The Plant Cell, Vol. 2, 71–83, January 1990

Transcriptional and Post-Transcriptional Regulation of RNA Levels in Maize Mitochondria

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Relatively little is known about the mechanisms that govern the expression of plant mitochondrial genomes. We have addressed this problem by analyzing the transcriptional activity of different regions of the maize mitochondrial genome using both in vivo and isolated mitochondrial pulse-labeling systems. The regions examined included the protein genes *atp*A, *atp*6, and *cox*II, the 26S, 18S, and 5S rRNA genes, and sequences surrounding the rRNA genes. The rRNAs were found to be transcribed at rates fivefold to 10-fold higher than the protein genes. These rate differences are comparable with the differences in abundance of these species in the total or steady-state RNA population. Pulse-labeled RNA unexpectedly detected transcription of all regions examined, including approximately 21 kilobases of presumed noncoding sequences flanking the rRNA genes for which stable transcripts were not detected. The results obtained with RNA labeled for short pulses in vivo and in isolated mitochondria were similar, suggesting that isolated mitochondria provide a faithful run-on transcription assay. Our results indicate that the absence in total RNA of transcripts homologous to a given region of maize mitochondrial DNA does not necessarily exclude transcriptional activity of that region and that both transcriptional and post-transcriptional processes play important roles in maize mitochondrial genome expression.

INTRODUCTION

Mitochondrial DNA (mtDNA) expression proceeds through diverse mechanisms in different organisms. In vertebrates, near-complete transcription of each template strand proceeds from only one or two closely spaced promoters located in the D-loop, or noncoding, region of the molecule (Clayton 1984; Attardi and Schatz, 1988). The production of individual mRNA, rRNA, and tRNA species is accomplished by extensive processing of the resulting polycistronic transcripts, which can extend up to full mitochondrial genome size in length. By contrast, in yeast mitochondria, transcription can initiate at 13 or more sites that are dispersed throughout the genome (Christianson and Rabinowitz, 1983; Attardi and Schatz, 1988). In many cases, these promoters also give rise to long, polycistronic transcripts, which are processed to yield mature RNA species. In each organism, mtRNA levels are regulated by differential rates of both synthesis and turnover (Gelfand and Attardi, 1981; Mueller and Getz, 1986; Wettstein-Edwards et al., 1986; Attardi and Schatz, 1988).

Mitochondrial transcription and processing are less well characterized in other organisms. In plants, for example, the characteristics of neither the promoter nor the transcription units are known. The identification of transcriptional initiation sites in regions lying immediately upstream of a number of different maize mitochondrial genes (Mulligan, Lau, and Walbot, 1988a; Mulligan, Maloney, and Walbot, 1988b) suggests that some individual genes may comprise separate transcription units. The occurrence of apparent precursor transcripts that encompass more than one coding region, however (Bland, Levings, and Matzinger, 1986; Dewey, Levings, and Timothy, 1986; Wissinger et al., 1988), suggests that other transcription units may be polycistronic and indicates that post-transcriptional events play important roles in mitochondrial gene expression. The processes regulating the steady-state concentrations of individual plant mtRNAs have not been established.

By analyzing maize mtRNA that has been pulse-labeled either in vivo or in isolated mitochondria, we show that an unexpected degree of transcriptional activity occurs over a large region of the mitochondrial genome that has no known coding function and for which stable transcripts are not detected. We also show that the rRNA genes are transcribed at rates 5 times to 10 times higher than those of three protein genes analyzed. Our results demonstrate that both transcriptional and post-transcriptional processes play important roles in governing the expression of the maize mitochondrial genome.

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RESULTS

Transcriptional Analysis of Specific Regions of Maize mtDNA

We have previously defined conditions whereby mtRNA can be specifically and efficiently pulse-labeled in isolated mitochondria with α-^{32}P-UTP (Finnegan and Brown, 1986, 1987). More recently, we have developed in vivo pulse-labeling techniques whereby ^{32}P-orthophosphate is incorporated into the mtRNA of Black Mexican Sweet maize cells (N cytoplasm) in suspension culture. The kinetics of label incorporation into total mtRNA and into a specific mitochondrial transcript, the 26S rRNA, are shown in Figure 1. Under conditions that allow labeling of mtRNA to high specific activity in isolated mitochondria, incorporation proceeds at an approximately linear rate for roughly 30 min. By contrast, label incorporation in vivo increases exponentially with time, as would be expected if progressive increases in the specific activities of the phosphate and nucleoside triphosphate pools were occurring. In both systems, labeling of total and 26S mtRNA proceeds with similar kinetics.

To evaluate the relative rates of accumulation for transcripts from specific regions of the maize mitochondrial genome and to compare these rates in isolated mitochondria with those in vivo, membrane-bound samples of plasmid DNAs containing mtDNA inserts were probed with maize cell suspension culture mtRNA that had been labeled either enzymatically with T4 polynucleotide kinase or pulse-labeled for various times in vivo or in isolated mitochondria. Mitochondria were partially purified following in vivo labeling, prior to mtRNA isolation, to minimize the potential contribution of nonmitochondrial sequences to the hybridization signals. We expected that, after short pulses of incorporation in vivo, the relative degrees of labeling of different mtRNA species should largely be a reflection of their relative rates of synthesis, whereas, after longer pulses, additional factors, such as transcript turnover, would play increasingly important roles (Gelfand and Attardi, 1981; Mueller and Getz, 1986). The relative degrees of labeling of different RNA species by polynucleotide kinase are expected to reflect the abundance of these species in the total or steady-state RNA population.

The mtDNA regions analyzed are shown in Figure 2 and have been characterized by others (Fox and Leaver, 1981; Iams and Sinclair, 1982; Chao, Sederoff, and Levings, 1983, 1984; Dale et al., 1984; Braun and Levings 1985; Dale, McClure, and Houchins, 1985; Dewey, Levings, and Timothy, 1985). They are described in detail in Methods. The analysis included: regions in the 26S rRNA gene (fragments D to F); the entire 18S and 5S rRNA genes (fragments M and L, respectively); regions of unknown function lying both upstream of the 26S rRNA gene (fragments A to C) and downstream of both the 26S and 5S

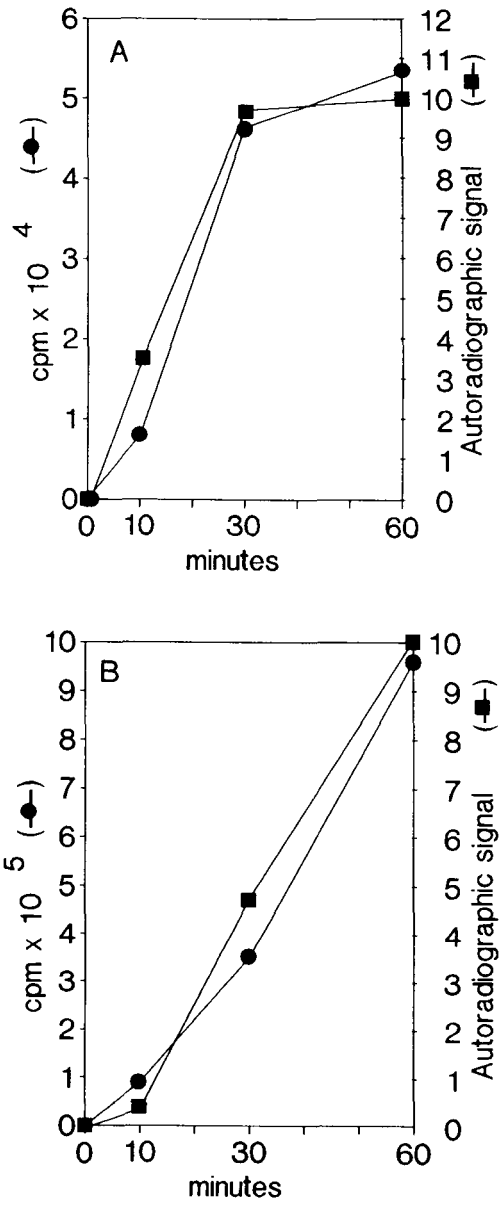

Figure 1. Kinetics of Label Incorporation into mtRNA in Vivo and in Isolated Mitochondria.

(A) Labeling in isolated mitochondria: At various times after the addition of α-^{32}P-UTP to mitochondria isolated from Black Mexican Sweet suspension culture cells, aliquots were removed and mtRNA was prepared.

(B) Labeling in vivo: At various times after the addition of ^{32}P-H_3PO_4 to Black Mexican Sweet cells suspended in phosphate-free medium, aliquots of the culture were removed, mitochondria were isolated, and mtRNA was prepared.

(●), cpm incorporated per 50 μL of incorporation mixture **(A)** or per milliliter of culture **(B)**. (■), incorporation into 26S rRNA fragment (fragment E, Figure 2) as determined from integration of autoradiographic signals. Signals from 60-min points for both **(A)** and **(B)** were arbitrarily assigned a value of 10.

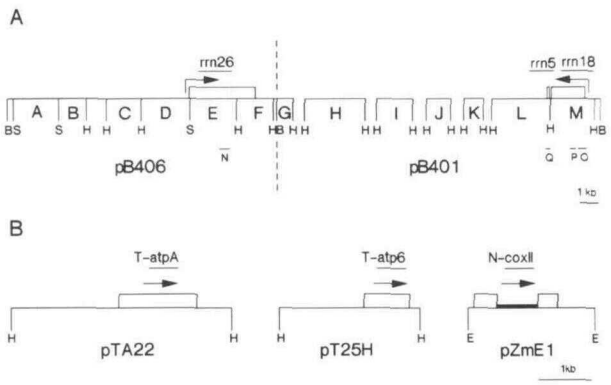

Figure 2. Regions of the Mitochondrial Genome Analyzed for Transcriptional Activity.

(A) Ribosomal RNA gene regions.
(B) Protein gene regions.
The restriction fragments used to obtain the fragments employed in the transcriptional analysis were cloned and characterized by others and include the 14.7-kb and 17.5-kb *BamHI fragments of* pB406 and pB401, respectively (Iams and Sinclair, 1982), as well as the 4.2-kb HindIII fragment of pTA22 (Braun and Levings, 1985), the 2.7-kb HindIII fragment of pT25H (Dewey et al., 1985), and the 2.4-kb EcoRI fragment of pZmE1 (Fox and Leaver, 1981). The locations of the genes indicated above the maps are shown by open boxes; the intron of *coxII* is shown by a heavy line on the map of pZmE1. The direction of transcription for each gene is indicated by an arrow above the map; the sites of transcription initiation for the rRNA cistrons (Mulligan et al., 1988b) are indicated by vertical lines at the tails of the arrows. Selected cleavage sites for several restriction enzymes are shown as vertical lines below the maps (B, BamHI; E, EcoRI; H, HindIII; S, SmaI). The subcloned fragments of pB401 and pB406 used in the slot-blot transcriptional analysis are indicated by the letters A through M between the delimiting restriction sites; those fragments used as templates for probe RNAs in the nuclease protection assays are indicated by horizontal lines, designated N through Q, shown below the maps. The order of fragments H to K on the mtDNA is not known. For further details, see Methods.

rRNA genes (fragments G to K); and fragments encompassing the mitochondrial *atp*A (pTA22, Braun and Levings, 1985), *atp*6 (pT25H, Dewey et al., 1985), and *cox*II (pZmE1, Fox and Leaver, 1981) protein coding regions. About 27 kb of the approximately 32 kb covered by the two contiguous BamHI fragments that encompass the rRNA genes (Iams and Sinclair, 1982) were analyzed. Autoradiographs from a typical experiment are shown in Figure 3.

The hybridization signals were quantitated by subjecting autoradiographs to scanning densitometry and determining the areas under the peaks generated by the different slots. Preliminary experiments showed the peak areas to be proportional to counts per minute over a relatively broad range of film response. Multiple film exposures were used to ensure that the signals used in the subsequent analysis represented this linear range of response. Areas from replicate samples were averaged and divided by the *mtDNA insert length* to compensate for differences in the sizes of the analyzed fragments. To allow comparison between signals generated at different labeling times and by different labeling procedures, values were expressed as a percentage of the total hybridization signal generated by each probe. The values obtained, referred to as relative

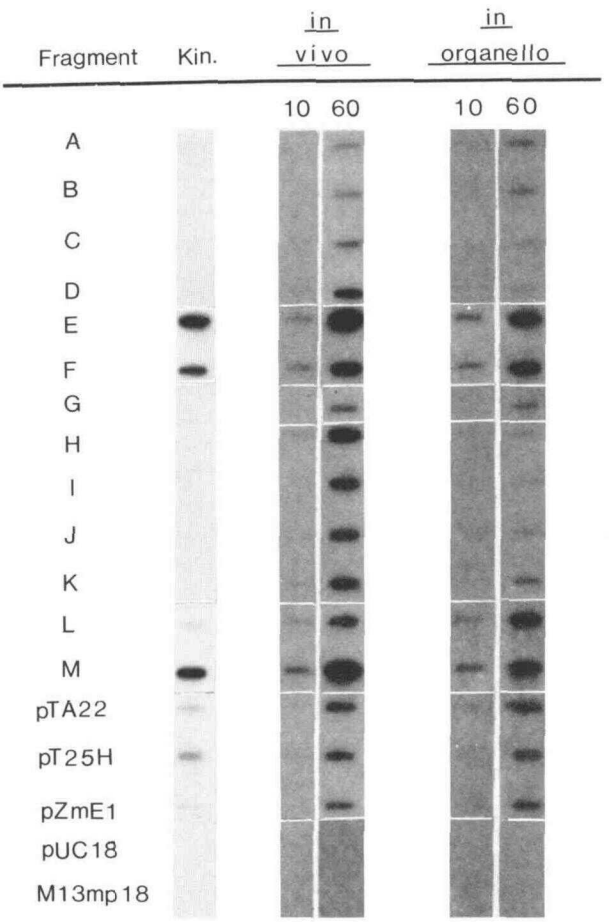

Figure 3. Hybridization of Pulse-Labeled or Enzymatically Labeled Maize mtRNA to Cloned Regions of Maize mtDNA.

Cell suspension culture mtRNA labeled with T4 polynucleotide kinase (Kin.) or for 10 min or 60 min in vivo or in isolated mitochondria was used to probe slot blots containing the indicated cloned mtDNA fragments. The fragment designations are as set out in Figure 2. The pulse-labeled probes used to produce the hybridization signals shown here were from the same in vivo or isolated mitochondrial labeling reaction. The hybridization signals shown in each column were the results of a single hybridization; the fragment order on the original autoradiographs has been changed for ease of presentation.

autoradiographic signals, are presented in Table 1 and Figure 4. They are a measure of the average relative rates of transcript accumulation for the respective regions during the labeling period examined.

Control experiments (not shown) indicated that the slot-blot hybridizations were quantitative according to the criteria of Marzluff and Huang (1984): the response was proportional to the amount of RNA probe added, and a 90% reduction in bound signal was seen when hybridized probes were used in subsequent hybridizations with identical blots. The hybridization signals were exclusively due to RNA because no hybridization was observed when the probe was treated with alkali overnight at 23°C. Because treatment of the hybrids with 10 μg/mL RNase A in 2 × SSPE had no significant effect on the hybridization patterns, the partial alkali degradation of the RNA probes was sufficient to prevent appreciable contribution of sequences contiguous with the hybridizing regions to the membrane-bound signal (not shown). Under the stringent washing conditions employed, hybridization to vector sequences was negligible.

rRNA and mRNA Transcription

The autoradiograph of Figure 3 demonstrates that, regardless of whether mtRNA is labeled in vivo, in isolated mitochondria, or with polynucleotide kinase and γ-^{32}P-ATP, the strongest hybridization signals are obtained from fragments E and M, which consist entirely (E) or primarily (M) of rRNA coding sequences. Other regions that give detectable signals using kinase-labeled RNA as probe include fragments D and F, which partly consist of 26S coding segments, fragment L, which partly consists of the 5S rRNA gene, and fragments carrying the genes for ATPase subunits F_1-α and 6 (pTA22, pT25H) and cytochrome oxidase subunit II (pZmE1).

Quantitation of the signals (Table 1) generated by the kinased RNA probe from fragments E, F, and M, when normalized to their rRNA coding content, indicates that the 18S and 26S rRNAs are each between 9 times and 13 times more abundant than sequences from the *atp*A region in mtRNA. A similar analysis of the signal from fragment L indicates that 5S rRNA sequences are approximately 18-fold more abundant than *atp*A transcripts. Because the *rrn*5 cistron comprises less than 3% of fragment L, it is possible that the slightly higher abundance figure for 5S rRNA arises from the signal generated by low-abundance transcripts extending into the 3′-flanking region of this gene (see below). Transcripts from the three protein genes are approximately equal in abundance.

Regardless of whether transcription is measured using the in vivo or isolated mitochondrial labeling systems, transcripts of the rRNA cistrons (fragments E and M, Table 1) are estimated to accumulate 5 times to 10 times faster than those from the *atp*A region. During in vivo labeling, 26S transcripts appear to accumulate somewhat more slowly than 18S transcripts. The rates of accumulation of transcripts from *atp*6 and *cox*II regions, measured either in vivo or in isolated mitochondria, are similar to those of *atp*A transcripts (Table 1). Thus, the data indicate that the rRNA genes are transcribed at considerably higher rates than the protein genes, and the differences in abundance of these two classes of mtRNA can be largely accounted for by differences in transcriptional rates.

Table 1. Sequence Representation in Total and Pulse-Labeled Mitochondrial RNA

mtDNA Fragment	Gene(s)	Fragment Length (kb)	Relative Autoradiographic Signal[a]				
				In Vivo		In Isolated Mitochondria	
			Kinase	10 min	60 min	10 min	60 min
E	*rrn*26	2.5	32.8	17.4	20.7	24.6	20.7
F	*rrn*26 + 3′ noncoding	1.9	16.8	20.1	12.6	16.8	21.2
FN[b]	*rrn*26 + 3′ noncoding	1.0[c]	31.9	38.2	23.9	31.9	40.3
L	*rrn*5 + 3′ noncoding	3.2	2.6	6.9	3.8	9.0	12.5
LN[b]	*rrn*5 + 3′ noncoding	0.13[c]	64.0	170.0	94.0	222.0	308.0
M	*rrn*18 + 5′, 3′ noncoding	2.6	36.4	33.0	28.5	25.3	18.8
MN[b]	*rrn*18 + 5′, 3′ noncoding	2.0[c]	47.3	42.9	37.0	32.9	28.4
pTA22	*atp*A	4.2	3.6	3.6	4.0	2.7	2.4
pT25H	*atp*6	2.7	3.4	4.4	3.8	2.9	2.6
pZME1	*cox*II	2.4	4.2	4.0	3.2	1.9	2.4

[a] All values in each set are normalized to an insert length of 1 kb and expressed as a percentage of the total hybridization signal obtained for that set.
[b] Values designated "N" have been normalized to 1 kb of the known coding capacity of the fragment.
[c] Length of known coding capacity.

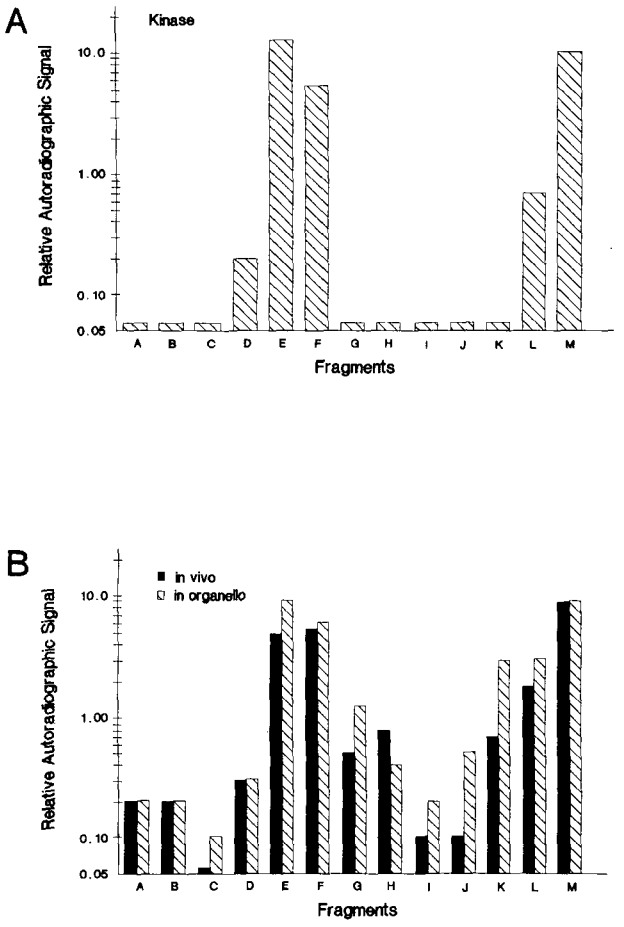

Figure 4. Relative Abundances of Transcripts from the Ribosomal RNA Gene Region in Total and Newly Synthesized Mitochondrial RNA Populations.

(A) Signals from mtDNA fragments A to M (Figure 2) produced from mtRNA labeled with polynucleotide kinase.
(B) Signals from mtDNA fragments A to M produced from mtRNA labeled for 10 min in vivo (solid bars) or in isolated mitochondria (hatched bars).
Signals were quantitated and values were divided by the quantitated signals from pTA22 after correction for differences in fragment length. As mentioned in the text, the order of fragments H, I, J, and K on the mtDNA is not known.

Transcription of Extragenic Regions

Analysis of the in vivo transcription of *rrn*26 fragments E and F shows that, after a 10-min pulse, labeled transcripts from the two fragments are roughly equal in abundance, whereas, after 60 min, transcripts from fragment E are nearly twice as abundant as those from fragment F (Table 1). A similar effect is observed during in vivo labeling of *rrn*18/*rrn*5 transcripts. Labeled transcripts of fragment L, which contains the *rrn*5 gene, decrease in abundance with time relative to those of the *rrn*18-containing fragment M. In both cases, the fragments for which labeling decreases with time are those that consist primarily of noncoding sequences, and, in both cases, relative abundance of the labeled transcripts approaches that found in total mtRNA as the time of labeling increases. It seems likely, therefore, that, during in vivo labeling, some fragment F and L transcripts initially accumulate in newly synthesized RNA but are then removed from the population. This would occur if transcription extends considerably beyond the 3′ ends of both *rrn*26 and *rrn*5 and the noncoding transcripts are subsequently degraded.

Direct evidence for extragenic transcription is obtained from analysis of regions lying upstream of the 26S rRNA gene (Figure 2A, fragments A, B, and C) and between the 26S rRNA gene and the 18S + 5S rRNA genes (Figure 2A, fragments G, H, I, J, and K). Although no coding functions have been assigned to these fragments, the autoradiographs of the slot blots (Figure 3) clearly show that each of these regions is transcribed both in vivo and in isolated mitochondria. Comparison of the ratios of the autoradiographic signals with those of pTA22 (Figure 4B; Table 1) indicates that transcripts from fragments G, H, and J accumulate at rates similar to those observed for the protein coding regions. Transcripts from fragment K accumulate at significantly higher rates, whereas transcripts from fragments A, B, C, and I accumulate at the lowest rates. Transcripts from all of these fragments are undetectable in total mtRNA (Figures 3 and 4A). These observations indicate that all the regions analyzed are actively transcribed and that the noncoding sequences are post-transcriptionally removed from the RNA population.

RNA Synthesis in Isolated Mitochondria versus in Vivo

The data of Table 1 and Figures 3 and 4B indicate that there is a general concordance between the extent to which transcripts are labeled in vivo and in isolated mitochondria. A few discrepancies are observed, however. In general, at early times after administration of the pulse, noncoding sequences, such as those on fragments I through K, are somewhat more abundant in RNA that has been labeled in isolated mitochondria (Figure 4B). Moreover, the observed decrease in abundance with time of in vivo labeled transcripts from fragments F and L (Table 1) is not observed when RNA is labeled in isolated mitochondria. In fact, the abundance of these transcripts, which largely represent 3′ noncoding sequences, increases, rather than decreases, with time. These observations suggest that processing activities, such as those postulated above to account for the in vivo disappearance of label from fragments F and L transcripts, are depressed in isolated mitochondria. Decreased RNA processing has previously been observed in both isolated yeast (Boerner,

Mason, and Fox, 1981) and human (Gaines and Attardi, 1984) mitochondria. In addition, the increase in abundance with time of sequences from the 3′ ends of the transcription units, observed when mtRNA is labeled in isolated mitochondria, may reflect decreased transcription initiation in this system. A similar observation has been made concerning RNA synthesis in isolated human mitochondria (Gaines and Attardi, 1984).

RNase Protection Analysis of Transcription and Processing

The decrease in abundance with time of in vivo labeled transcripts from fragments F and L could also be due to temporal changes in the pattern of label incorporation across the transcription unit instead of processing and turnover, as suggested. To rule out this possibility, and to provide an independent measure of transcriptional activity, we employed a nuclease protection assay similar to that used to quantitate transcription rates in yeast mitochondria (Mueller and Getz, 1986). Cell suspension culture mtRNA radiolabeled for 5 min, 20 min, or 40 min in vivo was quantitatively hybridized in solution to in vitro synthesized, nonradioactive probe RNAs complementary to regions within the 18S + 5S rRNA transcription unit. The nonhybridized RNA was then digested with RNase T1 under conditions of single-strand specificity. Three regions, designated O, P, and Q, were analyzed (Figure 2). Fragments O and P lie entirely within the 18S rRNA gene, whereas fragment Q encompasses the entire 5S rRNA gene plus the adjacent 67-bp 5′- and approximately 33-bp 3′-flanking sequences (Chao et al., 1983). A 703-bp probe complementary to 595 bp of the 26S rRNA was also included in the analysis (Figure 2, fragment N).

To compare the relative transcription rates of these rRNA segments, the probes were used in paired combinations. After quantitative hybridization and nuclease digestion, protected RNAs were subjected to electrophoresis, and autoradiographic signals from the gels were normalized to an insert length of 1 kb. As shown in Table 2, transcripts of the two 18S regions accumulate at approximately the same rate. We conclude that label incorporation rates in vivo along this cistron are uniform with time. This finding supports the idea that the decrease in abundance with time of in vivo labeled transcripts from 3′-proximal rRNA gene regions observed in the slot-blot analysis is due to rapid removal of sequences 3′ to the 26S and 5S rRNAs from the RNA population.

Table 2. Sequence Representation of RNA Regions in Pulse-Labeled RNA, as Determined by Nuclease Protection

mtDNA Fragment	Relative Autoradiographic Signal[a]		
	5 min	20 min	40 min
N	0.5	0.8	0.7
O	1.0	1.0	1.0
P	0.9	1.0	1.2

[a] The radioactivity in RNA fragments protected during nuclease T1 protection assays, using mtRNA labeled for the times shown in vivo as the target, was quantitated by scanning densitometry. The signals were normalized to a protected fragment length of 1 kb (see text). All values were then related to that obtained for fragment O (Figure 2).

We have consistently observed, using both filter hybridization and RNase protection assays, that 26S sequences accumulate at slightly lower rates than 18S sequences (Tables 1 and 2). Because, as expected, both species are equally abundant in total RNA, it is possible that 26S species are more stable than 18S species.

Regions P and Q correspond to 335 bp of *rrn*18 and 234 bp of *rrn*5 with surrounding sequences, respectively. As shown in Figure 5A, lane 1, hybridization of mtRNA labeled for 20 min in vivo to RNA probes of these regions results in the protection of two major radiolabeled RNA species of about 350 nucleotides (nt) and 130 nt in length. In addition, minor protected fragments, the largest of which is 250 bp, are observed. In contrast, when RNA labeled in isolated mitochondria is used as the target for the same probes, only the 350-nt and 250-nt species are detected (Figure 5A, lane 3). The RNA species of between 130 nt and 250 nt were specifically protected by the fragment Q probe (Figure 5B), whereas the 350-nt species was specifically protected by the fragment P probe (Figure 5A, lanes 2 and 4). The 130-nt RNA corresponds in size to the mature 5S rRNA (Chao et al., 1983) and the 250-nt protected species, therefore, represents an unprocessed form of the 5S rRNA.

To determine whether the 250-nt RNA might serve as a precursor to the 5S rRNA, the fragment Q-protected species in RNA labeled for longer and shorter periods were compared. In RNA that has been pulse-labeled for only 5 min, only the 130-nt band and a faint 250-nt band are detected in the protected products. In RNA pulse-labeled for 40 min, additional labeled species, the most prominent of which are 180 nt and 140 nt, are apparent. Densitometric analysis indicates that the ratio of the 250-nt to the 130-nt species is 4 times higher in the 5-min-labeled products than in the 40-min products, consistent with the view that the 250-nt RNA is a precursor to the mature 5S rRNA. The 180-nt and 140-nt species may represent processing intermediates. Interestingly, the 180-nt product corresponds to the approximate size of an RNA species that extends from the 5′ end of the mature 5S rRNA to a site or sites lying downstream of the fragment Q probe region.

The RNase protection analysis indicates that primary 5S transcripts extend at least 67 bp upstream and 33 bp downstream of the mature gene product and that processing of the in vivo labeled transcripts occurs sufficiently

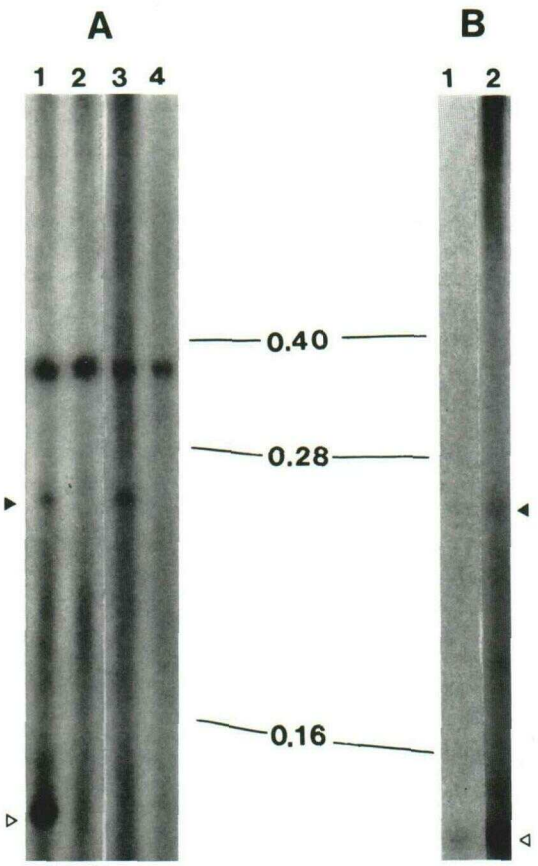

Figure 5. *RNase Protection Analysis of RNA Labeled in Vivo and in Isolated Mitochondria.*

(A) Cell suspension culture labeled for 20 min in vivo (lanes 1 and 2) or in isolated mitochondria (lanes 3 and 4) was digested with RNase T1 after hybridization in solution with RNA complementary to the coding strands of both fragments P and Q (lanes 1 and 3) or fragment P alone (lanes 2 and 4).

(B) Cell suspension culture mtRNA labeled in vivo for 5 min (lane 1) or 40 min (lane 2) was digested with RNase T1 after hybridization in solution with RNA complementary to fragment Q.

Protected RNA species were resolved by electrophoresis on 5% polyacrylamide/urea gels and subjected to autoradiography. Closed and open arrows indicate the positions of the 250-nt and 130-nt species, respectively, that are protected by RNA complementary to fragment Q.

rapidly to explain the labeling kinetics for downstream sequences observed in the slot-blot experiments. The presence of only unprocessed 5S rRNA sequences in the in organello products strongly indicates that processing of this RNA precursor does not occur efficiently in isolated mitochondria.

DISCUSSION

Transcription and Turnover of Extragenic Sequences

Previous investigations of plant mitochondrial transcription have been confined primarily to analysis of the total or steady-state population of mtRNA molecules. These studies have indicated that abundant mitochondrial transcripts are generally confined to coding regions although, in some cases, low-abundance transcripts extending into noncoding regions have been observed. A complete transcription map has been constructed for only one plant mtDNA, that of *Brassica campestris* (turnip, rapeseed) (Makaroff and Palmer, 1987). This investigation showed that approximately 30% of the 218-kb genome is represented in abundant transcripts, about half of which correspond to identified genes. Low-abundance transcripts covering a larger proportion of the genome are also detected. Because *B. campestris* mtDNA is among the smallest plant mitochondrial genomes thus far identified and because it seems unlikely that larger plant mtDNAs, such as those from maize (575 kb) or muskmelon (2000 kb), encode a significantly greater number of products, estimates of the extent of transcription of other plant mtDNAs, based solely on RNA gel blot-type analyses, are likely to be lower.

We show here that large regions (about 10 kb) of the maize mitochondrial genome that do not give rise to stable transcripts and are not known to possess coding functions may be transcriptionally active. Moreover, we show that such regions may be transcribed at rates comparable with or even greater than protein coding genes. We infer from this that maize mitochondria possess mechanisms for recognizing and selectively degrading extragenic transcripts. The existence of such mechanisms is also indicated by the observation that both the 26S and 18S rRNA transcripts initiate at sites about 200 bp upstream of their coding regions (Mulligan et al., 1988b). The present observations, however, indicate that these mechanisms may act on a considerably larger scale than these earlier findings alone would suggest. All the noncoding regions we have examined lie in the vicinity of the rRNA genes, and the extent to which transcripts outside the rRNA region are subject to this selective degradation remains uncertain. It seems likely, however, that this phenomenon pertains to other mtDNA regions as well because certain types of analysis of total mtRNA suggest that up to 70% of the sequences of some plant mtDNAs may be transcribed (Bendich, 1985; Stern and Newton, 1985).

The regions surrounding the rRNA genes that are actively transcribed are judged to be noncoding because stable transcripts cannot be detected either in cells in suspension culture or in coleoptilar tissue and because sequences homologous to mitochondrial genes, either from maize or other plants, do not map to these sites (Lonsdale, 1987). The sequences do not occur elsewhere

on maize mtDNA because hybridization of the cloned regions encompassing them to restriction digests of maize mtDNA identify only the corresponding restriction fragments, and the regions do not carry a known maize mitochondrial repeat (Iams and Sinclair, 1982; Stern, Dyer, and Lonsdale, 1983; Lonsdale, 1987; P. Finnegan and G. Brown, unpublished observations). Thus, the transcripts we have detected are not derived from other, functional parts of the genome. Although it is likely that these sequences consist primarily of noncoding DNA, it is possible that they contain genes for which more abundant transcripts occur in tissues other than those examined here. This would indicate that developmental regulation of gene expression in plant mitochondria could be achieved at the post-transcriptional level, as it can in plastids (Gruissem, 1989). It is unlikely, however, that such putative genes could account for all, or even most, of this unexpected transcription.

Carlson, Brown, and Kemble (1986a) and Carlson, Erickson, and Kemble (1986b), using an isolated mitochondrial labeling system different from that employed here, have detected transcripts homologous to all resolvable restriction fragments from both maize and *B. napus* mtDNAs, including regions carrying segments of transposed chloroplast DNA (cpDNA). None of the regions analyzed here carries cpDNA homologous sequences. The transcripts produced in our system, however, do not hybridize to all the restriction fragments resulting from digestion of maize mtDNA (not shown). Makaroff and Palmer (1987) have attributed chloroplast homologous transcripts in mtRNA preparations to cpRNA contamination. Consistent with this interpretation is our finding that even highly purified mitochondrial preparations from non-green tissue are contaminated to a small and variable extent by transcriptionally active plastids. Nevertheless, it seems likely that some transposed chloroplast sequences are transcriptionally active because functional mitochondrial $tRNA^{Trp}$ and $tRNA^{Pro}$ genes appear to be of chloroplast origin (Marechal et al., 1987; Wintz et al., 1988; Levings and Brown, 1989).

It should be emphasized that, although our results demonstrate that extensive extragenic transcription of mtDNA occurs throughout the examined regions, they do not necessarily indicate that all noncoding regions are transcriptionally active. Moreover, the transcription we observe appears to be highly asymmetric. We attempted to detect antisense transcripts of the fragments encoding the rRNA genes; a very low level transcription of the antisense DNA strand of fragment L, but not of other rRNA gene fragments, was detected, with probes labeled both in vivo and in isolated mitochondria. This low level of transcription may represent unattenuated 26S transcripts. No antisense transcripts to the fragment E were detected, suggesting that the observed transcription of the 26S rRNA upstream region is not the result of incomplete 18S/5S transcript attenuation.

It is not clear whether transcription of noncoding sequences results from initiation events occurring within these regions or from unattenuated transcripts of coding sequences, or both. The lack of a clear consensus sequence among the multiple initiation sites found upstream of several different mitochondrial genes has led to the suggestion that transcription initiation in maize mitochondria may be a relatively nonspecific process (Mulligan et al., 1988b) that, by extension to our results, may not be confined to functional regions. The products of RNA synthesis by isolated mitochondria are considerably larger than mature ribosomal and messenger RNAs (Finnegan and Brown, 1986, 1987) and, because identified maize mitochondrial transcription initiation sites tend to lie within a few hundred bases upstream of coding sequences (Mulligan et al., 1988a, 1988b), it seems likely that these long primary transcripts arise from termination at sites distant from the 3′ end of mature RNAs. In addition, the kinetics of in vivo labeling are consistent with the occurrence of long, unattenuated, rapidly turning over transcripts extending into regions downstream of both the 26S and 18S + 5S rRNA cistrons. Regardless of the mechanisms by which the extragenic transcripts are generated, their absence at detectable levels in total mtRNA indicates the importance of post-transcriptional events in the expression of the maize mitochondrial genome.

Differential Rates of rRNA and mRNA Transcription

On the basis of incorporation of pulsed radioactivity both in vivo and in isolated mitochondria, we estimate that the rRNA transcripts accumulate between 5 times and 10 times faster than the three mRNAs analyzed. Because the difference in abundance of these two classes of sequences in total RNA is approximately 10-fold, differential rates of transcript accumulation appear to be the primary factor affecting the levels of these RNA species. We have not analyzed the transcription of other protein genes, and it is possible that transcripts of some of these may accumulate at rates markedly different from those of the *atp*A, *atp*6, and *cox*II gene regions. Because rRNAs are transcribed 15 times to 60 times more rapidly than mRNAs in human mitochondria, and 3 times to 10 times more rapidly in yeast mitochondria (Gelfand and Attardi, 1981; Mueller and Getz, 1986) and because we estimate that transcripts of the three protein genes accumulate at nearly identical rates, it seems unlikely that analysis of additional protein genes will affect the general conclusion that rRNA transcripts accumulate considerably more rapidly than the mRNAs.

The relative rates of transcript accumulation given in Table 1 are expected to be an approximate measure of the transcriptional activity in the different analyzed regions. A number of factors that determine the relative transcriptional rates of the protein coding genes have not, however, been accounted for in these values. For example, although

the hybridization signals have been corrected to account for differences in the lengths of the analyzed regions, in some cases the locations and/or relative frequencies of transcriptional initiation and termination events are unknown; hence, the proportion of these regions that is transcriptionally active is unknown. This could lead to an underestimation of transcriptional rates. In the case of the *atp*6 gene, some transcription initiation events will occur at a site 617 bp to 624 bp upstream of the coding region (Kennell and Pring, 1989). Most *atp*6 transcripts, however, are 1.8 kb in size and originate upstream of the region included on pT25H. The values of Table 1, although not corrected for the contribution of transcripts originating at the more proximal site, are not, therefore, expected seriously to underestimate the rate of *atp*6 transcription. Even if the approximately −620 bp initiation site were used exclusively, the Table 1 values would underestimate the actual rate by only 33%.

The values for *atp*A could conceivably be underestimated more seriously because the site(s) at which the transcription of this gene is initiated is unknown. The longest of the gene's mature transcripts span the entire upstream region included in pTA22, but the shortest transcripts are less than one-half the length of the analyzed region (Braun and Levings, 1985; Isaac et al., 1985). We have attempted to determine the extent to which the Table 1 values underestimate *atp*A transcription by probing a sequence derived entirely from the *atp*A coding region with RNA pulse-labeled in vivo and comparing the hybridization response to that of pTA22. The results of this experiment (not shown) indicate that the relative rates of *atp*A transcription, measured using either pTA22 or the coding region alone, are identical. A serious error caused by this factor in the case of the *cox*II gene is also unlikely because the analyzed region is only 1.5 times larger than the *cox*II gene itself, and most *cox*II transcripts are longer than the analyzed region.

A second unaccounted factor that could contribute to the estimation of transcriptional rates is the copy number of the different genes. By probing slot blots of the different analyzed regions with nick-translated mtDNA under conditions of target DNA excess, it was determined that the copy numbers of all regions other than those represented on pTA22 are approximately equal. Copies of the pTA22 sequences in the mtDNA of these cells exceed those of the other regions by a factor of 1.4. This is not unexpected because this region is part of a duplicated region on the "master" mitochondrial chromosome representation of N mtDNA (Lonsdale, 1987). Thus, the values for pTA22 transcript accumulation in Table 1 overestimate its transcription by a factor of 1.4 when the rates are corrected for differences in gene copy number. On the whole, therefore, although the values of Table 1 do not represent the relative transcriptional rates of the protein genes in a strict sense, they provide a sufficiently close approximation of such rates to allow the conclusion that the rRNA genes are transcribed considerably more rapidly than protein genes.

In yeast and mammalian mitochondria, both different promoter strengths and attenuation contribute to the differential rates of rRNA and mRNA transcription (Attardi and Schatz, 1988). Because transcription initiation sites have been found immediately upstream of several mitochondrial genes (Mulligan et al., 1988a, 1988b), it seems likely that most maize mitochondrial transcription units encompass only single genes. If this is the case, attenuation is unlikely to play an important role in regulating maize mitochondrial transcription, and differences in promoter strengths, therefore, probably account for the observed transcriptional rate differences. Our analysis indicates that the strongest promoters lie in the regions upstream of the rRNA genes. These regions are, therefore, likely to prove the most useful for establishing bona fide in vitro transcription systems.

Run-On Transcription in Isolated Mitochondria

Isolated mitochondrial transcription systems such as that employed here have the potential to allow the transcriptional state of the mitochondrial genome to be assessed in a wide range of cell types and are more conducive to certain experimental manipulations than in vivo labeling systems. To make such investigations meaningful, however, it is necessary to demonstrate that transcription in the isolated organelle is an accurate reflection of transcription in vivo. We have addressed this problem by comparing the hybridization signals obtained when mtRNAs radiolabeled during short pulses of in vivo or in organello RNA synthesis were quantitatively hybridized to a variety of membrane-bound mtDNA sequences. The similarity in the resulting hybridization patterns indicates that transcripts accumulate in isolated mitochondria in a manner similar to that in vivo.

Although the relative abundances of the sequences synthesized during short pulses of RNA synthesis in vivo or in isolated mitochondria are generally similar, the newly synthesized mtRNA populations differ in several respects. These differences can be largely accounted for by the premise that transcript processing and possibly initiation are depressed in the isolated organelle. RNA synthesis in this system, therefore, seems mainly to be due to elongation of preinitiated RNA chains by transcription complexes that have remained bound to the mtDNA template during organelle isolation. This view is supported by the observation that the transcriptional activities of both isolated mitochondria and freeze-thaw mitochondrial lysates are not inhibited by heparin, a specific inhibitor of transcriptional initiation (Deng et al., 1987). In addition, the observation that the relative accumulation rates for several mtRNA sequences in isolated mitochondria are similar to those in vivo indicates that the amount of radioactivity

incorporated into an RNA sequence during the in organello reaction is a direct reflection of the in vivo transcription rate of that sequence. RNA synthesis in isolated maize mitochondria may, therefore, represent a run-on transcription system similar to those that employ isolated nuclei (Marzluff and Huang, 1984). Isolated mitochondrial systems may, therefore, prove useful in determining whether developmental regulation of plant mitochondrial gene expression (Newton and Walbot, 1985; Young and Hanson, 1987) is achieved at the transcriptional or post-transcriptional level.

METHODS

Materials

The "Bluescript" phagemid is a product of Stratagene. Recombinant bacterial plasmid DNAs were obtained from Dr. J.H. Sinclair, Indiana University (pB401 and pB406); Dr. T.D. Fox, Cornell University (pZmE1); and Dr. C.S. Levings III, North Carolina State University (pTA22 and pT25H). Other reagents and materials were from sources described (Finnegan and Brown, 1986, 1987).

Purification and Electrophoretic Analysis of Mitochondrial RNA and RNA Labeling in Isolated Mitochondria

Described experimental protocols (Finnegan and Brown, 1987) were modified as follows. Mitochondria were prepared from suspension cultures of the Black Mexican Sweet line of maize maintained in 2,4-dichlorophenoxyacetic acid-supplemented MS media (Chourey and Zurawski, 1981). Log-phase cells were harvested by vacuum filtration, washed with fresh, cold medium, and disrupted in 5 volumes of homogenization buffer with a glass/glass tissue homogenizer. Mitochondria from 30 mg to 60 mg of suspension culture cells were used in the labeling reaction, and UTP in the labeling reactions was adjusted to 2 μM (3000 Ci/mmol). Unlabeled RNA was obtained from mitochondria withdrawn from the RNA synthesis reaction prior to the addition of radiolabeled precursor. When mtRNA was used as an enzymatic substrate, aurintricarboxylic acid was omitted from the mitochondrial lysates.

In Vivo Labeling of mtRNA

Suspension culture cells harvested in log phase were washed four times with growth medium lacking KH_2PO_4 before resuspension in this medium at a final concentration of 25 mg of cells/mL. Labeling was initiated by adding neutralized, carrier-free ^{32}P-H_3PO_4 to a final concentration of 1.7 mCi/mL. Aliquots of 1 mL were withdrawn at indicated intervals, made 100 μg/mL in both ethidium bromide and actinomycin D, and used immediately for isolation of mitochondria at 4°C. Cells were collected from the labeling reaction by centrifugation for 2 min at 16,000*g*, resuspended in 1 mL of homogenization buffer (Finnegan and Brown, 1987) containing 100 μg of both ethidium bromide and actinomycin D, and disrupted with a glass/glass tissue homogenizer. Cell debris was removed by centrifugation of the homogenate for 2 min at 1300*g*. The supernatant was recentrifuged under these conditions before the mitochondria were collected at 5200*g* for 2 min.

DNase Treatment, Partial Degradation, and Enzymatic Labeling of Mitochondrial RNA

RNA preparations used in hybridization analyses were treated with 1 unit of RNase-free RQ1 DNase, extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1), and subjected to Sephadex G-50 spun-column chromatography (Maniatis, Fritsch, and Sambrook, 1982). The RNA was then precipitated with ethanol and partially degraded in 0.2 N NaOH for 20 min at 4°C. After neutralization with 1 M Hepes, the products were precipitated with ethanol, and their mean length (200 bp to 250 bp) was determined by agarose/urea gel electrophoresis (Finnegan and Brown, 1987).

Approximately 0.3 μg of unlabeled, DNase-treated, alkali-nicked RNA was 5′ end-labeled by γ-^{32}P-ATP and T4 polynucleotide kinase (Maniatis et al., 1982). RNA was recovered by phenol extraction, followed by Sephadex G-50 spun-column chromatography.

Characteristics of Analyzed Regions

Plasmids pB401 and pB406 represent contiguous maize mtDNA regions and encompass the 18S + 5S and 26S rRNA genes, respectively (Iams and Sinclair, 1982). Fragments of these plasmids were subcloned into the multiple cloning site of M13mp18 (Yanisch-Perron, Vieira, and Messing, 1985). Using the fragment designations set out in Figure 4, the subcloned fragments included SmaI fragment A; HindIII fragments C, F, H, I, J, K, L, and M; SmaI/HindIII fragments B, D, and E; and BamHI/HindIII fragment G.

The 26S, 18S, and 5S rRNA genes and surrounding regions that are located on these fragments have been sequenced (Chao et al., 1983, 1984; Dale et al., 1984, 1985). Fragment D carries sequences extending from the transcription initiation site of the 26S rRNA transcription unit, at about nucleotide −185 relative to the 5′ end of the mature rRNA (Mulligan et al., 1988a), to nucleotide +22 within the *rrn*26 cistron; the approximately 2.4-kb region that lies upstream of the initiation site on this fragment has no known function. Fragment E lies entirely within the 26S rRNA gene, from nucleotide +23 to +2539, whereas fragment F consists of 26S rRNA nucleotides +2540 to the 3′ terminus (+3491), as well as 0.9 kb of downstream sequences. Fragment L extends from approximately 2.9 kb downstream of the 5S rRNA gene to the HindIII site 67 bp upstream of the 5′ end of the mature 5S rRNA gene. The abutting fragment, M, encompasses the entire 1968-bp 18S rRNA gene, which ends about 111 bp upstream of the 5S rRNA gene. Also on this fragment is the transcription initiation site of the polycistronic 18S + 5S rRNA transcription unit, at about nucleotide −230 relative to the 5′ end of the mature 18S rRNA (Mulligan et al., 1988a), as well as 340 bp of upstream sequences. Fragments A to C and G to K have no known coding functions.

A 595-bp Sau3A fragment from fragment E, 435-bp and 335-bp TaqI fragments from fragment M, and a 234-bp HindIII/HaeIII fragment from fragment L were further subcloned into the multiple cloning site of the Bluescript M13 phagemid, which is flanked by

T3 and T7 RNA polymerase promoters. These fragments are designated N, O, P, and Q, respectively (Figure 2).

pTA22 carries a 4.2-kb HindIII fragment cloned in pUC9 that spans the region of T cytoplasm maize mtDNA encoding the *atp*A gene (Braun and Levings, 1985). This fragment falls within the 12-kb direct repeat of N cytoplasm mtDNA and is conserved in sequence, but not copy number, between N and T mtDNAs (Isaac et al., 1985; Fauron and Havlik, 1989). Three major *atp*A transcripts of 2.5 kb, 3.0 kb, and 4.5 kb have been observed (Isaac et al., 1985). Because the sizes of these transcripts are conserved among the maize cytoplasms, it is likely that they terminate within the repeated region of N mtDNA immediately downstream of the coding sequence. The transcript size differences, therefore, arise primarily from differences at the 5′ termini. The transcriptions of pTA22 and the *atp*A coding region alone were compared by probing the EcoRI/SstI fragment of the *atp*A gene cloned in the Bluescript vector together with pTA22 and dividing the hybridization signals obtained by the sizes of the respective insert DNAs.

pT25H carries a 2.7-kb HindIII fragment cloned in pUC9 that spans the region of T cytoplasm mtDNA encoding the *atp*6 gene (Dewey et al., 1985). This region is conserved between T and N mtDNAs (Fauron and Havlik, 1989). The major mature transcript of the gene in N mitochondria is 1.8 kb in length and is thought to be processed from a primary transcript initiated at a site lying upstream of the region included in pT25H (Kennell and Pring, 1989). A second, less abundant primary transcript of 1.6 kb is initiated at a site about 620 bp upstream of the coding sequence. pZmE1 (Fox and Leaver, 1981) carries a 2.4-kb EcoRI fragment carrying the coxII gene cloned in pBR322. Transcripts of the gene range from 2.6 kb to 3.5 kb in size; their sites of initiation and termination are unknown.

To confirm that the organization of these genes in the tissue culture cells employed is that expected of a typical N mtDNA, DNA gel blots of BamHI-, EcoRI-, and HindIII-digested Black Mexican Sweet suspension mtDNA were probed with nick-translated pB401, pB406, pTA22, pT25H, and pZmE1. In each case, the hybridizing restriction fragments were those predicted for N mtDNA.

Slot-Blot Assay for Transcriptional Activity

Ten μg of cloned mtDNA sequences were partially depurinated by treatment with 0.3 M HCl for 5 min at 23°C, denatured by addition of NaOH to 0.64 M and incubation for 15 min at 37°C, and neutralized with HCl and ammonium acetate. Duplicate 5-μg portions of this DNA were bound to a GeneScreen-*Plus* nylon membrane using a Hybri-Slot manifold (Bethesda Research Laboratories). The DNA was fixed to the membrane by exposure to UV light prior to prehybridization at 42°C in 10 mL of 50% deionized formamide, 5 × SSPE (1 × SSPE = 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 0.1 mM EDTA), 1% SDS, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 100 μg/mL heat-denatured salmon sperm DNA for at least 5 hr. Hybridizations were done under these same conditions for 60 hr to 80 hr with probes consisting of DNase-treated, alkali-nicked mtRNA pulse-labeled either in vivo or in isolated mitochondria or enzymatically labeled with T4 polynucleotide kinase. Blots were washed 30 min at 23°C in 1 × SSPE, 0.1% SDS; 30 min at 55°C in 1 × SSPE, 0.1% SDS; twice for 30 min each at 55°C in 0.1 × SSPE, 0.1% SDS; and autoradiographed. Preliminary experiments in which increasing amounts of rRNA coding fragments were probed with RNA labeled by the different methods showed that identical signals were obtained with 1 μg and 5 μg of plasmid DNA. Hence, all hybridizations were performed under conditions of DNA excess.

Analysis of Transcription by Solution Hybridization and Nuclease Digestion

DNA templates from Bluescript clones, digested with XbaI or XhoI, were transcribed with T3 or T7 RNA polymerase, respectively, according to the manufacturer's instructions. 100 ng of each of two in vitro synthesized RNA probes were combined with 50 ng of labeled mtRNA in 0.6 M NaCl, 0.1 M Tricine, pH 7.8, 2 mM EDTA. Samples were heated 5 min in boiling water, transferred to a 60°C to 65°C bath, and incubated 10 hr to 12 hr. The resulting hybrids were digested with 250 units of RNase T1 for 60 min at 37°C. The digestions were terminated by addition of 10 μg of proteinase K and incubation for 60 min at 37°C, followed by the addition of 40 μL of 2.5 mM aurintricarboxylic acid, 50 μg/mL *Escherichia coli* rRNA, 5 M ammonium acetate, 10 μL of 20% SDS, and 320 μL of isopropyl alcohol. After 20-min coprecipitation with *E. coli* rRNA in ethanol at 23°C, the products were collected, denatured by heating at 70°C in 80% deionized formamide, 1 × TBE (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA), 1 mM aurintricarboxylic acid, and analyzed on a 5% polyacrylamide gel containing 8 M urea.

It was determined that the RNase T1 treatment was sufficient for the quantitative conversion of a heterogenously sized, uniformly labeled target RNA into a protected fragment the same size as the complementary probe RNA. Because there was no reduction in the RNase-resistant signal when an rRNA probe was used at one-tenth the standard concentrations, the hybridizations were quantitative. RNase T1 digestion in the absence of probe RNA resulted in a homogeneous background similar to that seen in Figure 5.

ACKNOWLEDGMENTS

We thank Drs. Thomas Fox, Cornell University, C.S. Levings III, North Carolina State University, and John Sinclair, Indiana University, for providing cloned plasmid DNAs; Lucy Byrne, Rima Menassa, and Ming-da Zhang for assistance in preparation of the manuscript; Antonio Monroy for assistance on some experiments; and C.S. Levings III for comments on the manuscript. This work was supported by grants from the Natural Sciences and Engineering Research Council, Canada, and the Fonds pour le Formation de Chercheurs et l'Aide à la Recherche of the Province of Quebec.

Received June 5, 1989; revised November 6, 1989.

REFERENCES

Attardi, G., and Schatz, G. (1988). Biogenesis of mitochondria. Annu. Rev. Cell. Biol. **4,** 289–333.

Bendich, A.J. (1985). Plant mitochondrial DNA: Unusual variation on a common theme. In Genetic Flux in Plants, B. Hohn and E.S. Dennis, eds (Vienna: Springer-Verlag), pp. 111–138.

Bland, M.M., Levings, C.S., III, and Matzinger, D.F. (1986). The tobacco mitochondrial ATPase subunit 9 gene is closely linked to an open reading frame for a ribosomal protein. Mol. Gen. Genet. **204,** 8–16.

Boerner, P., Mason, T.L., and Fox, T.D. (1981). Synthesis and processing of ribosomal RNA in isolated yeast mitochondria. Nucl. Acids Res. **9,** 6379–6390.

Braun, C.J., and Levings, C.S., III (1985). Nucleotide sequence of the F_1-ATPase α subunit gene from maize mitochondria. Plant Physiol. **79,** 571–577.

Carlson, J.E., Brown, G.L., and Kemble, R.J. (1986a). In organello mitochondrial DNA and RNA synthesis in fertile and cytoplasmic male sterile *Zea mays* L. Curr. Genet. **11,** 151–160.

Carlson, J.E., Erickson, L.R., and Kemble, R.J. (1986b). Cross-hybridization between organelle RNAs and mitochondrial and chloroplast genomes in *Brassica*. Curr. Genet. **11,** 161–163.

Chao, S., Sederoff, R.R., and Levings, C.S., III (1983). Partial sequence analysis of the 5S to 18S rRNA gene region of the maize mitochondrial genome. Plant Physiol. **71,** 190–193.

Chao, S., Sederoff, R.R., and Levings, C.S., III (1984). Nucleotide sequence and evolution of the 18S ribosomal RNA gene in maize mitochondria. Nucl. Acids Res. **12,** 6629–6644.

Chourey, P.S., and Zurawski, D.B. (1981). Callus formation from protoplasts of a maize cell culture. Theor. Appl. Genet. **59,** 341–344.

Christianson, T., and Rabinowitz, M. (1983). Identification of multiple transcriptional initiation sites on the yeast mitochondrial genome by in vitro capping with guanylyltransferase. J. Biol. Chem. **258,** 14025–14033.

Clayton, D.A. (1984). Transcription of the mammalian mitochondrial genome. Annu. Rev. Biochem. **53,** 573–594.

Dale, R.M.K., Mendu, N., Ginsburg, H., and Kridl, J.C. (1984). Sequence analysis of the maize mitochondrial 26S rRNA gene and flanking regions. Plasmid **11,** 141–150.

Dale, R.M.K., McClure, B.A., and Houchins, J.P. (1985). A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: Application to sequencing the corn mitochondrial 18S rDNA. Plasmid **13,** 31–40.

Deng, X.-W., Stern, D.B., Tonkyn, J.C., and Gruissem, W. (1987). Plastid run-on transcription. J. Biol. Chem. **262,** 9641–9648.

Dewey, R.E., Levings, C.S., III, and Timothy, D.H. (1985). Nucleotide sequence of ATPase subunit 6 gene of maize mitochondria. Plant Physiol. **79,** 914–919.

Dewey, R.E., Levings, C.S., III, and Timothy, D.H. (1986). Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell **44,** 439–449.

Fauron, C., and Havlik, M. (1989). The maize mitochondrial genome of the normal type and the cytoplasmic male sterile type T have very different organization. Curr. Genet. **15,** 149–154.

Finnegan, P.M., and Brown, G.G. (1986). Autonomously replicating RNA in mitochondria of maize plants with S-type cytoplasm. Proc. Natl. Acad. Sci. USA **83,** 5175–5179.

Finnegan, P.M., and Brown, G.G. (1987). In organello transcription in maize mitochondria and its sensitivity to inhibitors of RNA synthesis. Plant Physiol. **85,** 304–309.

Fox, T.D., and Leaver, C.J. (1981). The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. Cell **26,** 315–323.

Gaines, G., and Attardi, G. (1984). Highly efficient RNA-synthesizing system that uses isolated human mitochondria: New initiation events and in vivo-like processing patterns. Mol. Cell. Biol. **4,** 1605–1617.

Gelfand, R., and Attardi, G. (1981). Synthesis and turnover of mitochondrial ribonucleic acid in HeLa cells: The mature ribosomal and messenger ribonucleic acid species are metabolically unstable. Mol. Cell. Biol. **1,** 497–511.

Gruissem, W. (1989). Chloroplast gene expression: How plants turn their plastids on. Cell **56,** 161–170.

Iams, K.P., and Sinclair, J.H. (1982). Mapping the mitochondrial DNA of *Zea mays*: Ribosomal gene location. Proc. Natl. Acad. Sci. USA **79,** 5926–5929.

Isaac, P.G., Brennicke, A., Dunbar, S.M., and Leaver, C.J. (1985). The mitochondrial genome of fertile maize contains two copies of the gene encoding the α subunit of the F_1-ATPase. Curr. Genet. **10,** 321–328.

Kennell, J.C., and Pring, D.R. (1989). Initiation and processing of *atp*6, T-urf 13, and ORF221 transcripts from mitochondria of T-cytoplasm maize. Mol. Gen. Genet. **216,** 16–24.

Levings, C.S., III, and Brown, G.G. (1989). Molecular biology of plant mitochondria. Cell **56,** 171–179.

Lonsdale, D.M. (1987). The physical and genetic map of the mitochondrial genome from the WF9-N fertile cytoplasm. Maize Genet. Coop. Newsl. **61,** 148–149.

Makaroff, C.A., and Palmer, J.D. (1987). Extensive mitochondrial specific transcription of the *Brassica campestris* mitochondrial genome. Nucl. Acids Res. **15,** 5141–5146.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Marechal, L., Runeberg-Roos, P., Greinenberger, J.M., Colin, J., Weil, J., Lejeune, B., Quetier, F., and Lonsdale, D.M. (1987). Homology in the region containing a $tRNA^{Trp}$ gene and a complete or partial $tRNA^{Pro}$ gene in wheat mitochondrial and chloroplast genomes. Curr. Genet. **12,** 91–98.

Marzluff, W.F., and Huang, R.C.U. (1984). Transcription in isolated nuclei. In Transcription and Translation: A Practical Approach, B.D. Hanes and S.J. Higgins, eds (Oxford: IRL Press), pp. 89–129.

Mueller, D.M., and Getz, G.S. (1986). Transcriptional regulation of the mitochondrial genome of yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **261,** 11756–11764.

Mulligan, R.M., Lau, G.T., and Walbot, V. (1988a). Numerous transcription initiation sites exist for the maize mitochondrial genes for subunit 9 of the ATP synthase and subunit 3 of cytochrome oxidase. Proc. Natl. Acad. Sci. USA **85,** 7998–8002.

Mulligan, R.M., Maloney, A.P., and Walbot, V. (1988b). RNA processing and multiple transcription initiation sites result in

transcript size heterogeneity in maize mitochondria. Mol. Gen. Genet. **211,** 373–380.

Newton, R.J., and Walbot, V. (1985). Maize mitochondria synthesize organ-specific polypeptides. Proc. Natl. Acad. Sci. USA **74,** 2904–2908.

Stern, D.B., and Newton, K.J. (1985). Mitochondrial gene expression in *Curbitaceae:* Conserved and variable features. Curr. Genet. **9,** 395–405.

Stern, D.B., Dyer, T.A., and Lonsdale, D.M. (1983). Organization of the mitochondrial rRNA genes of maize. Nucl. Acids Res. **10,** 3333–3340.

Wettstein-Edwards, J., Ticho, B.S., Martin, N.C., Najarian, D., and Getz, G.S. (1986). In vitro transcription and promoter strength analysis of 5 mitochondrial transfer RNA promoters in yeast. J. Biol. Chem. **261,** 2905–2911.

Wintz, H., Grienenberger, J.-M., Weil, J.-H., and Lonsdale, D.M. (1988). Location and nucleotide sequence of two tRNA genes and a tRNA pseudo-gene in the maize mitochondrial genome: Evidence for the transcription of a chloroplast gene in mitochondria. Curr. Genet. **13,** 247–254.

Wissinger, B., Hiesel, R., Schuster, W., and Brennicke, A. (1988). The NADH-dehydrogenase subunit 5 gene in *Oenothera* mitochondria contains two introns and is co-transcribed with the 5S rRNA gene. Mol. Gen. Genet. **212,** 56–65.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33,** 103–119.

Young, E.G., and Hanson, M.R. (1987). A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell **50,** 41–49.