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Transcriptional and Posttranscriptional Regulation of Maize Mitochondrial Gene Expression

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Lysed maize mitochondria synthesize RNA in the presence of radioactive nucleoside triphosphates, and this assay was utilized to compare the rates of transcription of seven genes. The rates of incorporation varied over a 14-fold range, with the following rank order: 18S rRNA > 26S rRNA > *atp1* > *atp6* > *atp9* > *cob* > *cox3*. The products of run-on transcription hybridized specifically to known transcribed regions and selectively to the antisense DNA strand; thus, the isolated run-on transcription system appears to be an accurate representation of endogenous transcription. Although there were small differences in gene copy abundance, these differences cannot account for the differences in apparent transcription rates; we conclude that promoter strength is the main determinant. Among the protein coding genes, incorporation was greatest for *atp1*. The most active transcription initiation site of this gene was characterized by hybridization with in vitro-capped RNA and by primer extension analyses. The DNA sequences at this and other transcription initiation sites that we have previously mapped were analyzed with respect to the apparent promoter strengths. We propose that two short sequence elements just upstream of initiation sites form at least a portion of the sequence requirements for a maize mitochondrial promoter. In addition to modulation at the level of transcription, steady-state abundance of protein-coding mRNAs varied over a 20-fold range and did not correlate with transcriptional activity. These observations suggest that posttranscriptional processes are important in the modulation of mRNA abundance.

The mitochondrial genome of normal (N), male fertile maize is very large (569 kb) and can be represented as a single circle (11, 26). The complement of genes encoded by maize and other plant mitochondrial genomes includes structural genes for subunits of the oxidative phosphorylation system, rRNAs, a number of tRNAs, ribosomal protein subunits, and additional genes of undefined function (37). There is also some evidence for differential regulation of genes in plant mitochondria. For example, tissue-specific gene expression has been observed in maize mitochondria at the protein level (38), and developmental expression of a cytoplasmic male sterility-related gene occurs during flowering in *Petunia* spp. (49). In addition, nuclear control over the expression of a cytoplasmic male sterility-related gene, *Turf-13*, has been described in the T cytoplasm of maize (22).

Progress in understanding the regulation of transcription of maize mitochondrial genes has been hampered by transcript complexity. Numerous transcripts are frequently present for a given gene, and these multiple size classes are the result of both multiple transcription initiation sites and in the case of rRNA processing sites as well (22, 33, 35). Multiple initiation sites can be distinguished from transcript processing, because only the primary transcripts can be labeled with guanylyl transferase (35). Guanylyl transferase utilizes [α-^{32}P]GTP and catalyzes the incorporation of GMP into a cap structure in a reaction that is specific for di- or triphosphorylated transcripts; thus, only primary, unprocessed transcripts are labeled by this reaction (29).

The DNA sequences at 11 confirmed transcription initiation sites in maize mitochondria have been examined, and although some sequence similarity existed between these sites, no well-conserved consensus sequence was found at or near all transcription initiation sites (33). In contrast, promoter sequences have been identified in many organisms and in some organelles, i.e., higher plant chloroplasts (16) and mitochondria of yeast (3, 6). In the latter case, a conserved nonanucleotide consensus sequence is located at transcription initiation sites for each yeast mitochondrial transcription unit (3, 6). In *Escherichia coli*, the best studied example, analysis of up- or down-regulated promoter mutants was used to confirm the consensus features of the promoter (17).

A relevant point to consider when comparing initiation sites in maize mitochondria is the strength of the promoter: weak initiation sites may deviate significantly from a consensus sequence and hence make identification of a consensus difficult. Examination of mRNA steady-state abundance on Northern (RNA) blots reveals that the individual transcripts from independent initiation sites of a gene, as well as the total transcript amounts from the various genes, differ in abundance (33, 35). These observations could result from differences in promoter strength. To address this possibility, we have utilized a run-on transcription system with purified maize mitochondria to quantitate the relative transcription rates for individual genes. In organelle labeling of maize mitochondrial transcription and translation products have been utilized for more than a decade to analyze the patterns of macromolecular synthesis (reviewed in reference 37). Recently, RNA synthesis is isolated maize mitochondria was shown to be quantitatively similar to that obtained in vivo with mitochondria in maize tissue culture cells (14). The rate of synthesis of rRNA transcripts was 5- to 10-fold greater than that of the protein-coding genes examined. We confirm this finding, and in addition, our analyses demonstrated that maize mitochondrial protein-coding genes are transcribed at different rates and that these differences may represent transcriptional regulation. Measurement of the steady-state abundance of mRNAs for these genes suggests that posttran-

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TABLE 1. Clones and subclones of maize mitochondrial genes

Gene	Clone	Restriction sites	Insert size (kb)	Characteristics	Source or reference
atp1	BP1.3	*Bam*HI-*Pst*I	1.3	Nontranscribed upstream sequence	Fig. 8A
atp1	BP3.0	*Bam*HI-*Pst*I	3.0	Transcribed upstream sequence	Fig. 8A
atp1	BP1.7	*Bam*HI-*Pst*I	1.7	Actively transcribed upstream	Fig. 8A
atp1	BH0.7	*Bam*HI-*Hin*dIII	0.7	Actively transcribed upstream	Fig. 8A
atp1	BH0.7d200		0.5	~200-nt deletion subclone of BH0.7	
atp1	BP2.0	*Bam*HI-*Pst*I	2.0	5′ flank and reading frame	Fig. 8A
atp1	BB2.7	*Bam*HI-*Bam*HI	2.7	Reading frame and 3′ flank	Fig. 8A
atp6	T25H	*Hin*dIII-*Hin*dIII	2.7	Gene and both flanks	T cytoplasm (9)
atp6	HE1.6	*Hin*dIII-*Eco*RI	1.6	5′ flank and reading frame	Cosmid N6D9
atp9	BHc0.5	*Bam*HI-*Hin*cII	0.5	Reading frame and 3′ flank	33
atp9	XhHc0.6	*Xho*I-*Hin*cII	0.6	5′ flank	33
atp9	117L	*Bam*HI-*Hin*dIII	1.7	Gene and both flanks	35
atp9	117F	*Eco*RI-*Eco*RI	2.9	*cob/atp9* intergenic region	35
cob	117B	*Hin*dIII-*Eco*RI	0.7	Reading frame	35
cob	117C	*Hin*dIII-*Hin*dIII	1.8	5′ flank and reading frame	35
cox1	pBN6601	*Eco*RI-*Bam*HI	3.9	Gene and both flanks	20
cox1	1RV0.8	*Eco*RV-*Eco*RV	0.8	5′ flank and reading frame	pBN6601
cox2	pZME1	*Eco*RI-*Eco*RI	2.4	Gene and both flanks	15
cox2	2Ex-2	*Hin*dIII-*Sal*I	0.6	Exon 2 and 3′ flank	pZME1
cox3	pTL42	*Xho*I-*Bgl*II	1.4	5′ flank	33
cox3	pTL36	*Bgl*II-*Xba*I	1.3	Gene and both flanks	33
cox3	pTL36d700		0.7	~700-nt deletion subclone of pTL36	
rrn26	26B	*Taq*I-*Nde*I	0.9	Precursor-specific	35
rrn26	26D	*Ava*I-*Ava*I	1.0	5′-mature rRNA-specific	35
rrn18	18B	*Bam*HI-*Hpa*II	0.7	Precursor specific	35
rrn18	18C	*Bam*HI-*Xho*I	1.2	5′ flank and mature rRNA	35
rrn18	18D	*Hpa*II-*Nde*I	0.1	18S + 5S rRNA intergenic sequence	35
rrn5	HAII0.3	*Hin*dIII-*Ava*II	0.3	5S rRNA and flanks	

scriptional processes may also regulate transcript abundance.

In our studies we have found that *atp1* is the most highly transcribed protein-coding gene. In order to better understand the process of transcription initiation in maize mitochondria, we have characterized the DNA sequence at the major transcription initiation site for *atp1*. With this new information and a ranking of apparent promoter strength, we have reevaluated the known initiation sites. Comparison of the DNA sequences at the transcription initiation sites relative to promoter strengths suggests that some sequence motifs are present at frequently used transcription initiation sites and may thus be important for transcription initiation.

MATERIALS AND METHODS

Designations. *rrn18*, 18S rRNA gene; *rrn26*, 26S rRNA gene; *rrn5*, 5S rRNA gene; *atp1*, gene for the alpha subunit of the ATP synthase; *atp6*, gene for subunit 6 of ATP synthase; *atp9*, gene for subunit 9 of ATP synthase; *cob*, gene for apocytochrome *b*; *cox1*, gene for subunit 1 of cytochrome *c* oxidase; *cox2*, gene for subunit 2 of cytochrome *c* oxidase; and *cox3*, gene for subunit 3 of cytochrome *c* oxidase.

Cloned DNA. Clones and subclones utilized in this study are shown in Table 1. Clones were kindly provided by C. J. Leaver (pBN6601, pZME1), C. S. Levings (T25H, TA22), and C. Fauron (N6D9). All clones shown in Table 1 are derived from B37N maize mitochondrial DNA (mtDNA) with the single exception of the T25H clone, which is from B73T maize mtDNA. Plasmids were purified by the alkaline-lysis extraction procedure (2).

Run-on transcription in lysed mitochondria. Mitochondria were isolated from etiolated seedlings of B37N maize, purified by centrifugation on a discontinuous sucrose density gradient, and resuspended in homogenization buffer containing 0.4 M mannitol (21). All procedures were performed at 4°C or in an ice bath, unless otherwise noted. Run-on transcription assays were initiated by lysis of intact mitochondria (25 μl) in a reaction mixture (475 μl) with no osmoticum. The reaction mixture contained 10 mM Tricine-NaOH (pH 7.2), 10 mM $MgCl_2$, 1.25 mM EDTA, 50 mM KCl, 2 mM dithiothreitol, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 50 to 200 μCi of [α-^{32}P]UTP (3,000 or 800 Ci/mmol), 20 μg of bovine serum albumin per ml, and purified mitochondria (185 μg of protein per μl). In some experiments, radiolabeled CTP or GTP were substituted. The reaction was terminated by the addition of 200 μg of proteinase K and 0.1% sodium dodecyl sulfate (SDS), followed by incubation at 37°C for 30 min. Carrier tRNA (10 μg) was added, and the nucleic acids were extracted with phenol-chloroform (1:1) and then precipitated and dissolved in 10 mM Tris hydrochloride (pH 8) and 1 mM EDTA.

Filter hybridization. Plasmid DNA (100 ng) was denatured and immobilized on a slot or dot blot to a nylon membrane (Hybond-N; Amersham). The membrane was prehybridized as previously described (35) and then hybridized with 50% of a run-on transcription assay (250 μl of the reaction). After hybridization for 12 h or more at 42°C, the blot was washed two times with 0.1% SDS–180 mM NaCl–1 mM EDTA–10 mM sodium phosphate (pH 7.7). Filters hybridized with run-on transcription products were treated with RNase A and T_1 to remove single-stranded RNA as previously described (35). The blots were exposed to preflashed X-ray film (A_{600} = 0.4) with two X-ray screens (Lightning Plus; Dupont) at −80°C for 12 to 36 h. Autoradiograms were scanned with a laser densitometer (Pharmacia UltroScan). The peak area for each transcription unit is expressed as the transcriptional activity per kilobase of transcribed sequence, obtained

by dividing the peak area by the number of nucleotides in the immobilized DNA sequence.

Nucleic acid quantitation. The relative stoichiometry of each mitochondrial gene was determined by immobilization of a dilution series of total mtDNA (i.e., 1,000, 400, and 160 ng) and of plasmid DNA carrying the gene of interest (i.e., 8, 4, 2, 1, 0.5, and 0.25 ng) to a nylon membrane. Hexamer priming and extension were used to prepare a probe for each gene sequence after digestion of the relevant plasmid to release the insert. The radiospecific activity of each probe was diluted to a constant value (5×10^8 or 1×10^9 cpm/μg of DNA in individual experiments) with unlabeled plasmid DNA. The filter was hybridized with 5×10^6 cpm of the mtDNA sequence of interest, washed at 42°C with a solution containing 0.1% SDS, 180 mM NaCl, 1 mM EDTA, and 10 mM sodium phosphate (pH 7.7), and autoradiographed with preflashed film. Appropriate exposures of the nylon membrane were obtained, and autoradiograms were scanned with a laser densitometer (Pharmacia UltroScan) or a light densitometer (Bio-Rad Model 620 Video Densitometer). The peak areas for the plasmid reconstruction were used to establish a standard curve for hybridization (peak area per nanogram of DNA). The peak areas from the scan of the autoradiogram of hybridized genomic mtDNA were divided by the standard peak area per nanogram of DNA to yield the nanograms of DNA sequence per microgram of mtDNA. This value was corrected to reflect the number of molar equivalents of gene sequence per microgram of DNA by division by the molecular weight of the hybridized sequence (number of base pairs × 660 g/bp). The results of three independent quantitation experiments were normalized and averaged.

RNA was quantitated by hybridization of 5'-end-labeled RNA to immobilized plasmid DNA. Mitochondrial RNA (mtRNA) was prepared as previously described (33), except the RNA was precipitated two times with 2.5 M LiCl to remove DNA and small RNA molecules. Thus, the very abundant tRNA molecules, which may compete in the subsequent labeling reaction, have been removed from this preparation. Two methods for labeling the RNA were utilized and gave similar results. In one procedure, intact mtRNA (10 μg) was dephosphorylated with 1 U of calf intestinal alkaline phosphatase (Boehringer-Mannheim) in the presence 80 U of RNasin (Promega). The reaction was terminated by treatment with 50 μg of proteinase K and 0.1% SDS. The RNA was extracted with phenol-chloroform (1:1) and precipitated. The dephosphorylated, intact RNA was 5' end-labeled by the forward reaction of T_4 polynucleotide kinase (28) and precipitated twice with ammonium acetate and ethanol. The RNA probe was hybridized to slot blots of maize mtDNA sequences, and the resultant autoradiograms were scanned as described above. Because intact RNA was utilized in these experiments, the radiospecific activities of all transcript species were equivalent and the total peak area for each gene was a direct measure of the abundance of the transcript. In a second method, mtRNA (5 μg) was partially hydrolyzed to an average size of ~200 nucleotides (nt) by incubation in 0.2 N NaOH for 20 min at 0°C and was neutralized with 1 M Tris. The mtRNA was precipitated and labeled as described above for intact mtRNA. Autoradiograms were scanned as described above, and the relative hybridization signal was expressed as peak area per 1,000 nt of transcribed sequence by dividing by the size of the transcribed region represented in the immobilized DNA sequence.

In vitro capping and RNase protection analyses. Radiolabeling of maize mtRNA by in vitro capping was performed as previously described (33, 35). Approximately 200 ng of in vitro-capped mtRNA and 200 ng of single-stranded DNA were denatured at 75°C in 10 μl of 80% (vol/vol) formamide, as described for nuclease S1 protection analysis (12), and then treated with RNase A or RNase T_1 as previously described (33). Protected RNA fragments were fractionated by electrophoresis on denaturing polyacrylamide gels, dried under vacuum, and visualized by autoradiography.

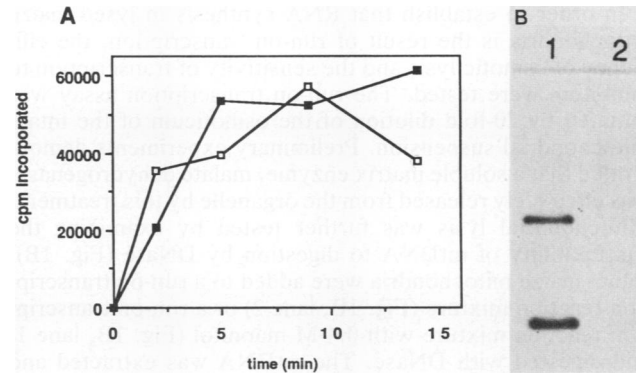

FIG. 1. RNA synthesis by lysed maize mitochondria. (A) Maize mitochondria (185 μg of protein per μl) were incubated in 200 μl of reaction mix at 25°C. Heparin was omitted (□) or present (■) at 250 ng/μl. Aliquots (20 μl) were removed at the indicated time, and the reaction was terminated with 0.1% SDS and 50 μg of proteinase K. Unincorporated nucleotides were removed by centrifugation through a column packed with P-10 (Bio-Rad). The incorporated ^{32}P was determined by the Cerenkov method. (B) Intact mitochondria (25 μl) were added to 475 μl of isosmolar run-on transcription buffer (0.4 M mannitol; lane 1) or hypotonic run-on transcription buffer (no osmoticum; lane 2). One unit of DNase I (RQ1, Promega) was added to the organelle suspension, and the suspension was incubated at 25°C for 30 min. The reaction was terminated with 50 mM EDTA, and the membranes were solubilized with 0.5% Triton X-100. Nucleic acids were extracted and precipitated. mtDNA was digested with *Bam*HI and analyzed by DNA blot hybridization with hexamer-labeled plasmid DNA (clone BB2.7).

Primer extension analysis. Primer extension analysis was done as previously described (33). A synthetic oligonucleotide (no. 6, an 18-mer; see Fig. 11) was radiolabeled by phosphorylation and hybridized to 10 μg of mtRNA at 45°C. The primed template was extended with 34 U of reverse transcriptase (Life Sciences, St. Petersburg, Fla.) for 30 min at 43°C. The assay was terminated with 20 mM EDTA and 20 μg of RNase A. The cDNA was extracted with phenol and precipitated. The cDNAs were electrophoresed on a 5% polyacrylamide sequencing gel. DNA sequence analysis was determined by the dideoxy-chain termination technique (39).

RESULTS

Properties of run-on transcription in isolated maize mitochondria. Purified mitochondria and chloroplasts have been used to assess organellar protein and RNA and DNA synthesis in many previous studies; macromolecular synthesis was dependent on temperature, pH, and substrate molecules (23). Our conditions for RNA synthesis in lysed maize mitochondria were a modification of published methods (1, 13). In our preparations, elimination of an energy source, succinate, had no effect on the incorporation of labeled nucleotides into RNA over a 60-min time course (data not shown). The incorporation of label reached a maximum at approximately 10 min (Fig. 1A).

In order to establish that RNA synthesis in lysed maize mitochondria is the result of run-on transcription, the efficiency of osmotic lysis and the sensitivity of transcription to inhibitors were tested. The run-on transcription assay was initiated by 20-fold dilution of the osmoticum of the intact mitochondrial suspension. Preliminary experiments demonstrated that a soluble matrix enzyme, malate dehydrogenase, was effectively released from the organelle by this treatment. Mitochondrial lysis was further tested by examining the susceptibility of mtDNA to digestion by DNase (Fig. 1B). Intact maize mitochondria were added to a run-on transcription reaction mixture (Fig. 1B, lane 2) or a run-on transcription reaction mixture with 0.4 M mannitol (Fig. 1B, lane 1) and digested with DNase. The mtDNA was extracted and digested with *Bam*HI, and a DNA blot was hybridized with a mtDNA probe, clone BB2.7 (Table 1). This probe includes a repeated sequence and unique flanking sequences; thus, the probe hybridizes to two restriction fragments of 2.7 and 5.6 kb (11). The hybridization of the probe to mtDNA extracted from osmotically lysed mitochondria demonstrated that most of the DNA was susceptible to DNase and that the organelles were effectively lysed (Fig. 1B, lane 2). The hybridization of the probe to mtDNA extracted from mitochondria maintained under isosmolar conditions demonstrated that mtDNA was resistant to DNase in the intact organelles (Fig. 1B, lane 1). The criterion of accessibility of the mtDNA to DNase establishes that the mtDNA is accessible to an exogenous protein molecule in osmotically lysed mitochondria.

Run-on transcription reflects the elongation of preexisting transcriptional complexes; thus, it is critical to establish that the incorporation of radiolabeled nucleotides is the result of transcript elongation and not transcription initiation. The role of transcription initiation may be tested by measuring the effect of factors that inhibit transcription initiation specifically but fail to inhibit RNA elongation, such as heparin, salt, or temperature (8, 18, 48). Heparin, a polyanion, inhibits transcription initiation by binding free RNA polymerase; this mechanism is universal against all RNA polymerases. Heparin at 20 ng/μl effectively inhibits transcription initiation with in vitro transcription systems from both chloroplast extracts (8) and bacteriophage T7 (18), although higher concentrations of heparin may be utilized in run-on transcription studies. In the lysed maize mitochondrial system, 250 ng of heparin per ml was not inhibitory to RNA synthesis (Fig. 1A). Thus, transcription initiation does not appear to be an important component of RNA synthesis in the lysed maize mitochondrial system, and the synthesis of RNA reflects run-on transcription.

Additional tests for transcription initiation are in agreement with this conclusion (34). In an attempt to develop a transient assay for maize mitochondrial gene expression, plasmid DNA with promoter regions of maize mitochondrial rRNA genes (*rrn18*, *rrn26*) or a protein-coding gene (*atp1*) were tested as templates for transcription with mitochondria permeabilized by high-field-intensity electric discharge (electroporation) or after lysis with 0.1% Triton X-100 or hypotonic treatment. Transcription of the exogenous plasmid DNA sequences was tested by hybridization of radiolabeled transcription products to vector DNA sequences or by nuclease S1 protection analysis with DNA probes labeled in the vector sequence. Each assay indicated that vector sequences were not transcribed, although the identical promoters of the endogenous genes were transcribed at high rates; thus, transcription initiation on the exogenous plasmids was not detected (34). These results, taken together with the inability of heparin to inhibit RNA synthesis, indicate that RNA synthesis in lysed mitochondria appears to be primarily the result of run-on synthesis.

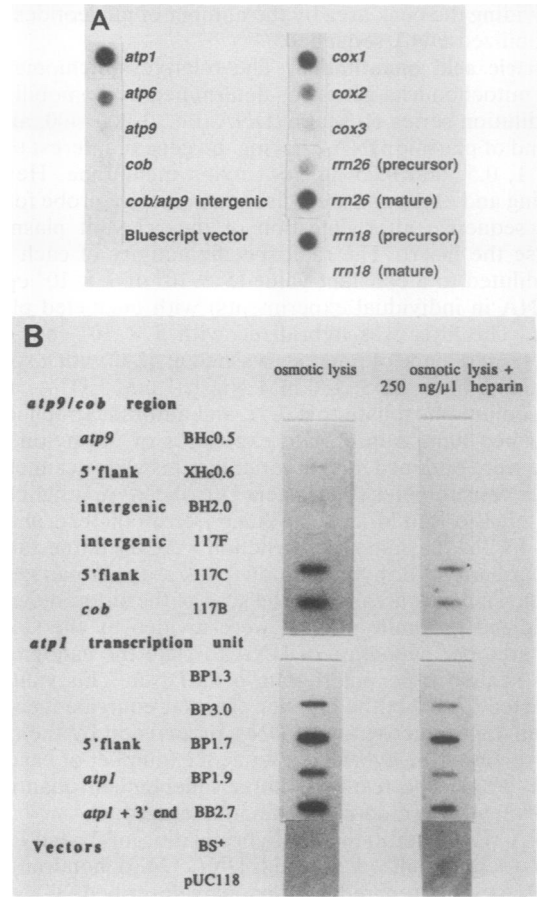

FIG. 2. Hybridization of run-on transcription products to maize mitochondrial genes. (A) RNA from a run-on transcription assay (250 μl) was hybridized to a nylon membrane with maize mitochondrial genes. The filter was treated with RNase and exposed to preflashed X-ray film as described in Materials and Methods. The clones represented are *atp1*-BP1.7, *atp6*-T25H, *atp9*-117L, *cob*-117B, *cob/atp9* intergenic-117F, Bluescript-Bluescript SK$^+$ vector, *cox1*-pBN6601, *cox2*-pZmE1, *cox3*-pTL36, *rrn26*(precursor)-26B, *rrn26*(mature)-26D, *rrn18*(precursor)-18B, and *rrn18*(mature)-18C. (B) Run-on transcription products from a 250-μl run-on transcription assay were hybridized to slot blots of the *cob/atp9* region and *atp1* transcription unit (clones described in Table 1; see also Fig. 8A). Heparin (250 ng/μl) was included or omitted from the reaction mixture as indicated.

Differential transcription of mitochondrial genes. Hybridization of the radiolabeled transcription products synthesized by isolated maize mitochondria to immobilized cloned sequences of maize mtDNA indicated that various genes were transcribed at different rates (Fig. 2A). The products of run-on RNA synthesis did not hybridize appreciably to the vector DNA controls (pUC118 or Bluescript) included in this experiment. Hybridization of in organelle-labeled RNA was detected to the 5′ flanking sequences of the 26S and 18S rRNA as well as to the mature rRNA regions (Fig. 2A). On the other hand, sequences without any detectable transcription products, such as the intergenic region between *atp9* and *cob*, were poorly transcribed (Fig. 2). In the case of *atp1*, run-on transcription products hybridized to the tran-

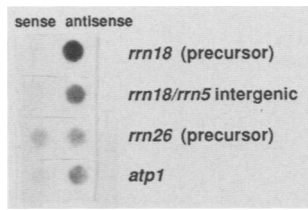

FIG. 3. The products of run-on transcription hybridize selectively to the antisense DNA strand. Single-stranded DNAs were prepared from mtDNA clones in the Bluescript + and − vectors with the helper phage M13KO7. Single-stranded DNA was immobilized on a nylon membrane and probed with run-on transcripts from a 250-μl reaction. The clones used in this hybridization assay are *rrn18*(precursor)-18B, *rrn18/rrn5* intergenic-18D, *rrn26*(precursor)-26B, and *atp1*-BP1.7.

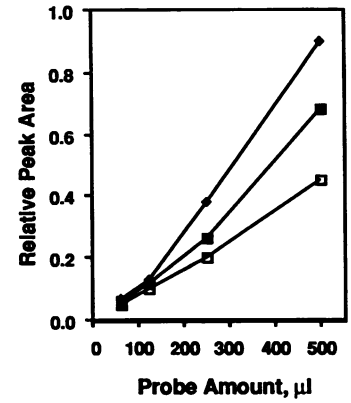

FIG. 4. Hybridization parameters for quantitative analysis. The linearity of the hybridization was tested by varying the amount of probe applied to four identical filters. Run-on transcription products were prepared from a 500-μl reaction mixture, and the probe was dissolved in 1 ml of 80% formamide; 500, 250, 125, or 62.5 μl or probe was hybridized to four slot blots. The target mtDNA clones contain the following: BP1.7, the *atp1* promoter region (◆); pBN6601, the *coxI* gene and flanking regions (■); and T25H, the *atp6* gene and flanking regions (□).

scribed 5′ flanking DNA sequences (BP3.0, BP1.7) but not to the nontranscribed upstream sequence, BP1.3 (Fig. 2B). In addition, the effect of heparin on run-on transcription was tested by hybridization of the products to mtDNA sequences for the *atp9-cob* region and the *atp1* transcription unit (Fig. 2B). The hybridization intensity of the run-on transcription probe was similar whether transcription was performed in the presence or absence of 250 ng of heparin per μl. These results indicate a strong bias in the organelle for incorporation into abundantly transcribed genes, consistent with the premise that incorporation represents elongation of preexisting transcriptional complexes.

The strand specificity of transcription in isolated mitochondria was examined by hybridization of the radiolabeled products of the run-on transcription assay to sense and antisense single-stranded DNA for several transcription units (Fig. 3). Four sense and antisense DNA sequences were tested: a clone specific for the precursor of the 18S rRNA, a clone specific for the precursor of the 26S rRNA, a clone specific for the spacer region between the dicistronic 18S+5S rRNA, and a clone for the promoter region of *atp1*. For three of these genes (pre-18S rRNA, 18S+5S spacer, and the *atp1* promoter), the products of run-on transcription hybridized specifically to the antisense DNA and not appreciably to the sense DNA (Fig. 3). Thus, the run-on transcription system reflects the strand specificity of the endogenous transcripts by faithful transcription of the coding DNA strand. In the case of the pre-26S rRNA, the probe hybridized strongly to the antisense DNA strand but more weakly to the sense DNA strand. The nature of the antisense transcript from the 26S rRNA gene is unknown, but a transcript in this region has been previously reported (14).

The relative rates of run-on RNA synthesis were quantitated by autoradiography of the dot blots followed by laser densitometry. Single-stranded RNA was removed from the membrane by treatment with RNase A and T_1 to limit hybridization to RNA colinear with the immobilized DNA sequences (8, 35). The sensitivity of the hybridization assay was confirmed by varying the amount of probe applied to the filters (Fig. 4). The peak areas showed a nearly linear response with the amount of probe utilized. Therefore, under the hybridization conditions used in these experiments, the amount of radiolabeled probe was limiting; thus, the signal intensity was approximately linear with the amount of probe present.

By using the densitometric data, the relative transcription rates per unit length of RNA (normalized to 1,000 nt) were compared (Fig. 5). The *rrn18* and *rrn26* genes were the most actively transcribed genes, followed by the protein-coding genes for *atp1*, *atp6*, *atp9*, *cob*, and *cox3*. The signal from the most weakly transcribed gene, *cox3*, was set to 1, and the transcription of other genes was expressed relative to this value. The *rrn18* and *rrn26* genes are the most abundantly represented transcription products, 14 and 9 times higher than *cox3*, respectively. The *atp1* signal ranks next at 6 times more abundant than *cox3* and is the most actively transcribed protein-coding gene tested. Differences in rates of run-on transcription in Fig. 5 are not corrected for differences in the abundance of the DNA sequences (see below); because the *atp1* gene is present at about twice the stoichiometry of the other genes examined, the rate of transcription of each copy is similar to the next most actively transcribed gene, *atp6*.

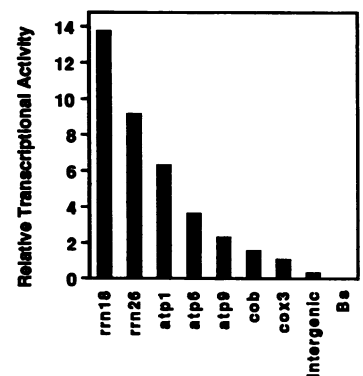

FIG. 5. Relative rates of run-on transcription in maize mitochondria. Maize mtDNAs were hybridized with [^{32}P]RNA from a run-on transcription assay as described in the legend to Fig. 2. The autoradiogram was scanned by laser densitometry, and the resulting peak areas were corrected for the length of the transcriptional unit represented in the clone (peak area per kilobase of transcriptional unit). The signal from *cox3* was set at 1 on the arbitrary scale. The relative transcriptional activities are not corrected for relative DNA sequence abundance. The clones utilized in this figure are the same as those listed in the legend to Fig. 2.

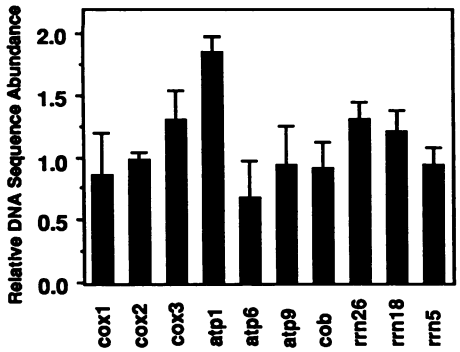

FIG. 6. Quantitation of mtDNA sequences. Mitochondrial gene sequences were immobilized on nylon filters and hybridized with random hexamer-labeled probes. The peak areas were obtained by densitometry and expressed as molar equivalents of DNA sequence per microgram of mtDNA. The hybridization experiments were repeated three times, and each hybridization experiment was normalized to the average of that experiment. The normalized results for each gene were averaged for three determinations, and the figure shows the average value and an error bar, which shows the standard error of the mean. The clones used in these experiments are as follows: *cox1*, 1RV0.8 (0.8 kb); *cox2*, 2Ex-2 (0.6 kb); *cox3*, pTL36d700 (0.7 kb); *atp1*, BH0.7 (0.7 kb); *atp6*, HE1.6 (1.6 kb); *atp9*, XhHc0.6 (0.6 kb); *cob*, 117B (0.7 kb); *rrn26*, 26B (1.0 kb); *rrn18*, 18B (0.7 kb); and *rrn5*, HAII0.3 (0.3 kb).

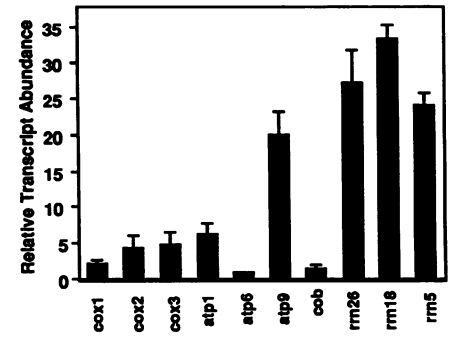

FIG. 7. Quantitation of mtRNA. The mean and standard error of the mean for three independent determinations of the relative quantity of mtRNA are shown. mtRNA was phosphorylated and hybridized to slot blots of cloned maize mtDNA sequences. Two of these determinations utilized 5'-phosphorylated intact mtRNA as a probe to the slot blot, and one experiment utilized 5'-phosphorylated mtRNA after partial alkaline hydrolysis. Autoradiograms were scanned, and the total peak areas for each gene are a measure of the abundance of the transcript. Details of the procedure are recorded in Materials and Methods. The clones used in these experiments are as follows: *cox1*, pBN6601; *cox2*, pZmE1; *cox3*, pTL42; *atp1*, BP1.7; *atp6*, BB6.5; *atp9*, 117L; *cob*, 117C; *rrn26*, 26B; *rrn18*, 18; and *rrn5*, HAII0.3.

Quantitation of abundance of mtDNA and RNA sequences. Although the B37N maize mitochondrial genome can be represented as a master circle of about 569 kb (11), the genome includes at least 10 direct repeats, and through recombination, the genome may exist as a number of subgenomic circular chromosomes. These subgenomic circular chromosomes bear subsets of genes, and if the circles replicate at different rates, variable gene stoichiometry would result. Indeed, differential abundance of specific maize mtDNA sequences has been reported: (i) sublimons are mtDNA sequences present in distinct genomic environments at very low stoichiometries compared with the main genomic DNA (42, 43), (ii) some inbred lines contain a higher copy number of some sequences (4), and (iii) the *atp1* gene is present in a direct repeat in some genomes (19). Differential amplification of mtDNA sequences could give rise to differential gene expression, independent of any intrinsic differences in promoter strength. Thus, it is critical to analyze the relative abundance of mtDNA gene sequences to evaluate whether differences in sequence representation could be responsible for the differences in gene expression detected among the maize mitochondrial genes.

The relative abundance of sequences for maize mitochondrial genes was determined by hybridization of labeled probes to genomic mtDNA (Fig. 6). Autoradiography and densitometric analyses indicated that most of the mtDNA sequences examined were present in approximately 1 equivalent. An exception is the gene for *atp1*, which was present at approximately 1.8 equivalents; this is in accord with the map of the B37N genome in which *atp1* is present on a direct repeat (11). Differential sequence amplification, however, cannot account for the differences in the rates of run-on transcriptions.

To determine whether the differences in gene transcription measured by run-on transcription were paralleled by differences in steady-state mRNA accumulation, total RNA was prepared from seedling mitochondria. The relative abundance of mtRNA was examined in three separate experiments by hybridization of 5'-labeled intact mtRNA or 5'-labeled mtRNA after partial alkaline hydrolysis to immobilized mitochondrial gene sequences. Autoradiography and densitometric analyses indicated that the steady-state abundance of mtRNA varied widely among genes (Fig. 7). As expected, transcripts for the rRNAs were most abundant. Transcripts for *atp9* were present at high abundance (10), while the transcripts for *atp1*, *cox1*, *cob*, and *atp6* were present at low abundance.

Identification of the transcription initiation site for *atp1*. Because the transcription signal from *atp1* was the strongest of the protein-coding regions tested in the run-on transcription assay, the most active transcription initiation site of this gene was characterized. The gene for *atp1* was isolated as a plasmid clone from a library of B37N mtDNA prepared by hybridization with the *atp1* gene from the T cytoplasm (5, clone TA22). Figure 8A shows a restriction map of *atp1* and the 5'-flanking region. This clone includes sequences of the direct repeat and 3'-flanking sequences, and it corresponds to copy N2 of this duplicated gene (19). Clones derived from the 5'-flanking region of *atp1* were used to probe Northern blots; the various transcript sizes detected are primarily the result of 5' heterogeneity (Fig. 8B). This is typical of several protein-coding genes in maize mtDNA and is the result of multiple transcription initiation sites (33, 35).

The 5'-flanking region and the *atp1* reading frame are both within the direct repeat. Thus, the contributions of the individual copies of *atp1* cannot be distinguished. Although there are multiple transcripts ranging in size from 1,000 to 6,000 nt, the most abundant transcript is approximately 4,000 nt. It hybridizes to subclone BP1.7, containing DNA sequence 1.3 to 3.0 kb upstream of the coding region; subclones from sequences further upstream failed to hybridize with this transcript. A small fragment of BP1.7 (clone BH0.7) was shown to include a transcription initiation site for *atp1* by hybridization of in vitro-capped mtRNA to a Southern blot of the 5'-flanking sequences of *atp1* (data not shown).

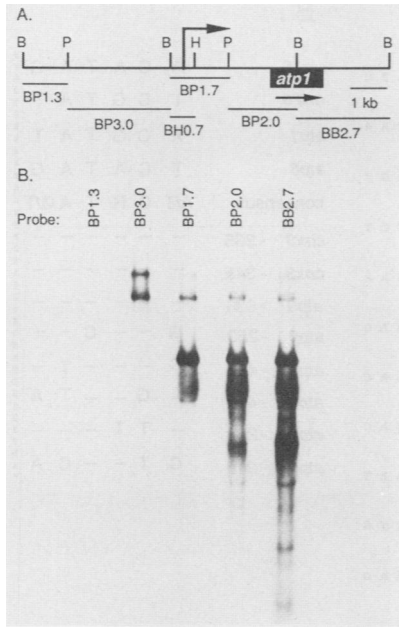

FIG. 8. (A) Restriction map of the *atp1* reading frame and flanking sequences. A restriction map of *atp1* and flanking sequences is shown. Abbreviations: B, *Bam*HI; P, *Pst*I; and H, *Hin*dIII. The subclones are shown underneath the DNA sequences they represent. (B) Hybridization of subclones to immobilized mtRNA. Plasmid DNA probes were hybridized to Northern blots of mtRNA.

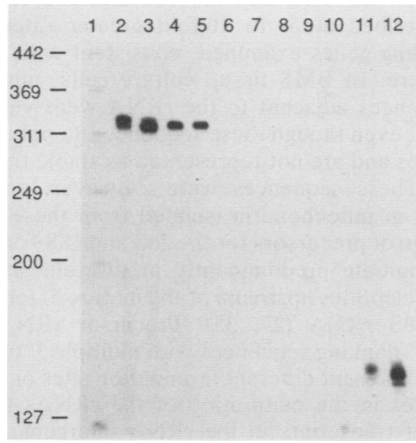

FIG. 9. RNase protection analysis of the transcription initiation site for *atp1*. Maize mtRNA was labeled by in vitro capping and hybridized with antisense single-stranded DNA from clone BH0.7 (lanes 2 to 5). The heteroduplex was digested with RNase A (50 or 250 ng, lanes 2 or 3, respectively) or with RNase T_1 (200 or 1,000 U, lanes 4 or 5, respectively). The sense DNA strand was substituted in lanes 6 to 9 and handled identically to the samples in lanes 2 to 5. Lanes 11 and 12 represent the protection of a capped RNA fragment by the antisense DNA strand of the 3′ deletion subclone, BH0.7d200. Samples for lanes 11 and 12 were digested with 50 ng of RNase A or 200 U of RNase T_1, respectively. DNA size standards are shown in lanes 1 and 10.

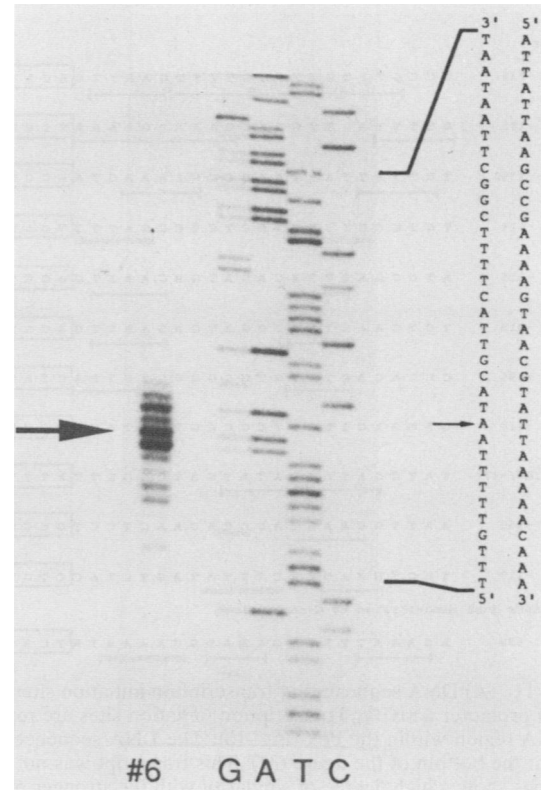

FIG. 10. Primer extension analysis of the 5′ terminus of *atp1* transcript. mtRNA (10 μg) was primed with a synthetic oligonucleotide (no. 6, an 18 mer) and extended with reverse transcriptase (lane #6). The DNA sequence (lanes G, A, T, and C) was obtained by priming the single-stranded DNA from clone BH0.7 with oligonucleotide no. 6 and extended with dideoxy sequence reactions. The products were electrophoresed on a 5% polyacrylamide sequencing gel.

Direct mapping of the transcription initiation site was accomplished by RNase protection analysis (Fig. 9). Capped RNA was hybridized with single-stranded DNA from clone BH0.7 (antisense DNA strand, Fig. 9, lanes 2 to 5; sense DNA strand, Fig. 9, lanes 6 to 9) and digested with RNase A (Fig. 9, lanes 2 through 3 and 6 and 7) or RNase T1 (Fig. 9, lanes 4 and 5 and 8 and 9). An RNA fragment of approximately 300 nt was protected from RNase by the antisense DNA, but no fragment was protected by the sense DNA strand. The position of the 5′ terminus of the primary transcript was more finely mapped with a subclone of BH0.7 generated by a 3′ deletion of approximately 200 nt (subclone BH0.7d200). The antisense DNA from this clone protected a capped RNA fragment of about 100 nt from both RNase A and T_1 digestion (Fig. 9, lanes 11 and 12, respectively). This position is approximately 2.3 kb upstream of the translational start site for *atp1*. These results are in accord with a series of nuclease S1 protection experiments that showed that this position represented the only major 5′ terminus within the 1.7-kb subclone, BP1.7 (data not shown).

Precise assignment of the 5′ terminus of the primary transcript was obtained by primer extension analysis and from DNA sequence data from subclone BH0.7 (Fig. 10). The primer extension products show a series of cDNAs which terminate over approximately 5 nt. The sequence context is similar to other maize mitochondrial initiation

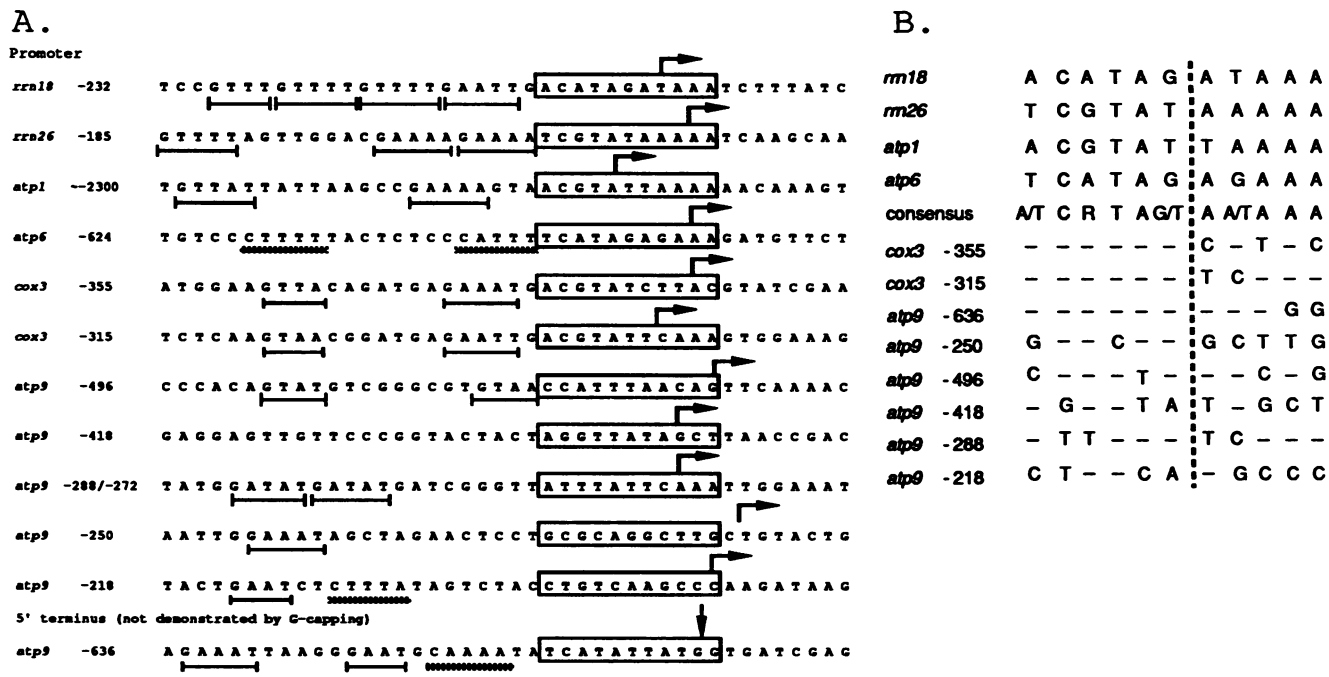

FIG. 11. (A) DNA sequences at transcription initiation sites for maize mitochondrial genes. The genes are ranked in order of strongest to weakest promoter activity. Transcription initiation sites are represented by the bent arrow; in the case of *atp1*, transcription initiates in the TATTAA region within the box (Fig. 10). The DNA sequence at the 5′ terminus of one of the most abundant transcripts of *atp9* (−636) is shown at the bottom of the figure (32). This transcript was not tested by G-capping analysis to determine whether it is a primary transcript, but it does show a high degree of similarity with the stronger promoters. Sequence similarity in an 11-nt region at the transcription initiation sites is shown within the rectangle. The $G(A/T)_{3-4}$ motifs on the sense strand are indicated by underlining; $C(A/T)_{3-4}$ motifs are indicated by the shaded underlining. As discussed in the text, these $R(Y)_{3-4}$ repeats may represent structural features of the promoter. (B) DNA sequences within the 11-nt region of panel A are compared for the three strongest maize mitochondrial promoters (top) and a consensus sequence. Promoter sequences for weaker promoters are listed in order of strength below the consensus sequence; the order of *atp9* sites is by transcript abundance. Nucleotide positions which do not deviate from the consensus are shown by a dash and positions which deviate from the consensus are identified by the nucleotide.

sites (Fig. 11). A sequence motif at or just 5′ of the initiation sites was identified by sequence comparison, and thus it seems likely that a consensus sequence for a maize mitochondrial promoter can be derived.

DISCUSSION

Run-on transcription reflects the elongation of preexisting transcriptional complexes; thus, the incorporation of radiolabeled nucleotides into transcription products reflects the relative rates of synthesis of various genes. In this study, a run-on transcription assay was utilized to rank promoter strength in maize mitochondria. Several parameters of the synthesis of transcription products were examined which substantiate the validity and fidelity of the run-on transcription assay. (i) RNA synthesis was insensitive to heparin, an inhibitor of transcription initiation, and was the result of elongation of preexisting transcriptional complexes. (ii) The transcription products hybridized preferentially to the antisense DNA strand and consequently show the proper asymmetry. (iii) The run-on transcription products hybridized to DNA sequences that contain transcriptional units with abundant endogenous products.

An earlier investigation of maize mitochondrial promoter strength utilized run-on transcription to examine the synthesis of the rRNAs and of several protein-coding genes in mitochondria isolated from Black Mexican Sweet (BMS) tissue culture cells and by in vivo labeling of intact cells (14). Their results indicated that the 18S and 26S rRNA genes were transcribed at 5- to 10-fold greater rates than the protein-coding genes examined, consistent with the results reported here. In BMS tissue culture cells, mitochondrial DNA sequences adjacent to the rRNA were very actively transcribed, even though these sequences do not encode any known genes and are not represented as stable transcription products. These sequences were, however, only weakly transcribed in mitochondria isolated from these cells. The transcription of precursors for the 26S and 18S+5S rRNAs is known to initiate predominantly at discrete sites several hundred nucleotides upstream of the mature 5′ termini of the 26S and 18S rRNA (27, 35). Precursor rRNAs include extensive 3′-flanking sequences with multiple 3′ termini, and these may represent different termination sites or processing intermediates in the maturation of the rRNAs (27). Thus, detection of transcripts in the rRNA intergenic regions in BMS tissue culture cells may result from inefficient transcription termination or different steps in precursor processing. In our study of seven transcription units, the actively transcribed regions corresponded with DNA sequences that are represented by abundant transcripts (Fig. 2). This apparent difference between the run-on transcription products obtained from etiolated seedling mitochondria and mitochondria in tissue culture cells may reflect some differences in transcriptional control by mitochondria in tissue culture.

The abundance of transcription products may be influenced by both the rate of synthesis and the stability of a

transcript. Our data support roles for both transcriptional and posttranscriptional regulation of gene expression in maize mitochondria. Differences in the rate of RNA synthesis in the run-on assay reflect transcriptional control, most likely exercised at the level of promoter strength. The incorporation of radiolabeled nucleotides into transcription products of isolated maize mitochondria varied 14-fold among the genes examined; similar differences have been observed in the expression of genes in chloroplasts (7) and yeast mitochondria (30, 31). Quantitation of the relative amount of mtDNA for each of the transcription units eliminates the possibility that differential gene expression is the result of differential amplification of mtDNA sequences; the differences in run-on transcription products appear to result from differences in promoter strength. rRNAs are required in large amounts by organelles, and these genes were consistently the most actively transcribed. Because the 26S and 18+5S rRNA transcripts are each derived primarily from single initiation sites, we conclude that very strong promoters regulate their expression. The mRNAs for protein-coding genes are required in smaller amounts and are synthesized less abundantly, apparently under the control of promoters of various strengths.

The relative abundance of maize mitochondrial transcripts varied widely among the genes examined. Transcripts for the *atp9* gene were very highly represented in the steady-state population, while other transcripts were present at much lower abundance (*atp6*, *cox1*, and *cob*). The differences in transcript accumulation do not reflect differences in the relative rate of synthesis by the run-on transcription assay. In fact, some of the most abundant transcripts for protein-coding genes, notably *atp9*, are poorly represented in the run-on transcription products, and some of the least abundant transcripts, notably *atp6*, are well represented in the run-on transcription products. These differences between the rate of synthesis and steady-state accumulation of transcripts suggest that transcript stability may differ substantially for these mRNAs; thus, posttranscriptional processes which govern transcript stability appear to play an important role in the accumulation of maize mitochondrial transcripts.

Chloroplast transcription products accumulate after exposure of plants to light. This process has been shown to arise from virtually constitutive expression of plastid genes in proplastids and etioplasts and from dramatic changes in the stability of the transcripts after exposure of plants to amounts of light sufficient enough to result in chloroplast differentiation (7, 32). Thus, light regulates chloroplast gene expression primarily by posttranscriptional processes that affect the stability of the transcripts. Many chloroplast transcripts terminate in an inverted repeat capable of forming a duplex of 10 to 30 nt. The inverted repeats have a role in the processing and stabilization of chloroplast transcrips (44, 45) and may play a role in the differential stability of chloroplast transcripts. Plant mitochondrial transcripts typically have two pairs of inverted repeats at the 3′ terminus (41), and it is possible that these stem and loop structures are also an important determinant of transcript stability in plant mitochondria.

Chloroplast promoters direct RNA synthesis with various efficiencies in vitro and in isolated plastids, and these differences reflect differences in the nucleotide sequence of the promoter (7, 16). Similarly, sequence analysis of promoters from regulatory mutants of *E. coli* has been useful in understanding the importance of DNA sequences at the promoter (17). Other investigators have discussed DNA sequences at the 5′ termini of plant mitochondrial transcripts but have not utilized transcript termini which are demonstrated to be polyphosphorylated by capping (25, 50). In our previous compilation of DNA sequences at transcription initiation sites (defined by in vitro capping) in maize mitochondria, we were unable to identify a well-conserved consensus sequence (33). From analysis of relative promoter strength by run-on assay, it is clear that we included many initiation sites from weakly transcribed genes (i.e., *atp9* was very poorly transcribed, and we sequenced six initiation sites in this upstream region). The DNA sequences at transcription initiation sites for maize mitochondrial genes are shown in Fig. 11A in decreasing order of apparent promoter strength. Substantial similarity exists between the DNA sequences at the transcription initiation sites for the stronger promoters; these sequences are shown within the box. A repeating motif [G(A/T)$_{3-4}$] is also present just upstream of the transcription initiation site in the stronger promoters; these sequences are underlined. For example, the strongest promoter, that of *rrn18*, includes 4 copies of the [G(A/T)$_{3-4}$] motif; this repeating motif is immediately adjacent to the conserved 11-nt consensus (boxed in Fig. 11A; discussed below). Weaker promoters contain fewer perfect copies of these repeats, and the repeats are separated by other sequences. It is not possible to establish the significance of the upstream [G(A/T)$_{3-4}$] motifs; however, AT-rich DNA does form a distinct double-helical structure (36), and this sort of structural motif may be involved in protein-DNA interactions (46). The upstream regions of promoters which lack these motifs on the sense DNA strand (*atp6* and some *atp9*) instead contain C(A/T)$_{3-4}$ repeats (shaded line). The C(A/T)$_{3-4}$ and G(A/T)$_{3-4}$ motifs may be similar structural features of maize mitochondrial promoters.

Examination of the DNA sequences for the four strongest promoters indicates that an 11-nt motif is present at the transcription initiation sites (Fig. 11B). We derived the consensus sequence [(A/T) C R T A (G/T) A (A/T) A A A] from these four promoters. The two ribosomal genes show a perfect match, while there is a single deviation for each of *atp1* and *atp6*: a T-for-A substitution at position 7 in *atp1* and a G-for-(A/T) substitution in position 8 of *atp6*. Comparison of the consensus sequence with DNA sequences for other promoters indicates that the 11-nt motif is also present in the moderate and weak promoters with increasing levels of deviance from the consensus. Furthermore, the consensus may be subdivided into two motifs. There is an upstream hexanucleotide sequence [(A/T) C R T A (G/T)] that is present in all of the promoters, including the −636 site of *atp9*. Second, there is an AT-rich motif in which transcription initiates and in which more deviations from the consensus are observed in the weak promoters. The known very weak promoters of *atp9* show serious mismatches to the consensus hexanucleotide. By Northern blotting analysis, the longer *atp9* transcripts are more abundant; this is also consistent with the −636 site being the best initiation site.

The relative importance of the 11-nt consensus and the [G(A/T)$_{3-4}$] motif cannot be evaluated from our data. It is clear, however, that promoters of high strength contain both of these elements. In contrast, the DNA sequences for most of the initiation sites in the *atp9* gene (33) and for *urf1* (47) show little homology with the DNA sequences discussed here, and the transcriptional activities of both of these genes were low (Fig. 5, *urf1*) (data not shown). If each of the six transcription initiation sites for *atp9* functions as an independent promoter, then the transcriptional activity of this gene is the sum of these promoters, and the individual promoter

strength at each transcription initiation site may be exceedingly low.

Another test of the consensus motif is its distribution. In searching the 34 kb of maize mitochondrial sequence available, we found only 28 instances of the 11-nt consensus (allowing one mismatch). Twelve of these cases are in the terminal inverted repeats of the 2.3-kb plasmid (24); transcription initiation does occur within this repeat. The other 16 cases are found in 32 kb of known sequence; several motifs are found in regions lacking stable transcripts (i.e., the 5-kb repeats), but for the most part, these motifs are located once or twice in the upstream region of known genes. Although this survey is not definitive, it does indicate that the 11-nt consensus sequence is relatively rare. Further studies of initiation sites could determine whether the sites identified by the computer search are in fact utilized as initiation sites.

The consensus sequence for maize mitochondrial promoters has some sequence similarity and positional similarity with the yeast mitochondrial consensus sequence (3, 6, 40). The maize mitochondrial consensus sequence can be aligned with the yeast consensus sequence [(A/T) TATAAGTA] with only 3 deviations in the nonanucleotide. The yeast consensus sequence is present immediately upstream of the transcription initiation site; transcription initiates at the last A of the consensus nonanucleotide. In addition, DNA sequences downstream of the transcription initiation site in yeast mitochondria have been shown to exert a strong effect on promoter strength (3, 40). Transcription initiation sites for maize mitochondrial genes were within a few nucleotides of the 3′ terminus of the consensus sequence. In many cases, primer extension experiments have indicated that multiple transcript termini exist which differ by a single nucleotide (e.g., Fig. 10); thus, transcription may initiate at one of several adjacent nucleotides in maize mitochondria. Thus, the yeast and maize consensus sequences are similarly located with respect to the transcription initiation site which is present at or near the 3′ terminus of the consensus sequence. This is in contrast to plastid and *E. coli* promoters which have consensus sequences at positions −10 and −35 relative to the transcription initiation site (16, 17).

Although the consensus motifs we propose for transcription initiation sites correlate with promoter strength, the contribution of individual parts of the motifs cannot be assessed. The role of these nucleotides must be tested through a functional assay of transcription in which nucleotide sequences are modified and analyzed. This will require developing an in vitro transcription system, a transient gene expression assay, or a transformation method for maize mitochondria.

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