and the dominant *Mct* nuclear gene (Zp cytoplasm in the A619 nuclear background), hybridized strongly with the 1.8 kb *Bam*HI fragment of the ZpA49 clone and with subcloned fragments RH0.6 and HRV0.4 (Figure 2A). Only the strong signals corresponding to the 1.8 kb *Bam*HI fragment and its internal *Eco*RI–*Hin*dIII 0.6 kb fragment remained after two washes containing RNase (Figure 2B), indicating the presence of a transcription initiation site within this fragment.

Attempts to show processed ends in this region by hybridization of 'kinased' RNA probes (radiolabeled using polynucleotide kinase) to DNA gel blots of subcloned fragments were negative (Figure 2C). Only two bands in lane 1, the 1.8 and 1.7 kb *Bam*HI fragments of the Zp49A clone, were detectable after two post-hybridization washes with RNase. Because the labeling reaction requires a 5′ hydroxyl group, which can arise from RNA processing, the hybridization with the 1.8 and 1.7 kb *Bam*HI fragments is probably due to the fact that they both contain intron splice sites. However, the *Eco*RI–*Hin*dIII 0.6 kb fragment (from RH0.6 and pB1.8) does not hybridize with the kinased probe, which suggests that it does not contain an RNA processing site. Because it only hybridizes strongly with the G-capped probe, we conclude that the *Zp-cox2* 1.5 kb mRNA is generated by transcription initiation within this *Eco*RI–*Hin*dIII 0.6 kb region.

In order to verify that a transcript was initiated within this region, protection experiments with *in vitro*-transcribed RNAs from the RH0.6 subclone were conducted. Unlabeled *in vitro* transcripts corresponding to the sense and antisense orientations of *Zp-cox2* RNAs were produced from the RH0.6 clone and hybridized with G-capped RNA. Following RNase A and T1 digestion, the protected products were analyzed in polyacrylamide sequencing gels (Figure 2D). Only the antisense RNA generated from the RH0.6 clone protected G-capped RNA from plants with Zp mitochondria in the *Mct* nuclear background (Figure 2D, lane 3). In addition, no protection of G-capped *Zp-cox2* RNAs isolated from *mct/mct* plants was found when either sense or antisense RH0.6 transcripts were used for RNase protection (Figure 2D, lanes 1 and 2). This result was expected because, in the absence of the *Mct* allele, no 5′ end can be localized within the RH0.6 region.

To identify the initiation site(s) responsible for production of the 1.9 kb *Mct*-independent transcripts, similar G-cap protection experiments were conducted using sense and antisense transcripts from an upstream clone, XH1EB (Figure 2E). Two G-capped protected RNAs were found. One of these (darker arrow) corresponds to the −907 major transcription initiation site determined by primer extension.

Identification of major transcript ends by primer extension

Primer extension experiments identified a unique *cox2* 5′ transcript end, consisting of three adjacent nucleotides, in Zp mtRNA from plants with the *Mct* allele (*cpc* in Figure 3A, lanes 1 and 2), but not in mtRNA from plants homozygous for the *mct* allele (Figure 3A, lane 3). This transcription initiation site lies within the *Eco*RI–*Hin*dIII 0.6 kb segment of DNA and was designated *cpc*, for conditional promoter of *cox2*. Inspection of the sequences in the *cpc* region did not identify a promoter that fitted any of the consensus sequences proposed for the characterized promoters of plant mitochondria (Mulligan *et al.*, 1991; Gray *et al.*, 1992; Rapp and Stern, 1992; Rapp *et al.*, 1993).

In addition to the conditional promoter end at 347 nt upstream of the translational start, a second major 5′ end was identified at −907 (Figure 3A and B, *upc*). This end corresponds to the 5′ end of the 1.9 kb transcript. Its upstream sequence has an excellent match (in two possible alignments) to the consensus for the previously characterized maize mitochondrial promoters (Rapp *et al.*, 1993), as shown in Figure 4. Thus, the 1.9 kb transcript appears to result from the action of an upstream promoter of *cox2*, or *upc*. One major primer extension band is seen in this region. After prolonged exposure of the autoradiographs, a very minor primer extension band could be detected at

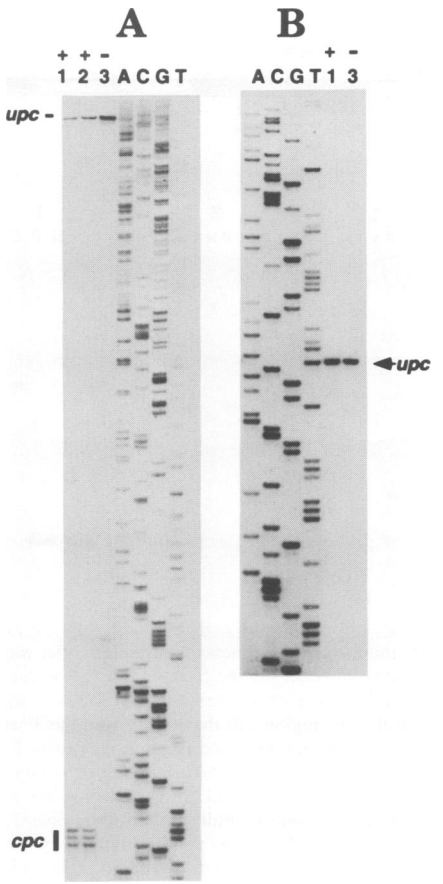

Fig. 3. Determination of the 5′ ends of the *Zp-cox2* transcripts by primer extension analysis. (**A**) Autoradiograph showing both the *upc* and *cpc* ends determined from reverse transcription with primer BWPR11. (**B**) Autoradiograph of the *upc* transcript end determined with primer BWPR13. RNAs were isolated from Zp-A619 plants (*Mct/Mct*, +, lanes 1), plants with Zp mitochondria in the hybrid A619/B73 nuclear background (*Mct/Mct*, +, lane 2) and Zp-W23 plants (*mct/mct*; −, lanes 3). The DNA sequences generated using the same primers with the pB1.8 clone are also shown (lanes A, C, G and T).

maize consensus	RARAANTRACGTAT
maize *atp1* (IVT)	RR__NNYNRYRTAT
teosinte *cox2* upc	AcGAAAaCtCGTATAATA
teosinte *cox2* upc	GAAAACTCGTATAaTA
teosinte *cox2* cpc	GAACGTgTGCATggAGAAAG

Fig. 4. Possible alignments of the upstream (*upc*) and the conditional (*cpc*) regions with the promoters derived from inspection of several maize mitochondrial transcription initiation sites (Mulligan *et al.*, 1991) and from the *in vitro* transcription (IVT) assay using *atp1* (Rapp *et al.*, 1993). Transcript initiation sites are indicated with bold letters (corresponding to the flags in Figure 1 for *upc* and *cpc*). Bases shown in lower case deviate from the maize IVT consensus and have a negative effect on transcription initiation at the *atp1* locus *in vitro*.

−959 (data not shown), which could correspond to another upstream start site predicted from the G-cap experiments (upper arrow in Figure 2E).

In vitro transcription experiments

The ability of both the *cpc* and the *upc* regions to act as promoters in the maize *in vitro* transcription system was tested. The fact that transcripts do initiate from the *upc* region has been confirmed by *in vitro* analysis. Figure 5A shows that transcripts are initiated from the upstream promoter (*upc*, Figure 5A, lane 2), but not from the conditional promoter (lane 1). The size of the *upc*-initiated transcript can be estimated from the markers (lane m) as ~145 nt. The expected size of the *cpc*-initiated transcript in this experiment was 200 nt and it was not detectable (lane 1). A clone containing the well-characterized *atp1* promoter (Rapp and Stern, 1992; Rapp *et al.*, 1993) was included as a control in the *in vitro* transcription experiment (lane 3). Because the *in vitro* transcription extract was made from a maize line that has the *Mct* allele in the nucleus (and maize N mitochondria), we had expected to see transcripts initiated from the conditional promoter of the *Zp-cox2* gene. However, we were unable to detect transcripts from the *cpc* region in this system.

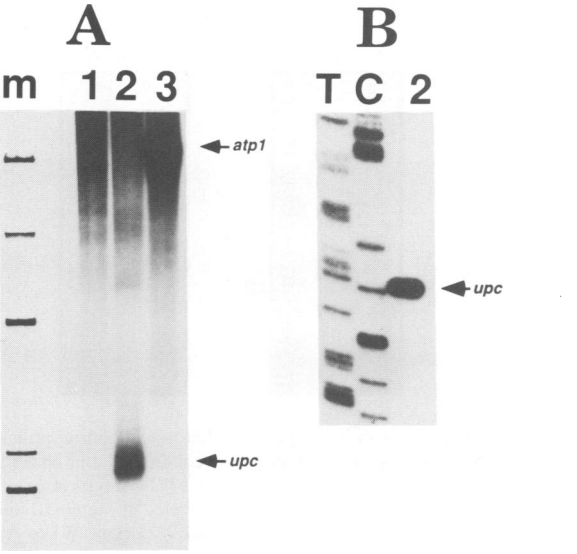

Fig. 5. *In vitro* transcription analysis. (**A**) Results of *in vitro* transcription using linearized plasmids. BP1.0 clone linearized with *Pst*I to detect the *cpc*-initiated transcript (lane 1); BP1.0 linearized with *Kpn*I to detect the *upc*-initiated transcript (lane 2); positive control clone BH0.7, containing the *atp1* promoter, linearized with *Hin*dIII (lane 3). (**B**) Primer extension of *in vitro* transcripts from the *upc* (see panel A, lane 2) using primer BWPR13.

Primer extension of the *in vitro*-transcribed product from the upstream *cox2* promoter showed a single 5′ end (Figure 5B). This end corresponds exactly to the transcript end identified by primer extension of the RNAs isolated from *Z.perennis* plants.

Proteins bind to the cpc and upc regions

Gel shift experiments were conducted in order to determine whether (i) proteins present in mitochondrial extracts can bind to the conditional and constitutive promoter regions and (ii) there is a difference in binding when extracts are prepared from *Mct/Mct* (A619) or *mct/mct* (W23) plants. DNA surrounding the *cpc* transcript initiation site was PCR-amplified, restriction fragment ends were fill-in labeled and the fragments were incubated with soluble extracts (S100 fractions from freeze−thaw lysates). Analysis in native polyacrylamide gels showed that proteins can bind to the labeled fragments and change their mobilities. In initial studies with W23 and A619 mitochondrial extracts from plants carrying maize cytoplasms, no differences in the mobilities of the shifted bands

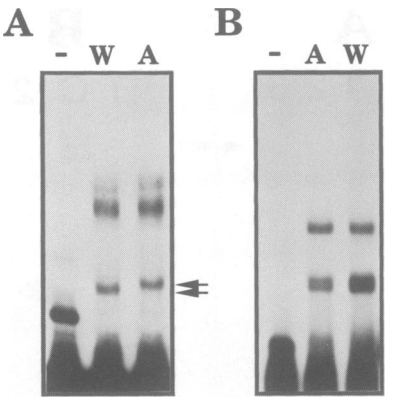

Fig. 6. Gel mobility shift assays show that mitochondrial proteins can bind to the DNA regions containing the conditional and constitutive promoter. (**A**) DNA binding assays using a PCR fragment extending from nt −436 to −250 (see Figure 1C) labeled at the *Hpa*II site (−436). S100 extracts were prepared from W23 (lane W) or A619 (lane A) plants carrying Zp mitochondria. The first lane (−) is a control incubated without added protein. Arrows indicate a band shift difference. (**B**) The *upc* region also binds mitochondrial proteins. A PCR fragment extending from nt −997 to −884 (Figure 1C) was labeled at the *Taq*I site (−884) and incubated with S100 extracts from A619 (lane A) or W23 (lane W) plants carrying Zp mitochondria. The first lane (−) is a control incubated without added protein.

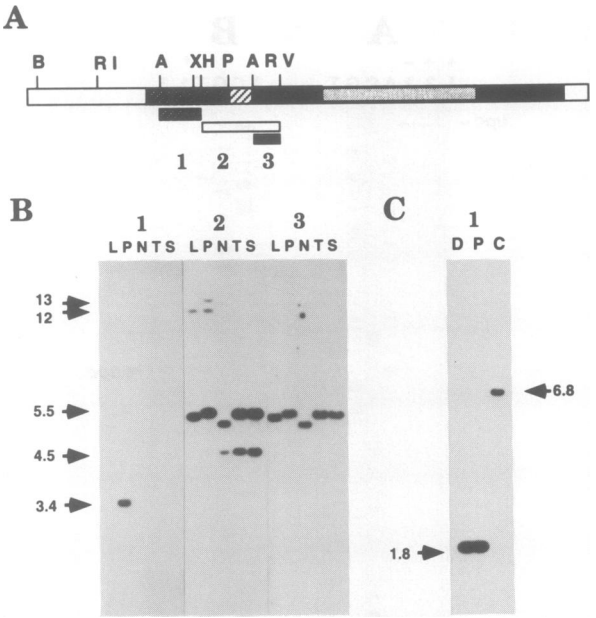

Fig. 7. A rare mitochondrial sequence that includes the conditional transcription start site occurs in the 5′ region of the perennial teosinte *cox2* genes. (**A**) Antisense riboprobes used for Southern analysis: (1) specific for the *cpc* region, (2) the region from the 3′ end of the *cpc*, the *atp6* homologous region and the 5′ end of exon 1 and (3) exon 1-specific. (**B**) DNAs from *Z.luxurians* (L), *Z.perennis* (P) and maize WF9N (N), B37T (T) and B37S (S) mitochondria were digested with *Xho*I and gel blots were hybridized with riboprobes 1, 2 or 3 as indicated. Because probe 2 includes the *atp6* homology, two genes hybridize: *cox2* (5.3–5.5 kb) and *atp6* (4.5 kb in maize, 12–13 kb in teosintes). (**C**) mtDNAs from *Z.perennis* (P), *Z.diploperennis* (D) and maize CMS-C (C) were digested with *Bam*HI and hybridized with probe 1. The sizes of hybridizing fragments listed along the sides (in kb) were estimated from comparison with the 1 kb ladder (BRL).

were detected, although there appeared to be differences in the relative intensities of the shifted bands (data not shown). However, a small difference in the migration of one of the shifted bands was seen more clearly when the extracts were made from W23 and A619 plants carrying *Z.perennis* mitochondria (Figure 6A, arrows). It will be of interest to determine if, upon further fractionation of the mitochondrial protein extracts, differences in *cpc*-directed transcription initiation can be correlated with the protein binding patterns. Proteins also bind to the *upc* region, but no differences have been seen in the mobilities of the shifted bands that correlate with the extract source (Figure 6B, lanes A and W).

The cpc region is not a common constituent of Zea mitochondrial genomes

In order to assay the conservation of the upstream region of the *Zp-cox2* gene, mtDNAs from different maize and teosinte sources were digested with restriction enzymes, fragments were separated in agarose gels and filter blots of the gels were probed with the cloned coding and 5′ regions (Figure 7A and B). The *cox2* gene is not repeated within the mitochondrial genome; only one restriction fragment hybridized with the exon 1-specific probe in most cases (see Figure 7, probe 3). Immediately upstream of the *cox2* start codon, repeated DNA was evident (Figure 7, probe 2) and sequence comparisons showed that this is the *atp6* homologous region (see Figure 1C). Probes made beginning 100 nt upstream from the translational start demonstrated that a portion of the *Zp-cox2* upstream region is absent from maize N, T and S and from *Z.luxurians* mtDNAs (Figure 7B, probe 1). However, DNA gel blot analyses showed that there is strong homology with the *Zd-cox2* gene (Figure 7C, lane D) and with the CMS-C mitochondrial genome of maize (Figure 7C, lane C). No evidence for homologies to maize nuclear or chloroplast DNAs could be found by hybridization experiments (data not shown).

Sequence comparisons to those in the data banks showed that this region had no significant homologies to other DNAs, including all reported chloroplast DNAs and most mitochondrial DNAs. The only exceptions were a very strong similarity (92% identity) to 442 bp 'of unknown origin', which is part of the *atp6* chimeric gene in CMS-C mitochondria (Dewey *et al.*, 1991), and lesser similarities to tobacco, Ogura radish and *Brassica napus atp6* sequences (Bland *et al.*, 1987; Makaroff *et al.*, 1989; Handa and Nakajima, 1992). Figure 8 shows the alignments between the *cpc* region of the *Zp-cox2* gene and the 'ORF (open reading frame) of unknown origin' of the *C-atp6* gene. Alignments with the maize *atp6* (*Zm-atp6*) and maize *cox2* (*Zm-cox2*) genes between the *cpc* region and the *cox2* translational start codon are also illustrated.

Discussion

We have shown that a DNA sequence that is not a normal constituent of plant mitochondrial genomes can act as a site of transcription initiation only if the appropriate dominant allele of a nuclear gene, designated *Mct*, is present. *Zea perennis* and *Z.diploperennis* mitochondria contain this sequence upstream of their *cox2* genes. No other tested genes in the mitochondria of *Z.perennis* or *Z.diploperennis* show differences in the sizes of their major transcripts correlated with the presence of the *Mct* allele (Cooper *et al.*, 1990). Therefore, the use of the

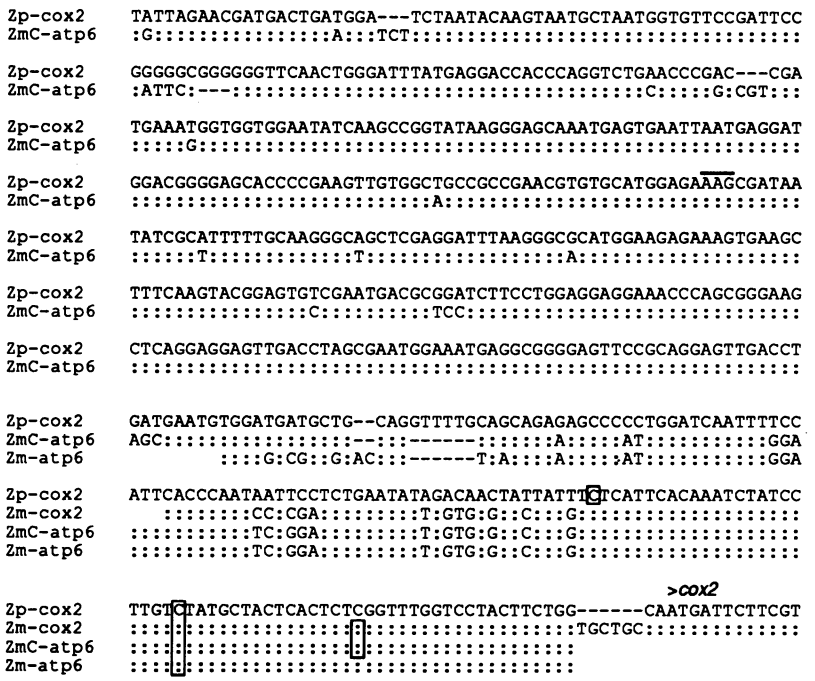

Fig. 8. Alignments between the *cpc* region of *Zp-cox2* and the 'ORF of unknown origin' of the *C-atp6* gene. In addition, the sequence alignments with the maize N cytoplasm *cox2* (*Zm-cox2*) and *atp6* (*Zm-atp6*) genes are shown. The *cpc* transcription initiation site is indicated by overlining and the RNA editing sites are boxed.

unusual mitochondrial DNA promoter appears to require both the novel sequence element at the locus and the appropriate allele of a nuclear gene.

The sequences surrounding the *Mct*-mediated transcription initiation site (located at −347 relative to the translational start) bear no resemblance to previously described plant mitochondrial promoters (see Mulligan *et al.*, 1991; Rapp *et al.*, 1993). In particular, the core YRTA motif associated with monocotyledon promoters is lacking. We have compared the conditional *cpc* promoter to a constitutive promoter of the *Zp-cox2* gene that lies further upstream. This *upc* sequence conforms closely to the proposed consensus for monocotyledon mitochondrial promoters (Rapp *et al.*, 1993). The first mitochondrial promoters analyzed from teosinte are the two that we report here. The *upc* is probably more typical of teosinte mitochondrial promoters, because, like most teosinte promoters, it does not require the dominant *Mct* allele to be functional.

Consistent with its sequence similarities to maize mitochondrial promoters, the *Z.perennis* constitutive *upc* promoter is active in the maize *in vitro* transcription extract described by Rapp and Stern (1992). It is interesting that the conditional promoter is not active in this extract, although the extract is made from plants that carry the dominant *Mct* allele. Not all promoters, including those for the mitochondrial ribosomal RNAs, are active in the *in vitro* transcription extract (see Rapp *et al.*, 1993), however, the *Zp-cox2 cpc* is the first promoter for a protein coding gene from maize or its relatives that has been shown to be inactive in this system.

The reasons for the lack of *in vitro* transcription initiation at the *Zp-cox2* conditional promoter are not yet clear. Although the sequences immediately surrounding the initiation site do not fit the maize mitochondrial consensus, the fact that transcripts initiate *in vivo* in this region in plants with the appropriate nuclear and mitochondrial genotypes is well documented by the G-capping data. The *in vitro* transcription system is a soluble protein extract further fractionated by differential ammonium sulfate precipitation and column chromatography; if the *Mct* locus encodes an integral membrane-bound protein or a protein that does not co-fractionate with the normal RNA polymerase activity, it would not be represented in the extract. An alternative explanation for the lack of *Mct*-mediated transcription *in vitro* would be that perennial teosinte mitochondria are required for the availability or activity of the MCT factor. So far, the only *in vitro* transcription extracts that have been tested were from plants with maize N mitochondria.

The origin of the 442 bp segment of DNA that is present in the *Zp-* and *Zd-cox2* upstream regions is unknown. We have shown that it is located downstream of a sequence implicated in recombination in cms-T mitochondria and immediately upstream of a portion of the *atp6* coding region that is also found in the same position 5′ to the maize N and T *cox2* genes. While the 442 bp sequence is not found in the mitochondrial, chloroplast or nuclear genomes of most maize and teosinte lines, a nearly identical sequence was reported upstream of the chimeric *atp6* gene of cms-C maize, where it was referred to as the 'ORF of unknown origin' (Dewey *et al.*, 1991; Kumar and Levings, 1993). Indeed, both the *Zp-cox2 cpc* and the corresponding *C-atp6* upstream regions could encode an in-frame polypeptide, but there is no evidence that this ORF is translated in either case.

We do not know the normal role of the product of the *Mct* gene in either maize or teosinte. Indeed, it is enigmatic that a maize nuclear gene product would appear to have specificity for a sequence that is absent from maize

mtDNA. However, we cannot exclude the possibility that a much shorter motif that includes the MCT site of action is found in one or several locations in maize mtDNA. Our genetic analysis has implicated *Mct* in the transcription of a novel conditional mitochondrial promoter. Other allelic products of this locus may be involved in the normal process of mitochondrial transcription. Our preliminary results indicate that the *cpc* region is recognized by proteins present in different types of mitochondrial extracts, however, binding is observed independently of the presence of *Mct*. Minor quantitative and qualitative differences were observed that did depend on the *Mct* genotype. Whether these binding activities involve the MCT product directly or indirectly and any understanding of their potential roles in transcriptional initiation will require further experimentation.

If the product of the *Mct* locus indeed represents a gene-specific transcription factor, this would have profound and unexpected implications. In the mitochondria of all animals and yeasts studied to date, a core polymerase and a single auxiliary factor are both necessary and sufficient to direct transcription initiation (reviewed by Jaehning, 1993). However, the mitochondria of these organisms differ from those of plants in that they have either two non-identical (animals) or ~20 highly conserved (yeast) promoters within their compact genomes. In maize, the genome complexity is greater than 500 kb (Lonsdale *et al.*, 1984; Fauron *et al.*, 1989) and, coupled with the very limited conservation of primary promoter sequences (Mulligan *et al.*, 1991; Rapp *et al.*, 1993; Stern and Rapp, 1993), this poses potentially unique challenges for the transcription apparatus. A more complex transcriptional machinery could reduce the frequency of aberrant initiation at 'promoter-like' elements that contain the core motif but lack other factor recognition sites. Alternatively, *cpc* may represent a new type of mitochondrial gene promoter and the *Mct* locus could encode a novel 'sigma-like' factor. Promoter switching has been documented for chloroplast promoters in the *psbD-C* region of several plant species, including maize and barley (Sexton *et al.*, 1990; Christopher *et al.*, 1992) and for the *psbE* operon of maize (Haley and Bogorad, 1990). These newly activated promoters do not resemble the prokaryotic promoters typically found in chloroplasts. Thus, there is a precedent for the activity of alternate promoters in plant organelles.

Materials and methods

Teosinte and maize stocks

The nuclear genome of the teosinte species *Z.perennis* (collected by Collins and Kempton) was replaced with that of maize by successively back-crossing with inbred lines A619 (*Mct/Mct*) or W23 (*mct/mct*). The initial five back-crosses were made at the University of Wisconsin by Jerry Kermicle and back-crossing was continued at the University of Missouri. Data presented here are from materials back-crossed at least 10 generations to the respective maize inbred lines. Some of the analyses were conducted on F1 material made by crossing Zp-A619 (plants that have *Z.perennis* cytoplasm and nuclear genes from A619) with pollen from B73 or a A188-B73-derived 'Hi-II' line ('tc'; see Armstrong, 1994), both of which also carry *Mct/Mct* alleles. Alternatively, hybrid plants were generated by crossing Zp-W23 plants with Mo17 (*mct/mct*) pollen. Ear shoots were used for the preparation of mitochondria; they were harvested from field or greenhouse grown plants when the silks had just emerged from the surrounding husk leaves.

Isolation and gel blot analysis of mitochondrial RNA and DNA

Mitochondrial RNA and DNA were prepared from 20–50 g of surface-sterilized ear shoots as detailed previously (Stern and Newton, 1986; Newton, 1994). The unpollinated ear shoots were harvested from field grown material when the silks had just emerged from the surrounding husk leaves. RNA samples were stored at −80°C and DNA samples at −20°C.

Total mitochondrial RNA (0.5 μg/lane) was electrophoresed through 1.2% agarose−6% formaldehyde gels, stained with ethidium bromide and blotted onto uncharged nylon filters (Magna 66; MSI) as previously described (Stern and Newton, 1986; Cooper *et al.*, 1990). RNA was UV-cross-linked to the filters in a BioRad GS Gene Linker. Estimation of RNA size was based on molecular size standards run alongside the samples (RNA Ladder; BRL).

Mitochondrial DNA was analyzed by digestion with restriction endonucleases and electrophoresis in 0.7 or 0.9% gels by standard techniques (Newton and Coe, 1986; Sambrook *et al.*, 1989) and estimates of restriction fragment lengths were based on comparisons with size standards (1 kb ladder; BRL).

Cloned restriction fragments containing the *Zp-cox2* gene were used to produce antisense and sense riboprobes (RNA Transcription Kit; Stratagene) according to the manufacturer's instructions. Standard conditions were used for probe hybridizations and washes (Stern and Newton, 1986; Sambrook *et al.*, 1989); stringent washes were with 0.1× SSC, 1% SDS at 65°C. The blots were exposed to X-ray film.

Cloning and sequencing of the Z.perennis cox2 gene and upstream regions

A 10.9 kb *Pvu*II fragment (from Zp-A619 mtDNA) containing the *Zp-cox2* gene was ligated into the *Hinc*II site of the pUC13 vector and transformed into SURE cells following standard procedures (Sambrook *et al.*, 1989). Subclones in pBluescript II KS$^-$ and SK$^-$ vectors (Stratagene) were generated from this ZpA49 clone and were propagated in XL1-Blue cells. The 1.8 and 1.7 kb *Bam*HI fragments were subcloned directly from ZpA49; the 0.6 kb *Eco*RI−*Hin*dIII and the 0.4 kb *Hin*dIII−*Eco*RV fragments were subcloned (designated RH0.6 and HRV0.4 respectively) from pB1.8. Single- and double-stranded sequencing reactions were performed using the Sequenase™ 2.0 kit (USB) according to the manufacturer's instructions. The sequence of the *Zp-cox2* gene and flanking regions has been assigned GenBank accession no. U16993.

RNA preparation, reverse transcription (RT)-polymerase chain reaction (PCR) and sequencing

Mitochondrial RNA was prepared from ear shoots as previously described (Cooper *et al.*, 1990). The mtRNA was treated with RNase-free DNase I and the elimination of any contaminating mtDNA was confirmed by PCR analysis. The DNA-free mtRNA was reverse transcribed with a primer TYPR19 (5′-ACGAGCGTTAGAGCTTTTTGAGCG; positions 1657–1634, assigning position 1 to the first nucleotide of the protein coding region), JYGPR2 (5′-AGAATGAGGAAGAAAAAGATATCGTGATGTA; 149–119) or TYPR32 (5′-TCGGAACACCATTAGCATTACTT; −522 to −544) (Figure 1C). This was followed by PCR with TYPR19 and TYPR18 (5′-ACAGAGAGCCCCCTGGATCATT; −130 to −107), JYGPR2 and TYPR11 (5′-CCGAGACTTCTGTTAATACAATCGCAA; −611 to −585) or TYPR32 and TYPR31 (5′-AGTCTCATTGGCCAAAGTAAAGT; −907 to −885). The PCR products were purified by agarose gel electrophoresis prior to the sequencing procedure. Sequencing was performed in the presence of detergent (0.5% Nonidet P-40) as originally described by Bachmann *et al.* (1990). The Sequenase™ 2.0 kit (USB) was used and Mn buffer was added to each reaction.

Analysis of end-labeled RNA

Aliquots of mitochondrial RNA (100 μg) were G-capped or labeled with polynucleotide kinase according to the methods of Mulligan (1994) and Mulligan *et al.* (1988b). The G-capped and kinased RNAs were hybridized with duplicate blots from 0.9% agarose gels following electrophoresis of the digested cloned DNAs. For RNase protection characterization of transcript initiation sites, slight modifications were made to Mulligan's methods. G-capping of mtRNA was performed in a 10 μl reaction containing 40–50 μg nucleic acid, 50 mM Tris−HCl, pH 7.9, 1.25 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.5 mM *S*-adenosylmethionine, 40 U RNasin (Promega), 4 U guanylyltransferase (BRL) and 125 μCi [α-^{32}P]GTP (3000 Ci/mmol). Reactions were incubated at 37°C for 30 min and then terminated. The capped RNA was extracted

once with phenol/chloroform (1:1), precipitated twice with 2.5 M ammonium acetate and 75% ethanol, to remove most of the unincorporated label, and resuspended in 20 µl of RNA hybridization buffer (40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA). Approximately 1–3×10^6 c.p.m. of capped RNAs were hybridized with sense and antisense RNAs transcribed from the *Zp-cox2* clones RH0.6 and HRV0.4. Protected initiation sites were detected by digestion with RNase A and RNase T1 and electrophoresis through 6% sequencing gels, with labeled *Hin*fI-digested ϕX174 DNA as molecular weight markers.

Primer extension analysis of RNA

To determine the 5′ ends of *cox2* transcripts, mtRNA from Zp-A619, Zp-W23 and the hybrids Zp-A619/tc (*Mct/Mct*) and Zp-W23/Mo17 (*mct/mct*) was reverse transcribed using primers BWPR11 (5′-AGATCCGCGTCATTCGACACTCCGTACTTG; positions −248 to −277 in Figure 1C) and BWPR13 (5′-ACTTTGAGATAGGGGTCGGCGTTGG; −765 to −789) utilizing standard protocols (Sambrook *et al.*, 1989). The primer was end-labeled with [γ-^{32}P]ATP and purified by electrophoresis through a 15% sequencing gel. After reverse transcription using Superscript Reverse Transcriptase (BRL), the products were electrophoresed through 6% sequencing gels. Sequencing reactions were carried out with the same end-labeled primers and with clone pB1.8 as template, using the one-step sequencing method (Ausubel *et al.*, 1992). The sequencing lanes were included on the gels with the primer extension products to identify the exact 5′ terminus.

In vitro transcription and primer extension reactions

In vitro transcription reactions contained 50 ng of template DNA and were carried out as previously described (Rapp and Stern, 1992; Rapp *et al.*, 1993). To determine the 5′ end of the *in vitro* transcription product, a 4-fold reaction was performed using *Pst*I-linearized BP1.0 DNA and the product was subjected to primer extension analysis (Rapp and Stern, 1992) using the 5′ end-labeled primer BWPR13. Annealing was performed at 50°C. Sequencing reactions were performed using the same end-labeled primer and a supercoiled plasmid DNA template.

Mobility shift DNA binding assays

To generate probes for the mobility shift assays, fragments were PCR-amplified using primers BWPR2 (5′-GGACCACCCAGGTCTGAA; positions −487 to −470 in Figure 1C) and KNPR3 (5′-ATCCGCGTCATTCGACACTCC; −250 to −270), for the *cpc* region, or TYPR36 (5′-CGATTCGTCTTTCTTGATCGGTT; −997 to −975) and BW12 (5′-GACATTTTTTGTAACGCATACACTAGAGC; −843 to −871), for *upc*. The PCR products were digested with *Hpa*II and *Taq*I respectively, labeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase and purified by PAGE as described in Ausubel *et al.* (1992). Purified mitochondria (see Newton, 1994), stored as pellets at −80°C, were used for freeze−thaw lysates. The mitochondrial pellets in a microfuge tube were resuspended in 1.5 volumes of TE (100:1) buffer containing 7.5% glycerol and 1 mM DTT and were frozen in liquid nitrogen, thawed three times and homogenized with a micropestle to yield a freeze−thaw lysate. The membrane fragments were removed by ultracentrifugation at 100 000 *g* for 30 min. The lysates were stored at −20°C after adding glycerol to a final concentration of 30%. Binding reactions (15 µl) containing ~10 µg of the mitochondrial protein, 12% glycerol, 4 µg poly(dI−dC):poly(dI−dC), 12 mM HEPES−NaOH, pH 7.9, 4 mM Tris−HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT were incubated for 5 min at room temperature, then 20 000 c.p.m. of labeled DNA was added and the incubation was continued for 30 min. The products of the binding reactions were assayed using high ionic strength PAGE as described (Ausubel *et al.* 1992).

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References

Allen,J., Emenhiser,G. and Kermicle,J. (1989) *Maydica*, **34**, 277–290.

Armstrong,C.L. (1994) In Freeling,M. and Walbot,V. (eds), *The Maize Handbook*. Springer-Verlag, New York, NY, pp. 663–671.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1992) *Short Protocols in Molecular Biology*. John Wiley & Sons, New York.

Bachmann,B., Lüke,W. and Hunsmann,G. (1990) *Nucleic Acids Res.*, **18**, 1309.

Bland,M.M., Levings,C.S. and Matzinger,D.F. (1987) *Curr. Genet.*, **12**, 475–481.

Brown,G.G., Auchincloss,A.H., Covello,P.S., Gray,M.W., Menassa,R. and Singh,M. (1991) *Mol. Gen. Genet.*, **228**, 345–355.

Christianson,T., Edwards,J.C., Mueller,D.M. and Rabinowitz,M. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 5564–5568.

Christopher,D.A., Minkyun,K. and Mullet,J. (1992) *Plant Cell*, **4**, 785–789.

Clayton,D.A. (1992) *Int. Rev. Cytol.*, **141**, 217–232.

Constanzo,M.C. and Fox,T.D. (1990) *Annu. Rev. Genet.*, **24**, 91–113.

Cooper,P. and Newton,K. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 7423–7426.

Cooper,P., Butler,E. and Newton,K.J. (1990) *Genetics*, **126**, 461–467.

Covello,P.S. and Gray,M.W. (1990) *Nucleic Acids Res.*, **18**, 5189–5196.

Covello,P.S. and Gray,M.W. (1991) *Curr. Genet.*, **20**, 245–251.

Dewey,R.E., Timothy,D.H. and Levings,C. (1991) *Curr. Genet.*, **20**, 475–482.

Doebley,J. and Iltis,H. (1980) *Am. J. Bot.*, **67**, 982–993.

Fauron,C., Havlik,M., Lonsdale,D. and Nicols,L. (1989) *Mol. Gen. Genet.*, **216**, 395–401.

Fox,T. and Leaver,C. (1981) *Cell*, **26**, 315–323.

Gracen,V. and Grogan,C. (1974) *Agronomics J.*, **66**, 654–657.

Gray,M.W., Hanic-Joyce,P.J. and Covello,P.S. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **43**, 145–175.

Gwynn,B., Dewey,R.E., Sederoff,R.R., Timothy,D.H. and Levings,C.S. (1987) *Theor. Appl. Genet.*, **74**, 781–788.

Haley,J. and Bogorad,L. (1990) *Plant Cell*, **2**, 323–333.

Handa,H. and Nakajima,K. (1992) *Curr. Genet.*, **21**, 153–159.

Hanic-Joyce,P. and Gray,M. (1991) *Mol. Cell. Biol.*, **11**, 2035–2039.

Hanson,M.R. and Folkerts,O. (1992) *Int. Rev. Cytol.*, **141**, 129–172.

Jaehning,J.A. (1993) *Mol. Microbiol.*, **8**, 1–4.

Kermicle,J. and Lonnquist,J. (1973) *Maize Genet. Coop. Newslett.*, **47**, 209–211.

Kumar,R. and Levings,C. (1993) *Curr. Genet.*, **23**, 154–159.

Laughnan,J. and Gabay-Laughnan,S. (1983) *Annu. Rev. Genet.*, **17**, 27–48.

Lonsdale,D.M., Hodge,T.P. and Fauron,C.M.R. (1984) *Nucleic Acids Res.*, **12**, 9249–9261.

Makaroff,C., Apel,I. and Palmer,J. (1989) *J. Biol. Chem.*, **264**, 11706–11713.

Mulligan,R.M. (1994) In Freeling,M. and Walbot,V. (eds), *The Maize Handbook*. Springer-Verlag, New York, pp. 559–565.

Mulligan,R., Lau,G. and Walbot,V. (1988a) *Proc. Natl Acad. Sci. USA*, **85**, 7998–8002.

Mulligan,R.M., Maloney,A.P. and Walbot,V. (1988b) *Mol. Gen. Genet.*, **211**, 373–380.

Mulligan,R.M., Leon,P. and Walbot,V. (1991) *Mol. Cell. Biol.*, **11**, 533–543.

Newton,K.J. (1994) In Freeling,M. and Walbot, V. (eds), *The Maize Handbook*. Springer-Verlag, New York, NY, pp. 549–556.

Newton,K. and Coe,E.J. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 7363–7366.

Rapp,W.D. and Stern,D.B. (1992) *EMBO J.*, **11**, 1065–1073.

Rapp,W.D., Lupold,S.L., Mack,S. and Stern,D.B. (1993) *Mol. Cell. Biol.*, **13**, 7232–7238.

Rottman,W.H., Brears,T., Hodge,T.P. and Lonsdale,D.M. (1987) *EMBO J.*, **6**, 1541–1546.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sexton,T.B., Christopher,D.A. and Mullet,J.E. (1990) *EMBO J.*, **9**, 4485–4494.

Stern,D.B. and Newton,K.J. (1986) *Methods Enzymol.*, **118**, 488–496.

Stern,D.B. and Rapp,W.D. (1993) In Brennicke,A. and Kück,U. (eds), *Plant Mitochondria*. VCH, Weinheim, Germany, pp. 181–197.

Yang,A.J. and Mulligan,R.M. (1991) *Mol. Cell. Biol.*, **11**, 4278–4281.

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