The Maize Mitochondrial cox2 Gene Has Five Promoters in Two Genomic Regions, Including a Complex Promoter Consisting of Seven Overlapping Units*

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Plant mitochondrial genes are often transcribed into complex sets of RNAs, resulting from multiple initiation sites and processing steps. To elucidate the role of initiation in generating the more than 10 cox2 transcripts found in maize mitochondria, we surveyed sequences upstream of cox2 for active promoters. Because the cox2 coding region is immediately downstream of a 0.7-kb recombination repeat, cox2 is under the control of two different sets of potential expression signals. Using an in vitro transcription assay, we localized four promoters upstream of the coding region in the so-called master chromosome, and two promoters upstream of the coding region in the recombinant subgenome. Ribonuclease protection analysis of labeled primary transcripts confirmed that all but one of these promoters is active in vivo. Primer extension was used to identify the promoter sequences and initiation sites, which agree with the consensus established earlier for maize mitochondria. This study identified two unusual promoters, the core sequences of which were composed entirely of adenines and thymines, and one of which was a complex promoter consisting of seven overlapping units. Deletion mutagenesis of the complex promoter suggested that each of its units was recognized independently by RNA polymerase. While each active promoter fits the maize core consensus sequence YRTAT, not all such sequences surveyed supported initiation. We conclude that in vitro transcription is a powerful tool for locating mitochondrial promoters and that, in the case of cox2, promoter multiplicity contributes strongly to transcript complexity.

The transcriptional strategies of vascular plant mitochondrial genomes differ from those of their fungal and metazoan counterparts, with a major region being genome structure. Plant mitochondrial genomes are relatively large and the genes are mostly dispersed (1, 2). In contrast, metazoan genomes are compact and have a single promoter for each DNA strand, and the Saccharomyces cerevisiae mitochondrial genome is 75 kb, and contains about 20 promoters (reviewed in Ref. 3). The maize mitochondrial genome, the expression of which we have studied, can be mapped as a 570-kb circle with numerous repeated elements mediating recombination, leading to a complex set of overlapping molecules (4). Based on recent microscopic studies (5), these molecules are likely to be linearly permuted.

Promoter analysis in plant mitochondria has been accelerated in the last several years by the development of in vitro transcription systems from wheat (6), maize (7), and pea (8). Extensive mutational analysis of the maize atpA and cox3 promoters showed that the only universally-present sequence required for transcription initiation in vitro was YRTAT (Y = T or C and R = G or A), located at or immediately upstream of the start site (9, 10). Even this degenerate sequence, however, is not found near all 5' termini identified as transcription start sites based on their ability to be capped in vitro by guanylyl transferase (11). While some of these promoters may be recognized by specific transcription factors (12), the frequency of active promoters has not been surveyed over large regions of any plant mitochondrial genome. Such an approach might aid in identifying contextual features other than YRTAT that constitute functional promoters, at least in maize mitochondria.

Maize cox2, which encodes subunit II of cytochrome c oxidase, was the first plant mitochondrial gene to be cloned and sequenced (13). RNA filter hybridization analysis of cox2 transcripts in maize mitochondria identified at least 11 distinct messages. Since cox2 contains only a single intron, multiple 5' and 3' ends must account for most of these RNA species. Here we investigate how genomic context and promoter multiplicity contribute to cox2 transcript diversity, and show that in vitro transcription is a useful means to identify previously unknown promoters.

**EXPERIMENTAL PROCEDURES**

*Plasmid and Cosmid Clones—Cosmids N5G8 and N6A6 (Fig. 1; Ref. 4) were obtained from Dr. Christiane Fauron (University of Utah). The BamHI and XhoI fragments illustrated in Fig. 2 were gel-purified and inserted into plasmid vectors, generating plasmids pN1, pN2, pN4, and pN7. To generate antisense single-stranded DNA for mapping of capped transcript 5' termini, the subfragments shown in Fig. 2 were inserted into pBluescript (Stratagene, Inc.) in the appropriate orientation. These plasmids were pN1VV (from the EcoRV site in pBS to the EcoRV site in pN1), pN2AR (from the XhoI to RsaI sites in pN2), and pN7F (from the ApaI site in pBS to the AatII site in pN7).

Oligonucleotides SL5 and SL6 (region B) and SL7B and SL9 (region A) were designed based on unpublished sequences provided by Dr. D. Lonsdale (John Innes Institute, Norwich, United Kingdom) or on sequence data that we generated from the region A cosmid, respectively. SL5 begins 804 bp upstream of the 0.7-kb repeat and has the sequence ACTGACTCTCTGATG, and SL6 begins 163 bp upstream of the repeat and has the sequence GATTAAACGGAGACTC. SL7B begins approximately 3190 bp upstream of the repeat and immediately upstream of the EcoRV site, and has the sequence CAACATGAGAAGATC. SL9 begins 10 bp upstream of the repeat and has the sequence AACGCGTTTGTCCCAT.
To generate the deletions of the complex promoter illustrated in Fig. 7, pN2 was linearized with MloI, which cleaves immediately upstream of promoter A2a. 10 μg of plasmid DNA was digested with 5 units of BstXI for 10–45 s at room temperature. The pooled products were repaired with the Klken fragment of DNA polymerase and religated. Escherichia coli colonies were screened by the polymerase chain reaction for inserts of the approximate size desired, and the precise deletion end points were determined by DNA sequencing.

In Vitro Transcription—Subclones of the cosmids were linearized with various restriction enzymes as shown in Fig. 3, and in vitro transcription was carried out as described previously (7). The sizes of transcripts were estimated by comparison to a DNA size ladder made using 32P-labeled X174 RF DNA digested with HindIII.

Capping of mtRNA and Primer Extension Analysis—RNA was isolated from gradient-purified mitochondria of 4-day-old dark-grown maize seedlings. 100 μg of RNA was capped using 15 units of guanylyl transferase and 250 μCi of [γ-32P]ATP. Sequence ladders were generated from appropriate plasmid clones using the same primers.

RESULTS

Recombination Across the 0.7-kb Repeat Places the cox2 Coding Region Downstream of Two Different Sets of Potential Expression Signals—Fig. 1 shows that the cox2 coding region is located immediately downstream of a 0.7-kb direct repeat sequence, present in two copies in the maize mitochondrial master chromosome. Recombination across these repeats generates the two postulated subgenomes shown at the right of Fig. 1. In the master chromosome, cox2 is preceded by sequences which we have designated region B, whereas in subgenome 1, cox2 is downstream of region A. Conversely, in the master chromosome region A is upstream of sequences of unknown function (designated NC), whereas region B is upstream of NC in subgenome 2.

Since the master chromosome and subgenomes co-exist in vivo (4),2 promoters driving cox2 transcription could be located in regions A and B, and/or in the repeat sequence. Fig. 2 shows a more detailed map of the A, B, cox2, repeat and NC regions, and the plasmid clones that were made in order to systematically search these regions for promoters using in vitro transcription. While most of region B, the repeat, and the cox2 coding sequence are present in the data base, region A is not, and therefore clones were mapped for restriction endonuclease cleavage sites. Closely spaced sites were required because our in vitro transcription assay best resolves 50–500-nt transcripts, thus our goal was to find unique sites 0.5 kb apart or less. Clones linearized at each unique site were used as templates for in vitro transcription.

Fig. 3 (top) shows examples of in vitro transcription reactions in which promoter activity could be identified. Other plasmid restriction endonuclease combinations did not yield resolvable transcripts. A summary of the clones tested and the transcripts obtained is given in Table I. Fig. 3 (bottom) summarizes the locations of promoters deduced from in vitro transcription. Two promoters are found in region A: A1, which is located approximately 3 kb upstream of the 0.7-kb repeat; and A2, which is immediately upstream of the repeat. Remarkably, A2 (hereafter referred to as the "complex promoter") gave rise to seven transcripts differing in size by 5 nt. Four promoters were found in region B, all clustered within 1 kb of the 0.7-kb repeat. No promoters were detected within the repeat. Taken together, these results suggested that cox2 could be transcribed from either the master chromosome or subgenome 1, and that up to six promoters, including a complex promoter, might contribute to RNA accumulation in vivo.

Five of the Six Promoters Identified in Vitro Are Active in Vivo—It was possible that in vitro transcription would give artifactual promoter activity at a site that was not used in vivo. To determine whether the in vitro start sites coincided with primary transcript 5' ends, we used a combination of in vitro guanylyl transferase capping (G-capping) and RNase protection. Since plant mitochondrial transcripts are not capped in vivo, primary transcripts (those with di- or triphosphate ter-

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Mini) can be labeled in vitro with $[^{32}\text{P}]\text{GTP}$. Total maize mitochondrial RNA was labeled in this way and then annealed to ssDNA complementary to the $\text{cox2}$ regions containing putative promoters. The hybrids were digested with RNase A and analyzed by gel electrophoresis.

**Fig. 3. Mapping of $\text{cox2}$ promoters using in vitro transcription.** The top panels show in vitro transcription products for each of the active templates, which are labeled by the number of the subclone and the restriction endonuclease used to linearize it (e.g. 7T is the N7 subclone linearized at the AatII site). The transcript size in kb and promoter identified are indicated at the left for each set of lanes. The bottom panels are maps of the regions searched for promoters for $\text{cox2}$, templates used, transcripts obtained, and promoters identified. The promoters are shown as bent arrows and are labeled based on the region in which they are found and order in which they occur, and the 0.7-kb repeats are represented by gray arrows. See the legend to Fig. 2 and Table I for additional details.

**Fig. 4.** A shows the protected primary transcripts corresponding to promoters A1, A2, B1, B3, and B4. The promoter identifications were based on the sizes of protected transcripts, coupled with knowledge of the in vitro start sites (Fig. 3) and primer extension (see below). Fig. 4B summarizes the verified promoters and the ssDNAs used to protect the primary transcripts. Of the promoters identified by in vitro transcription, only B2 was not identified by capping. This suggests that either B2 is an in vitro artifact, or that the primary transcript from B2 is rapidly processed in vivo. Although we cannot distinguish between these two alternatives, the weak usage of B2 in vitro (Fig. 3) suggests that at best, B2 is a marginal promoter in vivo. Taken together, these results confirm that $\text{cox2}$ is transcribed by multiple promoters in two distant genomic regions, and that the maize mitochondrial in vitro transcription system can be
reliably used to locate promoters in sequences of unknown function.

Each cox2 Promoter Fits the Previously Established Consensus—We have previously determined the essential features of maize mitochondrial promoters by using several types of tagenesis (7, 9, 10). A key feature of these promoters is the so-called core sequence, which has the consensus YRTAT; mutations in the core severely reduce or abolish transcription 

Table 1: In vitro transcription templates and their products

| Region | Clone | Enzyme | Transcript size | Promoter(s) |
|--------|-------|--------|-----------------|-------------|
| A      | N1    | XbaI   | None            | None        |
| A      | N1    | EcoRI  | 200             | A1          |
| A      | N1    | NsiI   | None            | None        |
| A      | N1    | XhoI   | None            | None        |
| A      | N2    | BglII  | 500             | A1          |
| A      | N2    | XhoI   | 250             | A2          |
| B      | N4    | BglII  | 300             | B1          |
| B      | N4    | EcoRI  | 400             | B1          |
| B      | N7    | SpeI   | None            | None        |
| B      | N7    | Stul   | None            | None        |
| B      | N7    | XhoI   | 280             | B1          |
| B      | N7    | AccI   | 500             | B1,B2       |
| B      | N7    | AatII  | 500             | B3,B4       |

The promoter sequences and initiation sites are summarized in Fig. 6, and compared with the consensus sequence derived for the atpA promoter (9). All of the promoters had sequences within 5 bp upstream of the start site that fully matched the consensus sequence for the core region, either CGTAT or TATAT. Their match with the overall consensus sequence was

![Image of Fig. 4: Identification of cox2 primary transcripts by G-capping. A. results of RNase protection of guanylyl-transferase-labeled primary mitochondrial transcripts by the ssDNAs indicated at the top of each panel. 250 or 50 ng of RNase A were used in each experiment. HindIII-digested plX174 markers are in the left lanes of each panel and approximate sizes (in nt) are shown at the left. The promoters verified by the protected primary transcripts in each panel are indicated at the right. B, a diagram summarizing the results. The promoters are shown as bent arrows, the 0.7-kb repeats are represented by gray arrows, and the arrowed lines below each diagram represent the ssDNAs used for RNase protection. See the legend to Fig. 2 for additional details.](image-url)
variable, ranging between 79% and 100%. The importance of the consensus sequence apart from the core is suggested by the fact that promoter A1, which was the weakest promoter in vitro, had the lowest percent match. Interestingly, promoters A2b–g and B3 are the first mitochondrial promoters identified in which the core sequences are composed of only A and T nucleotides.

Deletion Mutagenesis Suggests That the Complex Promoter Is Recognized Stochastically—The complex promoter A2 is highly unusual and consists of one “typical” CGTAT core sequence promoter, followed by seven copies of the repeated sequence GTATATA. This repeated sequence contains a putative TATAT promoter, followed by seven copies of the repeated sequence GTATATA. This repeated sequence contains a putative TATAT core sequence, and gives rise to six additional overlapping promoters, with the overlap coming between the core and the purine-rich upstream domain (see Fig. 6).

We envisioned two models for recognition of the complex promoter by the transcriptional machinery, as diagrammed in Fig. 7C. In the scanning model, the first (CGTAT core) promoter nucleates the transcriptional apparatus, which then either initiates transcription or scans downstream sequences for an alternative promoter. In the stochastic model, the transcription apparatus can independently recognize each individual promoter, although since the mitochondrial genome is multipartite this could be occurring on separate molecules. The RNase protection and primer extension data (Figs. 4 and 5) show that approximately twice as many transcripts initiate at the first promoter as at each of the downstream promoters, which yield similar amounts of transcripts. These results could favor the stochastic model, if one assumes that CGTAT is a stronger core sequence than TATAT, or if one assumes that the downstream promoters are disfavored because they are overlapping. However, a scanning model also predicts increased initiation at the first promoter, but also a successive reduction in initiation at each following promoter, which is seen in Fig. 3 (in vitro) but not in Figs. 4 and 5 (in vivo).

To determine whether the first promoter was required to anchor transcription as proposed by the scanning model, we generated Bal31 deletion mutants lacking the first promoter and variable amounts of the downstream promoters, as shown in Fig. 7A. These constructs were used for in vitro transcription analysis, as shown in Fig. 7B. In each case, the remaining promoters are transcriptionally active, which rules out a scanning model dependent on the CGTAT-containing promoter. In the analysis of these deletion mutants, we noted that there was some ambiguity in the assignment of start sites. In particular, in the WT lane there is an additional diffuse band migrating slower than the band that we believe represents promoter A2a, and in the deletion constructs the number of residual start sites did not always coincide with the expected number. For example, we expected two additional start sites in mutant Δ3 versus Δ5, but if this is the case the two largest transcripts from Δ3 were unexpectedly weak. These subtleties may result from sequence contexts arising out of the ligation of different bidirectional deletions.

DISCUSSION

Here we have looked globally at promoters potentially driving maize mitochondrial cox2 transcription. The screen for promoters was carried out using an in vitro transcription system, and the reliability of this approach was confirmed in five of six cases using analysis of mtRNA. Each promoter conforms to a previously established consensus sequence for maize mitochondrial promoters, although there are some with unusual features. This promoter multiplicity can help account for the complexity of accumulating cox2 transcripts.

Approximately 8.4 kb of DNA was examined for promoter activity in this study. Of the six promoters active in vitro, each was flanked by a sequence fitting the consensus YRTAT, with little homology upstream (see Fig. 6). Clearly, if YRTAT were the only requirement for promoter activity, a much larger number should have been detected. For example, in the part of region B that we surveyed (4.75 kb), YRTAT appears 18 times, including 5 matches of CGTAT, the most common core motif in maize mitochondrial promoters (11). The number of YRTAT matches is close to that expected by chance, suggesting that there is no bias to exclude such sequences from the genome. These results suggest that YRTAT is necessary, but not sufficient for promoter activity in our assay system. It is possible that other features such as DNA bending, which occurs at human and yeast mitochondrial promoters (14, 15), contribute to promoter recognition and usage in maize mitochondria.

Promoter B2 was active in vitro but not in vivo. While this result could be explained by a rapid processing of B2-initiated transcripts in vivo, it is also possible that this promoter-like sequence is not recognized in the cellular environment. For example, a conserved sequence that acts as an 18 S rRNA promoter in Arabidopsis, potato, and Oenothera is inactive in pea (16). This again points to features other than the primary sequence that mediate promoter site selection. Interestingly, the yeast mitochondrial genome also contains many promoter-like sequences that are not active in vivo but are active in vitro (17). Analysis of these variants suggests that they are inactive in vivo not because RNA polymerase is unable to bind, but instead because elongation does not occur. Thus, multiple criteria must be satisfied if transcription initiation is to occur successfully.

Some of the promoters identified in this study are far from the cox2 coding region, such as A1 (>4 kb) and B1 (>1.5 kb).
The fact that these are used for cox2 transcription in vivo is consistent with the lengths of some cox2 transcripts (13) as well as reverse transcription-polymerase chain reaction results in which cox2-primed cDNAs could be amplified with primers downstream of each promoter.² While such a distance between coding region and promoter is unusual, it is not unprecedented. For example, the atpA promoter is more than 2 kb upstream of the coding region, and there may be another promoter even further upstream (11). Furthermore, several other maize mitochondrial genes have multiple promoters (18, 19). So far, however, the combination of promoter distance and number, along with the presence of the coding region downstream of a recombinant repeat, places cox2 in a unique category.

The 0.7-kb repeat upstream of cox2 contains no promoters, and does not flank the cox2 gene in other maize cytoplasms such as cms-T and NA188 (1). Thus, the configuration in the genome we examined (NB37) is the only one of the three in which repeat-driven transcription could express cox2. In the maize ancestor teosinte, the cox2 transcription pattern is simple (20) and driven by two promoters, one of which is dependent on a nuclear locus that may encode a specific transcription factor (12). These data highlight the plasticity of plant mitochondrial genomes, and the multiplicity of promoters may ensure the expression of genes despite frequent mtDNA rearrangements during evolution (21).

Two unusual promoters were uncovered by this study, namely A2 and B3. Both of these promoters have the novel core consensus TATAT, except A2a, which has a classic core motif. While we have not tested the importance of each of these positions in the cox2 promoters by mutagenesis, there is no other nearby sequence that matches the consensus, and indeed more than 10 bp upstream of promoter B3 consist of A or T. The ability of all-A+T sequences to function as mitochondrial promoters is illustrated indirectly by the recovery of two yeast petite mutants which are able to replicate, yet possess mitochondrial genomes consisting only of A-T base pairs (22). Since replication is most likely initiated with an RNA primer (23), one can infer that transcription initiation occurs within these A-T sequences.

Promoter A2 consists of seven units punctuated by guanosines, and these units overlap if one includes the upstream purine-rich region. Each of these units can be recognized both in vitro and in vivo, which is most consistent with them acting independently. Our results do not address the question of whether more than one transcription complex can simultaneously bind to this region. This complex promoter could have arisen by slippage during DNA replication, a mechanism that probably can generate short tandem repeats in chloroplasts (24). Overlapping promoters are somewhat unusual in the literature, and we have not found anything closely

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**Fig. 6. Transcription initiation regions for cox2.** Numbering at the top is with reference to the consensus sequence derived from analysis of atpA, as described in the Introduction, where +1 is the transcription initiation site. The columns marked “txn start” and “promoter” refer to the initiation site relative to the +1 position of the atpA-based consensus sequence and the cox2 promoter identifier, respectively. Bases not matching the consensus are shown in lowercase, and the boxes containing them are shaded. In the consensus sequence, R = G or A, Y = C or T, N = no base preference, and “?” indicates that the position was not tested by mutagenesis (9).

| -13 | -12 | -11 | -10 | -9 | -8 | -7 | -6 | -5 | -4 | -3 | -2 | -1 | +1 |
|-----|-----|-----|-----|----|----|----|----|----|----|----|----|----|----|
| **upstream domain** | **promoter core** | **→ consensus** |
| R | R | ? | N | N | Y | N | R | Y | R | T | A | T | ? |
| G | T | A | A | C | A | A | A | C | G | T | A | T | +3 |
| G | A | G | A | A | A | C | G | T | A | T | +5 | A2a |
| A | G | T | A | T | A | G | T | A | T | +2 | A2b-g |
| A | A | G | A | A | A | T | G | C | T | A | T | +5 | B1 |
| A | T | A | T | A | T | A | T | A | T | +4 | B3 |
| A | G | A | A | A | A | A | T | G | C | T | A | T | +5 | B4 |

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**Fig. 7. Deletion mutagenesis of the complex promoter A2.** A, sequences of the wild-type complex promoter and its deletion derivatives. The promoters are shown as bent arrows with the CGTAT core promoter in black and the others in gray. The CGTAT core and one of the repeat units are underlined in the WT sequence. B, in vitro transcription results for WT and deletion mutants A1–A5. The transcripts are labeled with the corresponding promoter from which they originate. An alternative interpretation is that the diffuse band above that labeled “a” is in fact the A2a transcript from WT, which would imply that the band labeled “g” is an artifact. The identification of bands in this experiment for the WT construct was based on its resemblance to earlier experiments, such as the one shown in Fig. 3 C, two models for complex promoter recognition, as discussed under “Results.” RNA polymerase is represented as having two subunits, based on the known characteristics of the yeast mitochondrial enzyme.
rearrangements, including intragenomic recombination, as to maintain gene expression in the face of frequent genome possibility. Another reason to maintain multiple promoters is been shown to vary between tissues, consistent with this chondrial transcripts (27) and translation products (28) have translated regions influence translational yield. Maize mitochondrial transcripts are a consequence of a promiscuous transcription patterns are a consequence of multiple promoters, contrasting point of view to the above is that complex transcriptional stability as another key control point in gene expression. A coding regions in maize mitochondria (31) points to RNA plast genes (30). Evidence for transcription of known non- for gene-specific promoters, as is the case for some chloro- with RNA processing events could substitute in some cases transcription termination may not be efficient in plant mito- maize. One could speculate that this arrangement lends flex- mitochondria, and seems to be particularly prevalent in remain to be determined.

In summary, promoter multiplicity is not uncommon in mitochondria, and seems to be particularly prevalent in maize. One could speculate that this arrangement lends flexibility to modes of gene expression, particularly if promoters are differentially used and if the resulting different 5′-untranslated regions influence translational yield. Maize mitochondrial transcripts (27) and translation products (28) have been shown to vary between tissues, consistent with this possibility. Another reason to maintain multiple promoters is to maintain gene expression in the face of frequent genome rearrangements, including intragenomic recombination, as discussed above. However, in vitro experiments suggest that transcription termination may not be efficient in plant mitochondria (29), meaning that read-through synthesis along with RNA processing events could substitute in some cases for gene-specific promoters, as is the case for some chloroplast genes (30). Evidence for transcription of known non-coding regions in maize mitochondria (31) points to RNA stability as another key control point in gene expression. A contrasting point of view to the above is that complex transcriptional patterns are a consequence of multiple promoters, which themselves are a consequence of a promiscuous transcriptional machinery.

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