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Genomic context influences the activity of maize mitochondrial *cox2* promoters

(mitochondrial recombination/transcription initiation/reverse transcriptase–PCR)

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ABSTRACT **Plant mitochondrial genomes are highly recombinogenic, with a variety of species-specific direct and inverted repeats leading to *in vivo* accumulation of multiple DNA forms. In maize, the *cox2* gene, which encodes subunit II of cytochrome *c* oxidase, lies immediately downstream of a 0.7-kilobase direct repeat, which is present in two copies in the 570-kilobase master chromosome. Promoters for *cox2* exist upstream of both of these copies, in regions we have termed A and B. Three region B promoters are active for *cox2* transcription in the master chromosome, whereas two region A promoters are active for *cox2* transcription after recombination across the direct repeats. We have measured the proportion of genomes carrying region A or B upstream of *cox2* in maize seedlings and found a ratio of approximately 1:6. Promoter strength, based on run-on transcription assays, shows a ratio of 1:4 for region A to region B promoters. These data allowed us to predict the relative contributions of region A and B to mitochondrial transcript accumulation, based on a simple product of genome-form abundance and promoter strength. When promoter use was determined by using quantitative reverse transcriptase–PCR, however, we found that region A promoters were used at an unexpectedly high rate when upstream of *cox2* and used less than expected when not upstream of *cox2*. Thus, the use of this set of promoters seems to respond to genomic context. These results suggest a role for intragenomic and intergenomic recombination in regulating plant mitochondrial gene expression.**

Plant mitochondrial genomes are approximately 200–2,000 kilobases (kb) in size, and for many of the genomes that have been physically mapped, all of this DNA can be represented as a single circular molecule or master chromosome (reviewed in ref. 1). However, most plant mitochondrial genomes contain recombinationally active repeated sequences (2), spawning a variety of inversions or excisions that have led to complex models to represent extant genome structure (3–7). Furthermore, extensive analysis of plant mtDNA with a variety of gel electrophoresis techniques suggests that linear, rather than circular, forms are most predominant *in vivo* (8). These different forms may be present in widely differing stoichiometries (9), and recent evidence suggests, under certain circumstances, relative copy number may determine plant phenotype (10).

Plant mitochondrial recombination also plays an important role in generating novel genomes. Most commonly, recombination across short direct repeats (and possibly nonhomologous recombination) generates novel ORFs whose products disrupt mitochondrial function and cause cytoplasmic male sterility (reviewed in ref. 11). In other cases, recombination can cause rearrangements or deletions that affect plant development, e.g., in the nonchromosomal stripe mutants of maize (12, 13) or in the chloroplast mutator background in *Arabidopsis* (14). The role of recombination in normal organelle gene expression and function, however, is largely unexplored.

The master chromosome of maize mitochondria can be represented as a 570-kb circle containing multiple direct and inverted repeated elements (15). Among these, one copy of a two-copy direct repeat of 0.7 kb lies immediately upstream of the *cox2* gene, which encodes subunit II of cytochrome *c* oxidase, with the second copy approximately 20 kb distant (16). Because this repeat element contains no detectable promoter activity (17), *cox2* transcription relies on sequences upstream of the repeats. In the master chromosome, *cox2* is flanked by what we have termed region B, which contains three nearby promoters, whereas in recombinant forms of the genome, *cox2* is flanked by region A, which contains two more widely spaced promoters (17). The region A promoter closest to *cox2* is an unusual, complex promoter, containing a small repeated element giving rise to multiple initiation sites. The genome configurations and promoters are summarized in Fig. 1.

In this paper, we use *cox2* as a model to study the influence of genome configuration on mitochondrial transcription in maize. To do this study, we have measured the relative copy numbers of master and recombinant genomes, *cox2* promoter strength, and actual promoter use based on quantitative reverse transcriptase–PCR (RT-PCR). Our results indicate that genomic context can indeed influence transcription, supporting our and others' contentions that mitochondrial promoter strength is not based solely on primary sequence (18, 19) and raising the possibility that recombinational equilibria may modulate mitochondrial gene expression in plants.

MATERIALS AND METHODS

Nucleic Acids, DNA Filter Hybridizations, and Run-On Transcription Assays. The cosmid and plasmid subclones of the *cox2* region have been described (17), except for pN6, which is shown in Fig. 1*B*. pN6 was subcloned into pBluescript SK(−) (Stratagene) from cosmid N6A6 as a 5.35-kb *Xho*I fragment. pN6 was sequenced with primer 17 (Fig. 1*B*) to locate the *Stu*I site in the NC region, to find the actual border of the 0.7-kb repeat, and to determine the DNA sequence for the design of primer 19. DNA (20) and RNA (21) were purified from mitochondria of 4-day-old dark-grown maize seedlings as described. For the filter hybridization shown in Fig. 2, a repeat-specific *Xho*I–*Eco*RI fragment (Fig. 1*B*) was used. After hybridization, the final wash was in 0.2× SSC (0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.1% SDS at 65°C. Labeling of mitochondrial transcripts in purified organelles was carried out as described by Mulligan *et al.* (22). The 3× probe shown in Fig. 3 contained approximately 3×10^7 incorporated cpm. The final wash for this filter was performed

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Abbreviations: kb, kilobase; RT-PCR, reverse transcriptase–PCR; NC, noncoding.

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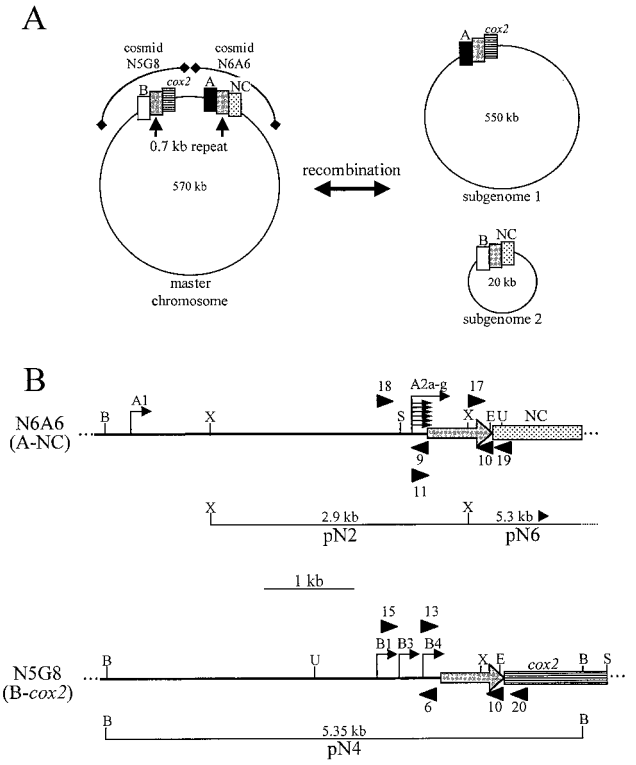

FIG. 1. Maize mitochondrial genome and *cox2* promoter structure. (*A*) The master chromosome of maize mtDNA represented as a circle with relevant DNA elements discussed in the text shown (not to scale). The two cosmid clones used to analyze *cox2* sequences and expression are shown at the top; they were obtained from Christiane Fauron (University of Utah, Salt Lake City, UT). (*B*) Detail of regions A and B, containing *cox2* promoters. Only the master chromosome configurations are shown. Arrowheads represent oligonucleotide primers, which are not shown to scale but accurately reflect their locations relative to promoters and the 0.7-kb repeat. Plasmid subclones are shown below the maps; they have been described elsewhere (17) or in *Materials and Methods*. Relevant restriction sites shown are B, *Bam*HI; X, *Xho*I; S, *Sal*I; E, *Eco*RI; and U, *Stu*I. NC is an apparently noncoding region.

as described immediately above. Hybridized filters were treated with RNase; they were incubated for 2 h at 37°C in 10 mM Tris·HCl, pH 7.7/5 mM EDTA/300 mM NaCl containing 125 μg of RNase A and 17 units of RNase T1. The filters were again washed in 0.2× SSC/0.1% SDS at 65°C.

PCR and RT-PCR. Oligonucleotide primers were obtained commercially. Their positions are shown in Fig. 1*B*, and their purposes are described in the text or figure legends. Their sequences are available on request. For the PCR experiment shown in Fig. 4*A*, conditions were as described below for amplification of cDNAs. For the experiment shown in Fig. 4*B*, conditions were identical, except reactions were carried out in 100 μl by using 1.5 mM $MgCl_2$ and 0.2 μl of *Taq* DNA polymerase (Promega). The annealing temperature used for primer 20 was 45°C, and that for primer 19 was 50°C; the 45°C temperature for primer 20 gave more consistent results.

RT was carried out in 44 μl of PCR buffer containing 1.5 mM $MgCl_2$ (23) in the presence of 1 unit/μl RNAsin (Promega) and 220 ng (5 ng/μl) DNase I-treated mtRNA. After addition of the RT primer, 10% of the reaction was stored on ice as a negative control, which never generated a product in the subsequent PCR (data not shown). To the remainder of the reaction, 1 μl of Superscript AMV RT (GIBCO/BRL) was added, and incubation was carried out for 1 h at 37°C.

To carry out the quantitative PCR step, different amounts of cDNA, as detailed in the legend to Fig. 5, were added to a

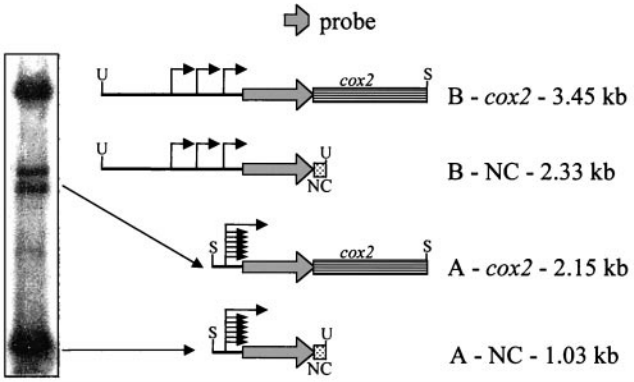

FIG. 2. Analysis of *in vivo* recombination across the 0.7-kb repeat. mtDNA (10 μg) was digested with *Sal*I and *Stu*I, separated in a 1.2% agarose gel, and blotted to a nylon membrane. The filter was probed with the repeat-specific *Xho*I–*Eco*RI fragment (Fig. 1*B*) as described in *Materials and Methods*. The four repeat-containing genome configurations are shown at the right, and their relative amounts were determined by using a PhosphorImager. This experiment was repeated twice with results in close agreement. The faint hybridizing fragment of approximately 1.6 kb is most likely caused by a short sequence in the probe that is present at an unknown location elsewhere in the mitochondrial genome. Such short and possibly degenerate repeats have been observed previously in plant mtDNA (54).

reaction with a final volume of 50 μl of PCR buffer, which contained 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.3 μl of *Taq* DNA polymerase. The reaction was denatured at 94°C for 3 min, and then 25 cycles were carried out with the following parameters: 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. A final elongation step of 72°C for 7 min was carried out before analysis of the products in 1.2% agarose gels that were stained with ethidium bromide. To quantify the products, the gels were photographed with an Eagle Eye II (Stratagene), and the stored files were analyzed with IMAGEQUANT software (Molecular Dynamics). In one case, the gel was filter blotted and hybridized with a repeat-specific *Xho*I–*Eco*RI fragment as a probe (Fig. 1*B*) to ensure that densitometry reflected the amount of PCR product accurately.

Multiple experiments were carried out to ensure the lack of DNA contamination in RNA preparations and to show that the amount of PCR product was linear with respect to the amount of cDNA added. In addition, one experiment was carried out in which the repeat primer (primer 10; Fig. 1*B*) was 5′ end labeled with polynucleotide kinase and [γ-^{32}P]ATP before PCR. The gel-separated products were quantified by using a PhosphorImager (Molecular Dynamics), and the results agreed with those obtained by using the nonradioactive method detailed above (data not shown).

RESULTS

Genomic Locations of *cox2* Promoters. Fig. 1*A* shows the genomic configurations upstream of the *cox2* coding region either in the master chromosome or in two hypothetical subgenomic circles, which would be derived by recombination across the directly repeated 0.7-kb element (16). However, there are five additional repeated elements in the maize mitochondrial genome (15), and thus an essentially infinite number of recombination products is possible. For the purposes of this paper, we are concerned with *cox2* transcription, and thus the key point is whether *cox2* is downstream of region B, as shown in the master chromosome, or downstream of region A, as it would be in any configuration product in which the two 0.7-kb repeats had recombined. For convenience, the master chromosome configurations are referred to as A-NC and B-*cox2*, and the recombinant configurations are referred to as A-*cox2* and B-NC. Fig. 1*B* shows the locations of known

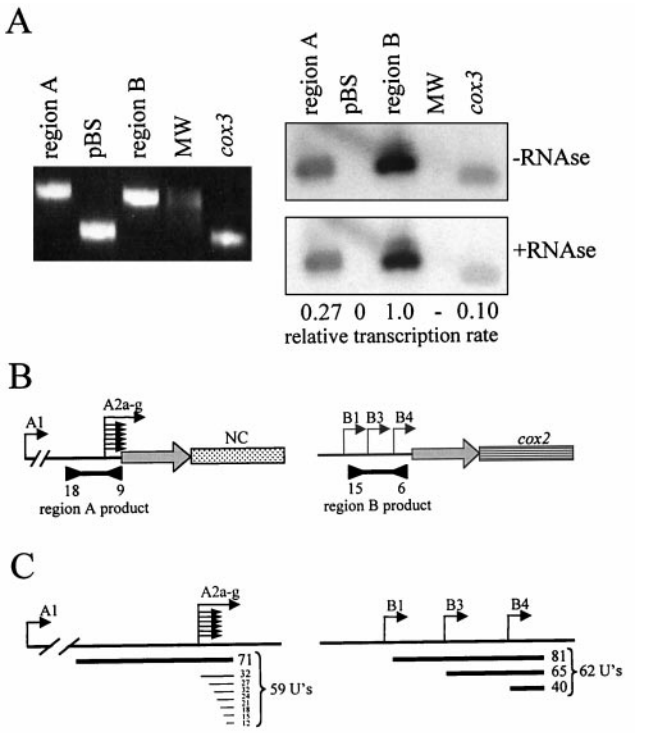

FIG. 3. Run-on transcription measurements of *cox2* promoter activity. (*A Left*) An ethidium bromide-stained 1% agarose gel of PCR fragments representing region A promoters (pN2 and primers 9 + 18), (*A Right*) pBluescript KS(+) (pBS; primers T3 and T7), region B promoters (pN4 and primers 15 + 6), and *cox3* (primers BR20 + GA355; ref. 19). (*A Right*) ^{32}P-labeled transcripts from isolated mitochondria were hybridized with a filter blot of a similar gel and treated as described in *Materials and Methods*. The relative transcription rates were calculated by using a PhosphorImager. MW, molecular weight markers. (*B*) Maps of the master chromosome configurations in regions A and B, showing locations of the products amplified by PCR. The region A product is 287 bp and the region B product is 277 bp. Note that the stained and probed blots in *A* are different, and thus the migration is not identical. (*C*) Uridine content of initiated transcripts within each PCR product. The thickness of each line represents relative promoter strength as discussed in *Results*. To the right of each hybridizing segment, the number of uridines is indicated. The number outside of the brackets (59 or 62) indicates the average number of uridines in region A or B hybridizing transcripts, as discussed in *Results*.

promoters; these promoters were identified based on *in vitro* transcription and capping analysis of mtRNA (17). For the promoters, only the master chromosome configuration is shown. In Fig. 1*B*, a map of relevant clones, restriction sites, and oligonucleotide primers also is shown (see also *Materials and Methods*).

Ratio of Master and Recombinant Genomes. To determine the relative numbers of master and recombinant genomes in the mitochondria of etiolated maize seedlings, mtDNA was digested with *Sal*I and *Stu*I, and a DNA filter hybridization was performed by using a repeat-specific *Xho*I–*Eco*RI fragment (Fig. 1*B*) as a probe. A representative result is shown in Fig. 2. Master chromosome configurations A-NC and B-*cox2* are represented by the smallest and largest hybridizing fragments, respectively, and clearly constitute a majority of the repeat-hybridizing DNA. The recombinant configurations are represented by the two middle hybridizing fragments. Quantitation of this and another gel blot with a PhosphorImager revealed that the proportion of the recombinant-to-master configuration was 1:6. This configuration suggests that recombination across the 0.7-kb repeat is relatively infrequent and/or that the recombination products are underreplicated or less stable.

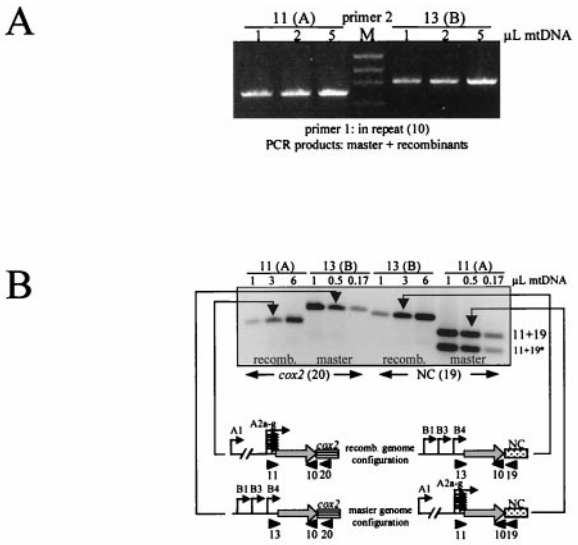

FIG. 4. Control reactions for RT-PCR analysis, with mtDNA at a concentration of 1.25 ng/μl. (*A*) Experiment showing that both primer pairs amplify mtDNA with approximately equal efficiency. Both reactions contained primer 10, and the second primer and its upstream promoter set are shown above each set of three lanes. The amount of mtDNA in each reaction is given in microliters. M represents molecular weight markers (ØX174 digested with *Hae*III). (*B*) Experiment showing that primer pairs amplify the correct genome configurations in the expected stoichiometry. The primer pairs are indicated diagrammatically at the bottom, as well as above and below each set of lanes. An additional band for primer pair 19 + 11 of unknown origin is marked with an asterisk; the upper band is the expected one based on its size. The unknown band appears at different Mg^{2+} concentrations and annealing temperatures (data not shown). The amount of mtDNA in each reaction is indicated in microliters.

Strength of *cox2* Promoters. To determine the overall strength of region A and region B *cox2*-proximal promoters, we employed a run-on transcription assay. This assay (22) measures the incorporation of labeled ribonucleotide triphosphates into preinitiated transcripts, and the short time of labeling minimizes transcript degradation (see also ref. 24). In addition, because the labeled transcripts are used as probes, even partially degraded RNAs will give hybridization signals.

To measure *cox2* transcription, isolated mitochondria were pulsed for 10 min with [α-^{32}P]UTP, and the labeled transcripts were hybridized with filter blots containing PCR-generated DNA fragments corresponding to region A, region B, and two controls: a vector fragment as a negative control [pBluescript KS (+)] and a fragment of the *cox3* gene. The region A and B PCR products were of similar size, and their locations are shown in Fig. 3*B*. To ensure that the filter-bound DNA was in excess, blots were hybridized with two dilutions of the probe. We found that the same relative signals were obtained with 3-fold different amounts of probe, indicating that the PCR products were in excess during the hybridization reaction (data not shown). To detect any signals from labeled transcripts extending beyond the end of the PCR products, we compared signals from blots either treated or not with RNase A after hybridization. Again, no differences in relative signal were seen, suggesting that any such effects were equivalent for each region tested (compare −RNase and +RNase in Fig. 3*A*). Finally, it is important to note that in this experiment, there is no way to distinguish between promoter activity in master or recombinant genomes. Thus, what is being measured is the overall relative strength of region A vs. region B promoters.

Fig. 3*A* shows the result of a typical run-on experiment. As expected, no hybridization was seen with the vector [pBluescript KS(+)] control or the molecular weight markers, whereas hybridization was seen with both regions A and B, as

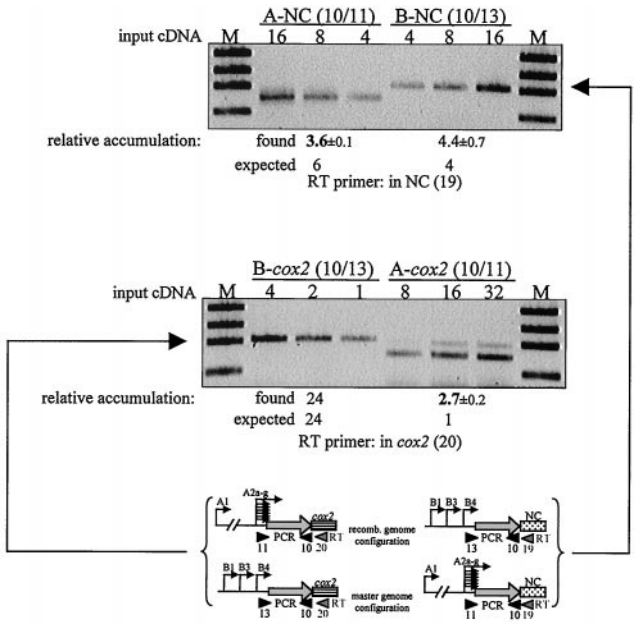

FIG. 5. Quantitative RT-PCR analysis of *cox2* transcripts was performed as discussed in *Materials and Methods*. The amount of input cDNA is given in microliters and was adjusted to give PCR products in similar quantities. The expected values for RNA accumulation, calculated as discussed in *Results* and the found values, as averaged from this and 14 other experiments, are shown below each panel. The boldface values are those that differ significantly from what was expected. At the bottom of the figure, the various RT-PCR primer sets are shown diagrammatically.

well as with *cox3*. Region A promoters are clearly weaker than those of region B, as determined by this assay, and quantitation of this and other filters, including those hybridized with more diluted probe, yielded the relative rates of approximately 1:4 shown at the bottom of Fig. 3*A*. In other words, the three region B promoters are together approximately four times more active than the two promoters in region A.

In interpreting the results shown in Fig. 3*A*, two factors need to be considered. One assumption is that there is not substantial differential RNA stability between region A- and region B-derived transcripts during the 10-min labeling. Although the half-lives of maize mitochondrial transcripts have not been measured directly, the facts (*i*) that the maize run-on system shows active transcription of intergenic regions that accumulate no stable transcripts (24); (*ii*) that relative promoter strengths measured by run-on transcription and *in vitro* transcription are in general agreement (22, 25); and (*iii*) that run-on transcription for chloroplasts shows near wild-type rates even in *Chlamydomonas* nuclear mutants in which RNAs are strongly destabilized and fail to accumulate (26, 27) together argue that a significant effect of differential RNA stability in the interpretation of this experiment can be discounted.

A second factor is evident from the diagram in Fig. 3*B*, i.e., that different lengths of initiated transcripts will hybridize with each PCR product and that individual promoters have different strengths. For example, transcripts initiated at promoter A1 will be fully colinear with the region A product, whereas transcripts initiated at promoter A2 will hybridize only with the 3′ portion of the PCR product. In this experiment, the length of hybridizing RNA can be considered most simply as its uridine content, because the transcripts are labeled with UTP. As shown in Fig. 3*C*, the hybridizing portions of transcripts from region A have 12–71 uridines, whereas those from region B contain 40–81 uridines. One must next take into account the relative strengths of the individual promoters. Our best measurements of the strengths of the individual promoters come from the direct quantitative measurements of primary transcripts that we recently published (17). These measurements suggest that promoter A1 is approximately three times stronger than the collective A2 promoters, whereas each of the region B promoters gives rise to a similar number of primary transcripts. Taking uridine content and promoter strength into account, we thus assume that, from region A, 75% of hybridizing transcripts will contain 71 uridines, and 25% will contain 12–32 uridines. Initiation from promoter A2a is complex, with A2a giving rise to approximately twice as many primary transcripts as the other promoters. Carrying out the resulting calculation gives an average hybridizing number of uridines from region A transcripts of 59 (Fig. 3*C*). The calculation for region B is much simpler, because the average number of hybridizing uridines is simply the average of those in B1, B3, and B4 transcripts, i.e., 62 (Fig. 3*C*). In effect, we conclude that differences in hybridizing length have little impact on interpreting the measurements in Fig. 3*A*. Thus, the conclusion holds that region A promoters are approximately four times less active than region B promoters *in vivo*.

Use of *cox2* Promoters *in Vivo* PCR Controls. Knowing the relative steady-state levels of the master (A-NC and B-*cox2*) and recombinant (A-*cox2* and B-NC) configurations of the maize mitochondrial genome (Fig. 2) and the relative strengths of region A and region B promoters (Fig. 3), it is possible to make a simple calculation as to the expected steady-state accumulations of the resultant transcripts. This calculation assumes that the relative strengths of A and B region promoters are unaltered by their genomic context, i.e., whether they are part of a master or recombinant genome. It is this assumption that we wished to test.

In terms of genome configuration, our DNA filter hybridization data (Fig. 2) gave a recombinant:master ratio of 1:6. For promoter strength, the average of two experiments gave a A:B ratio of 1:4 (Fig. 3). These two experiments were in close agreement. Thus, the following predictions would be made for approximate relative steady-state transcript levels: A-NC, 6 (1× promoter strength × 6× genomes); A-*cox2*, 1 (1× promoter strength × 1× genomes); B-*cox2*, 24 (4× promoter strength × 6× genomes); and B-NC, 4 (4× promoter strength × 1× genomes). Because the *cox2* transcript pattern is exceedingly complex (28), we chose to quantify the four classes of transcripts by using a quantitative RT-PCR assay.

To set up a quantitative RT-PCR assay, we first carried out two types of control experiments, as shown in Fig. 4. PCR was carried out by using three dilutions of total mtDNA, primer 10 in the 0.7-kb repeat, and two different upstream primers, each downstream of the *cox2* promoters in region A or B (see diagram at the bottom of Fig. 4*B*). The purpose of this experiment was to show that each primer pair was equally efficient at amplifying mtDNA and therefore a putative *cox2* cDNA product. As Fig. 4*A* shows, each primer pair yielded similar amounts of product, and the yield was in a linear range with respect to the amount of mtDNA added.

Fig. 4*B* shows a second type of experiment, in which PCR yields should reflect the relative quantities of master vs. recombinant genomes. In this experiment, the first primer was either in *cox2* (primer 20) or in NC (primer 19), and then the same two primers shown in Fig. 4*A* were used as second primers. We expected a lower yield of product from the 6-fold less abundant recombinant genomes, i.e., primer 11 with primer *cox2* and primer 13 with NC. To illustrate this difference, we used a series of mtDNA dilutions in which the amount amplified by the recombinant genome primer pair was always 6-fold more than that amplified by the master genome primer pair. For example, when we used primer 19 (Fig. 4*B*, right six lanes), 6 μl of mtDNA with primer 13 gives nearly the same amount of product as 1 μl of mtDNA with primer 11, and 1 μl with primer 13 gives an equivalent amount to 0.17 μl with

primer 11. A similar result was seen for primer 20 reactions, although the amount of recombinant chromosome was slightly lower than expected. Taken together, the data shown in Fig. 4 suggest that the primers chosen would fairly represent the steady-state levels of different *cox2*- or NC-containing cDNAs.

Use of *cox2* Promoters *in Vivo* RT-PCR. RT-PCR was carried out by using a NC (primer 19) or *cox2* (primer 20) primer to prime cDNA synthesis and then a second pair of primers consisting of one within the 0.7-kb repeat (primer 10) and a second specific to a set of promoters, primer 11 in the case of A1/A2 and primer 13 in the case of B1/B3/B4. This strategy is illustrated at the bottom of Fig. 5. While the NC or *cox2* cDNA primer generated specific pools of cDNA, PCR was carried out with the repeat primer to make the greatest effort to equalize primer efficiency in the different reactions.

Fig. 5 shows a representative RT-PCR result that quantifies region A or region B promoter activity in a specific genome configuration; such experiments were carried out multiple times and with two different mtRNA preparations. Although there was some variability in the results, including the failure of certain PCRs, we feel confident in the results presented in Fig. 5. Note that in these experiments, the amount of input cDNA was adjusted to give similar amounts of PCR product. This adjustment allowed us to quantify products within a linear range, and we simply corrected the amount of product, as determined by densitometric analysis of such gels, for the amount of input. For example, the greatest amount of input was needed for the A-*cox2* reaction, as expected because this reaction is a combination of the weaker promoter set with the less abundant genome configuration.

Below each gel in Fig. 5, the relative RNA accumulations based on RT-PCR are shown (found) and compared with the theoretical results discussed above (expected). We arbitrarily normalized the results so that B-*cox2* gave the same amount of transcript as expected; because we are measuring relative transcript accumulations from different genomic environments, the relative results are unaffected by this assumption. The results that differ substantially from those predicted are shown in boldface. For A-NC, we found a relative accumulation of 3.6 instead of the expected 6. However, for A-*cox2*, we found a relative accumulation of 2.7 instead of the expected 1. Thus, promoters A are relatively underused when upstream of NC and approximately 3-fold overused when upstream of *cox2*. We therefore suggest that the *cox2* promoter(s) A1 and/or A2 respond(s) to intragenomic or intergenomic recombination across the 0.7-kb repeat by modulating initiation activity.

DISCUSSION

The major finding in this work is that *cox2* promoter activity seems to respond to genomic context, although we cannot completely rule out an effect of RNA stability, as discussed below. We found that region A promoters are used more than predicted when upstream of the *cox2* coding region in the recombinant configuration and less than predicted when upstream of region NC in the master configuration. These results are based on numerous RT-PCR experiments with two different RNA preparations.

It is important to consider the possibility that differential RNA stability, rather than differential transcription, is responsible for the effects we have seen. In this scenario, the combination of region A 5′ ends with *cox2* 3′ ends would have an increased stability relative to region A 5′ ends with NC 3′ ends. However, if region NC 3′ ends are destabilizing, we should have seen a similar effect for region B, i.e., that B-NC is relatively unstable and apparently undertranscribed compared with B-*cox2*. Based on our RT-PCR results, this alternative explanation is not the case. By similar reasoning, we cannot conclude that region A 5′ sequences specifically destabilize A-NC transcripts; in this case, both A-*cox2* and A-NC would be underrepresented.

Based on the data we have presented, we also cannot absolutely differentiate between increased transcription of A-*cox2* relative to A-NC and increased stability of A-*cox2* (but not B-*cox2*) transcripts. In this case, differential stability would have to be imparted by a specific interaction between region A transcribed sequences and region *cox2* or NC. Although RNA stability determinants in organelles can be quite short (D. C. Higgs, R. S. Shapiro, K. L. Kindle, and D.B.S., unpublished work, and refs. 29–31), in no case have the functions of these elements been shown to depend on distant sequences. For example, *Chlamydomonas* chloroplast RNA stability determinants for *petD* and *psbD* function identically whether in their normal locations or when fused to downstream reporter genes (30, 32). Furthermore, because we observed increased stability of A-*cox2* but not B-*cox2* transcripts, we cannot invoke a translation-linked stabilization. In summary, we argue that the effects we see are transcriptional and specifically caused by the combination of A region promoters and their downstream regions.

Our measurements of transcription activity for the collective region A or region B promoters were based on run-on transcription, which is a well established method for measuring plant organellar transcription rates (22, 33–36). Based on the results presented in Fig. 3, we concluded that region A promoters were four times less active than region B promoters, partly by using indirect arguments. It is important to point out that, even if these measurements were inaccurate, this inaccuracy does not change our qualitative conclusions. For example, if region A and B promoters were equally active, the expected values shown in Fig. 5 would be different; however, the relative transcription of A-NC and A-*cox2* would still be different than expected, because this difference does not depend on the absolute activity of the region A promoters.

A final consideration in our data analysis can be found in Fig. 4*B*; therein, it seems, based on PCR, that the product of primers 11 and 20, i.e., A-*cox2*, seems to be less than expected. If indeed this recombinant genome is underrepresented by PCR relative to DNA filter hybridizations (Fig. 2), it only strengthens the assertion that A-*cox2* is overtranscribed, because the A-*cox2* RT-PCR products in Fig. 5 might also be underrepresented. Thus, although the compilation of data by using various methods does raise difficulties that must be addressed, in the case of the studies shown here, these difficulties do not suggest that our conclusions can be discounted.

If one accepts that region A promoters are more active in the recombinant vs. master genome configuration, it is worthwhile to address why this difference might exist. Although a simple picture of genome structure (Fig. 1*A*) would provide a convenient context for explaining such effects, plant mtDNA is largely linear *in vivo* (8). Indeed, the maize mitochondrial *in vitro* transcription system depends on linear templates (25), and the maize mitochondrial RNA polymerase resembles that of bacteriophages with linear genomes (37). However, regardless of linear vs. circular topology, it is possible that certain sequence combinations would affect transcription initiation rates. For example, in *Chlamydomonas* chloroplasts, inhibitors of topoisomerase I differentially affect transcription from separate genomic regions (38), and in yeast mitochondria, DNA packaging and bending affect promoter use *in vitro* (39). For maize *cox2*, one possibility is that long-range protein–DNA interactions affect local DNA structure and thus modulate initiation rates. In this regard, it is interesting to note that *cox2* contains an intron in maize and most other species. Intronic enhancers of transcription and RNA accumulation are known in many nuclear genes, including those of plants (40–43). On the other hand, mitochondrial introns in fungi are optional and have no defined role in gene expression but instead are thought to play a role in genome evolution (44, 45).

Whether mitochondrial recombination plays an important role in *cox2* expression *in vivo* remains to be addressed. Various studies have addressed the differential accumulation of mitochondrion-encoded proteins and RNAs during plant development, particularly during gametogenesis (46–49); however, in most cases, whether the regulation is transcriptional or posttranscriptional is unknown. Environmental effects on DNA topology have been noted in *Chlamydomonas* and barley chloroplasts, where light has been shown to affect chromosome structure, as determined by their ability to intercalate crosslinking agents into the genome (50, 51). Mutants affecting *cox2* expression may help elucidate its role during plant development (52), including the role of specific promoters (53).

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