A Novel Pea Mitochondrial in Vitro Transcription System Recognizes Homologous and Heterologous mRNA and tRNA Promoters*

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To elucidate the mechanism involved in the transcription initiation process in mitochondria of dicotyledonous plants, an in vitro transcription system was established for pea (Pisum sativum L.). The partially purified mitochondrial protein extract initiates transcription on homologous pea templates as well as on heterologous mitochondrial DNA from other dicot plant species. In vitro transcription begins within the nonanucleotide 5'-7CRTAAGAGA(-10)3' (transcription start site is underlined) conserved at most of the identified transcription initiation sites in dicot plant mitochondria. In vitro transcription initiation at promoters of protein as well as of tRNA coding genes identifies a conserved nonanucleotide motif which is essential for transcription initiation for different types of RNA. The competent recognition of different heterologous templates supports a general functional role of the nonanucleotide in mitochondrial promoters of dicotyledonous plants. Initial studies of the promoter structure by deletion analysis in the 5' region of the pea atp9 promoter show that in addition to the conserved nonanucleotide, which is essential for transcription initiation in vitro, sequences up to 25 nucleotides upstream of the transcription start site are necessary for an efficient initiation event.

The resident mitochondrial genomes encode essential genes, whose expression is indispensable for the function of the mitochondria and thus for the survival of the eukaryotic cell (Levings and Brown, 1989). Towards understanding the regulatory control mechanisms involved in mitochondrial gene expression, the biochemistry of transcription of the mitochondrial DNA has been intensively studied in animals and fungi. The mammalian 16-kilobase mitochondrial genome is symmetrically transcribed from two major promoters, one for each of the two different DNA strands. Transcription of the Saccharomyces cerevisiae mitochondrial DNA is initiated at about 20 copies of a highly conserved promoter motif (Christianson and Rabinowitz, 1983). In vitro transcription studies showed that in both yeast and mammals at least two protein components are engaged in specific and efficient transcription initiation. Although in yeast all genes and the encoded proteins of the mitochondrial transcription machinery are described in detail, only the gene for the transcription factor h-mtTF1 has been identified in human mitochondria (Shadel and Clayton, 1993). Compared with animals and fungi the mode of transcription initiation in mitochondria of plants remains elusive. Their much larger and more complex mitochondrial genomes and the frequently observed complex transcription patterns of individual genes impede the identification and analysis of promoters in mitochondria of plants (Levings and Brown, 1989; Newton, 1988). Several transcription initiation sites have clearly been identified by in vitro capping analyses in mitochondria from both monocot and dicot plant species. Inspection of sequences at these potential promoter regions identified distinct sequence motifs to which promoter function has been attributed (Mulligan et al., 1988a, 1988b, 1991; Covello and Gray, 1991; Brown et al., 1991; Binder and Brennicke, 1993a, 1993b).

The successful development of in vitro transcription systems for the monocot plant species wheat and maize confirms the competence of several promoters of protein coding genes in vitro (Hanic-Joyce and Gray, 1991; Rapp and Stern, 1992). Detailed investigation by deletion analysis, linker-scanning mutagenesis, and site-directed mutagenesis revealed the atp1 promoter in maize to be composed of a central domain extending from -7 to +5 and a 3-base pair upstream domain located between positions -10 and -12. The central element contains the highly conserved 5'-YRTA-3' core element that is consistent with the 5'-CRTA-3' motif defined by sequence inspection at the transcription initiation sites (Rapp et al., 1993; Mulligan et al., 1991). This motif is observed at most of the identified monocot mitochondrial promoters, which otherwise show only limited similarity between different species (Mulligan et al., 1991; Gray et al., 1992).

In dicot plant mitochondria, in vitro capping analysis of primary transcripts indicates a much better conservation of most of the potential promoter sequences. A conserved nonanucleotide motif 5'-7CRTAAGAGA(-10)3' has been defined by comparison of sequences surrounding mRNA, rRNA, and tRNA transcription initiation sites. With monocot promoters, only the tetranucleotide 5'-CRTA-3' appears to be conserved. Because the nonanucleotide motif is common to transcription initiation sites of different dicot plant species, it may represent a general core element of mitochondrial promoters in this plant group (Brown et al., 1991; Binder and Brennicke, 1993a, 1993b). However, several transcription initiation sites have been determined that show no sequence similarity to this conserved nonanucleotide motif or any other known plant mitochondrial promoter. Structure and function of these most likely gene- and/or species-specific potential promoters remain unclear (Brown et al., 1991; Binder et al., 1994).

To extend our knowledge about the mode of transcription initiation in dicot plant mitochondria we have now established an in vitro transcription system for pea. The in vitro results indicate the conserved nonanucleotide motif to be essential for...
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transcription initiation and confirm its function as a general promoter motif in different dicot plant species. In addition to mRNA coding with the pea mitochondrial extract, transcription initiation was observed at a RNA promoter suggesting that the transcription activity in this in vitro system is not restricted to protein coding genes.

MATERIALS AND METHODS

Recombinant DNA Templates—Plasmid clones patp9SC500 and patp9SK630 contain sequences covering the most distal 5’ mRNA end of the atp9 gene subconed from the type I allele of the 4 different atp1/atp9 arrangements present in pea mitochondrial DNA (Marikami and Nakamura, 1987, 1993). For deletion analysis of the pea atp9 promoter region, DNA fragments were amplified by PCR using patp9SK630 as template and the following oligonucleotides: clone Δpatp9–67, de–76 (5′-GTGGATCCTTATGTGAGCTTTTCTCC-3′) and de–355 (5′-TGGTATACCTCAGGAGATGTTGGGG-3′); clone Δpatp9–35, de–35 (5′-GTGGATCCTTGGTGATGACTGCAG-3′) and de–355; clone Δpatp9–25, de–25 (5′-GTGGATCCTTACCAGAAATAATAG-3′) and de–355; clone Δpatp9–7, de–7 (5′-GTGGATCCCTAATAAGAAGGATAGTTGG-3′) and de–355; and clone Δpatp9+2, de–2 (5′-GTGGATCCTAGATTGGGACAATTGAG3′) and de–355. Designation of the upstream oligonucleotides reflects, with respect to the transcription start site, the position of the most 5’ nucleotide within the oligonucleotide, which is identical with the mitochondrial sequence. Apatp9 promoter fragment was inserted into each upstream oligonucleotide for cloning. The downstream oligonucleotide de+355 covers a KpnI site contained in the mitochondrial DNA sequence. Amplified DNA fragments were digested with the respective restriction enzymes and cloned into pBluescriptII KS(−) vectors.

The soybean mtDNA clones KM57BK3 and KM2F2EH containing transcription initiation sites for the atp9 and RNA b/c gene, respectively, were kindly provided by Dr. Gregory Brown (Brown et al., 1991). Clone satp9XR482 is a 0.48-kilobase XbaI/Rsal subclone of KM57BK3. Clone opeBH500 contains the transcription initiation site identified upstream of the Oenothera trNA\textsuperscript{m} gene on a DNA fragment obtained by PCR with oligonucleotides OP-1 (5′-GGAATTCGATAAGAGAAGATATTGG-3′) and RNAsatK1 (5′-GGAATTCGAGATTCCATAAATGATGTTGG-3′) and de+355. Designation of the upstream oligonucleotides reflects, with respect to the transcription start site, the position of the most 5’ nucleotide within the oligonucleotide, which is identical with the mitochondrial sequence. Apatp9 promoter fragment was inserted into each upstream oligonucleotide for cloning. The downstream oligonucleotide de+355 covers a KpnI site contained in the mitochondrial DNA sequence. Amplified DNA fragments were digested with the respective restriction enzymes and cloned into pBluescriptII KS(−) vectors.

Preparation of Mitochondrial Protein Extracts—Pea seedlings (Psilium sativum L., var. Progress No. 9 and var. Lancet) were grown in soil (Vilmorin-Andrieux, France) and harvested after 5 days of growth. Mitochondria were isolated by differential centrifugation at 100,000 × g for 30 min (50% AS fraction). The supernatant (S100) was diluted with 1 volume of buffer V containing 90 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 0.5% glycerol, and hydrophobic proteins were precipitated by the addition of 0.224 g of solid (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} per ml of diluted solution and centrifugation at 16,000 × g for 30 min (20% AS fraction). The remaining protein was precipitated from the supernatant by the addition of 0.41 g of solid (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} per ml of solution and centrifugation at 16,000 × g for 90 min (50% AS fraction). The protein pellet was then resuspended in 1 ml of buffer A (10 mM Tris-HCl pH 8.0, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 7.5% glycerol) containing 50 mM KCl and dialyzed for several hours against 3 liters of the same buffer. For further purification by affinity chromatography, this protein fraction was applied to a MonoQ column (Pharmacia Biotech Inc.) equilibrated with buffer A (with 50 mM KCl added). Bound proteins were eluted from the column with 100, 200, 250, 300, 400, 500, and 750 mM KCl in buffer A. Fractions of each elution step were pooled, dialyzed against 3 liters of buffer A containing 10 mM KCl, concentrated by ultrafiltration using Centricon 10 (Amicon), and stored at −80 °C. Protein concentrations were measured using a Bio-Rad (Bradford) protein assay.

In Vitro Transcription Assays—In vitro transcription reactions were performed in a total volume of 12.5 μl of reaction mixture containing 10 mM Tris-HCl, pH 7.9, 10 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, 20 mM KCl, 500 μM each of ATP, CTP, and GTP, 25 μM UTP, 40 units of RNase inhibitor (Boehringer Mannheim), 10 μCi of [3H-UTP (3000 Ci/mmol), and 100–500 ng of linearized template DNA. Reactions were started by the addition of 30–80 μg of the protein extract and were incubated for 30 min at 30 °C. After the addition of 37 μl of stop mix (4.8 M urea, 0.4 M sodium acetate, 5.3 mM aurintricarboxylic acid, 30 μg/ml RNA (wheat germ), and 0.8% (w/v) SDS), reaction mixes were extracted with phenol/chloroform, and nucleic acids were precipitated with ethanol.

RESULTS

Development of an In Vitro Transcription System for Dicot Plant Mitochondria—The recent identification of several mitochondrial transcription initiation sites of soybean and Oenothera by in vitro capping analysis (Brown et al., 1991; Binder and Brennicke, 1993a, 1993b) provided the DNA templates required for the preparation of a transcriptionally active mitochondrial extract. The isolation of such transcriptionally active extracts from dicot plant mitochondria was additionally guided by the successful development of an in vitro transcription system for monocot plants (Hanicj et al. and Gray, 1991; Rapp and Stern, 1992). Because data on functional transcription initiation sites were only available for Oenothera and soybean, the isolation of transcriptionally active protein fractions was started in these two plant species. It soon became evident, however, that the impossibility of isolating large quantities of mitochondria from Oenothera or soybean and the high content of nucleases in protein extracts of these plant species prohibited the large scale preparation of transcriptionally active lysates. Attempts to prepare protein extracts from large amounts of mitochondria isolated from potato tubers likewise did not yield the desired activities, possibly owing to the basically low transcriptional activity in resting tissues like tubers (data not shown).

The search for a suitable dicot plant species and tissue type was finally successful in identifying pea seedlings as a suitable source for reasonable quantities of transcriptionally active mitochondria. Because no in vitro capping analysis had been carried out with pea transcripts, only little data were available on transcription initiation sites in mitochondria of this plant species. Homologous transcription assays thus relied on a DNA fragment containing the most distal 5’ mRNA terminus of the atp9 mRNA sequence. The pea atp9 mRNA promoter region includes a putative RNA-promoter sequence. Amino acid sequence of the first 12 amino acids of the predicted peptide encoded by the most distal 5’ end of the mRNA is identical with the mitochondrial sequence. Apat9 promoter fragment was cloned into pBluescriptII KS(−) vectors.
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Specific transcription initiation by a pea mitochondrial protein extract—The KpnI-linearized patp9SK630 template containing the region described above (Fig. 1A) was used in homologous in vitro transcription assays to test all protein fractions obtained during the enrichment procedure (see “Materials and Methods”). A weak signal corresponding to a transcript of the predicted size (Fig. 1A, 355 nt) was detected in the assay performed with the 50% ammonium sulfate fraction (Fig. 1B, 50% AS). The additional fractionation of the 50% AS cut resulted in enrichment of the specific transcription initiation activity. The majority of the specific activity could be detected in the 250 mKCl MonoQ fraction represented by the signal in the size range expected for the correct run-off transcript. Additional weaker transcription initiation activity was also present in the 300 mKCl MonoQ fraction, as indicated by a faint band of the predicted size (Fig. 1B, 250 and 300 mKCl).

No specific transcription products were observed in assays with only vector sequences or without any DNA template, indicating their dependence upon mitochondrial promoter sequences. The absence of any transcription product in a reaction carried out in the presence of RNase A showed the products of these assays to be indeed RNA resulting from genuine transcription events (data not shown). Because the majority of the specific transcription initiation activity eluted at 250 mKCl, this fraction was used for the detailed investigation of the accuracy of the initiation process in vitro and for heterologous transcription assays.

To confirm that transcription is indeed initiated within the mitochondrial insert, two differently linearized pea atp9 templates (patp9SK630 and patp9SC550, see “Material and Methods”) were tested in the transcription assays. In all instances run-off transcripts of the predicted sizes were observed in these reactions, and the lengths of the in vitro synthesized RNAs differed by the distances of the respective restriction sites used for linearization of the DNA templates confirming correct initiation within the mitochondrial insert (data not shown).

Transcription is initiated on heterologous templates from soybean—The conservation of the potential promoter sequences identified by in vitro capping assays of soybean and Oenothera transcripts and by the above in vitro studies of the pea atp9 gene indicates similar transcription initiation complexes most likely including homologous protein components within these dicot plant species. This hypothesis was tested by heterologous transcription assays with mitochondrial extracts and DNA templates from different dicot plant species. Clones KM57BK3 and satp9XR482 containing the mitochondrial promoter of the soybean atp9 gene were assayed for transcription initiation in pea protein extracts (Fig. 2A). Both templates direct transcription of RNAs corresponding to the predicted sizes, indicating accurate initiation in these heterologous reactions (Fig. 2B, lanes 1 and 2).

In addition to the atp9 promoter clone, KM2F2EH carrying two transcription initiation sites for RNAs of unidentified function (RNA b, c, and d) (Brown et al., 1991) was subjected to heterologous assays (Fig. 3). Although the common transcription initiation site for RNA b and c conforms to the consensus sequence element, sequences at the transcription start site for RNA e show no similarity to this promoter motif (Brown et al., 1991). Transcripts with the sizes predicted for correct initiation at the promoter for RNA b and c were observed with two differently linearized templates (Fig. 3). However, no tran-

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**Fig. 1.** Enrichment procedure for promoter specific in vitro transcription activity in pea mitochondrial extracts. A, structure of the genomic fragment containing the atp9 coding region present in the type I allele in pea mitochondria (Morikami and Nakamura, 1987, 1993). Three 5' ends ((1)-(3), indicated by bent arrows) have been mapped upstream of the coding region. A SfiI/KpnI fragment containing the most distal 5' end ((1)) located 1048 nt upstream of the start codon was subcloned (patp9SK630) and used for in vitro transcription reactions. A 355-nt-long run-off transcript (dotted arrow) is expected for correct transcription initiation on the KpnI-linearized DNA template. The sequence from -7 to +2 surrounding this 5' end (underlined) shows high similarity to the putative promoter consensus sequence from mitochondria of dicot plants (Brown et al., 1991; Binder and Brennicke, 1993a, 1993b). Restriction sites are indicated for Clal (C), KpnI (K), and SfiI (S). B, gel analysis of in vitro transcription assays with different fractions of the pea mitochondrial protein extracts and KpnI-linearized patp9SK630 DNA templates. Fractions used in the assays are lysed mitochondria (mitochondria), S100 supernatant (S100), 20% and 50% ammonium sulfate fractions (20% AS and 50% AS), and fractions eluted from the MonoQ column with 50, 100, 200, 250, 300, 400, 500 and 750 mKCl. Sizes of DNA length standards are given in nucleotides.

mapped in the upstream region of the atp9 gene (Fig. 1A, terminus (1)) (Morikami and Nakamura, 1993). The sequence surrounding this 5' end (5'-(<sup>-7</sup>CATAAGAGA<sup>1-2</sup>)-3'), which was determined by primer extension and S1 protection analysis, shows high similarity with the conserved nonanucleotide element detected at primary 5' transcript termini in mitochondria of other dicot plants (Brown et al., 1991; Binder and Brennicke, 1993a, 1993b).

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scripts of the respective sizes were observed for RNA e, suggesting that this promoter is not recognized by the pea extract.

To extend the analysis of heterologous transcription initiation, cox2 and atp1 templates from Oenothera were tested with the pea extract. In these assays distinct albeit weak signals corresponding to run-off transcripts of the predicted sizes showed correct recognition of these heterologous templates by the pea extract (data not shown).

In Vitro Transcription of a tRNA Gene Promoter—In vitro capping analysis of primary transcripts covering tRNA genes encoded in Oenothera mitochondria had identified a transcription initiation site located upstream of three alleles of the gene for tRNA^{A} and a gene for tRNA^{A}. The sequence at this transcription initiation site is consistent with the conserved sequence element derived from protein coding and rRNA genes (Binder and Brennicke, 1993b). To test whether this promoter is also recognized and transcribed by the pea extract, done opheBK500 containing a PCR-amplified 500-nt-long DNA fragment with the complete gene for TtRNA^{A} was used in the transcription analysis. A run-off transcript of 301 nt is expected from correct transcription initiation at this tRNA promoter and full-length elongation along the HindIII-cleaved template (Fig. 4A). Analysis of the transcription products by polyacrylamide gel electrophoresis shows a RNA of the expected size to be synthesized in the in vitro reaction (Fig. 4B). This result confirms common features for promoters of both protein coding and tRNA genes.

Precise Mapping of 5′ Ends of in Vitro Synthesized RNAs—The accuracy of the in vitro transcription initiation event was determined by primer extension analysis carried out with specific transcripts synthesized in both homologous and heterologous in vitro transcription reactions.

In the analysis of homologous pea atp9 transcripts, the 5′ ends of the in vitro transcribed RNAs are scattered around the transcription start site used in vivo. In addition to the 5′ end of
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Deletion Analysis of the Pea atp9 Promoter—Analysis of the pea atp9 promoter focused on the contribution of the region upstream of the transcription start site and on the role of the conserved nonanucleotide as a functional element in mitochondrial promoters of dicot plants. For this purpose a series of constructs with progressively deleted sequences upstream of the respective transcription start site were generated from clone patp9SK630 (Fig. 6A). The DNA templates were linearized with KpnI and tested for their ability to direct transcription initiation. A 355-nt RNA species is expected upon correct initiation at the atp9 promoter on this template (Fig. 1A). Transcription product analysis shows that deletion of sequences upstream of nucleotide −25 has no significant effect on the initiation process in vitro (Fig. 6B, Δpatp9 −67 to Δpatp9 −25). Deletion of additional sequences reduces specific transcription initiation almost completely (Fig. 6B, Δpatp9 −7 to Δpatp9 +2). This indicates that sequences required for transcription initiation in vitro are clustered immediately upstream of the transcription start site within the 25 nucleotides that are directly upstream. Although these sequences contain the conserved nonanucleotide, the conserved sequence element alone is not sufficient to efficiently promote transcription, because deletion of the sequence between the nonanucleotide and nucleotide −25 reduces the rate of in vitro transcription almost completely (Fig. 6B, Δpatp9 −7).

**DISCUSSION**

In Vitro Transcription System for Mitochondria of Dicot Plants—In vitro transcription systems have been successfully established for mitochondria of a number of different organisms including the two monocot plant species wheat and maize (Edwards et al., 1982; Walberg and Clayton, 1983; Bogenhagen and Yozza, 1986; Kennell and Lambowitz, 1989, Hanic-Joyce and Gray, 1991; Rapp and Stern, 1992). One of the major difficulties during the development of an in vitro transcription system for dicot plants was encountering the identification of a plant species amenable as a source for the isolation of large amounts of transcriptionally active mitochondria. We have now established such a system for pea mitochondria that allows the investigation of various features of the transcription initiation process in dicot plants.

Fractionating pea mitochondrial extracts on a MonoQ column showed that the formation of transcription initiation complexes is favored by the enrichment of RNA polymerase and potential transcription factors in an optimal stoichiometric proportion in the 250 mM KCl elution step. The vast majority of nonspecific transcription activity (i.e. RNA polymerase separated from specificity factors) elutes with the 400 mM KCl fraction. Nonspecific transcription activity is additionally enhanced by the contamination of this fraction with endogenous mtDNA, which elutes from the MonoQ column at KCl concentrations higher than 300 mM. Co-fractionation intact mtDNA-RNA polymerase complexes possibly also contribute to background activity. High molecular weight transcripts detected in all transcription assays are most likely due to nonspecific initiation events. Similar transcripts were also observed in other in vitro transcription systems and are attributed to initiation events at ends or nicks of the linear DNA templates (Hanic-Joyce and Gray, 1991; Rapp and Stern, 1992).

Further purification of the active protein fractions supplemented by DNA binding assays will be necessary to characterize the individual protein components involved in the transcription initiation process.

**Accuracy of the Pea Mitochondrial Transcription System—**
Every in vitro system should clearly and accurately represent the in vivo process investigated. In this respect the precise initiation in an in vitro transcription system should be identical with the transcription start site determined in vivo.

In the pea system described here, transcription is indeed initiated precisely on the soybean atp9 template (Fig. 5B). With the soybean RNA b/c template, a 5' end is detected that is very close to the 5' transcript terminus determined by an in vitro capping analysis (Fig. 5C). We assume that the differences between 5' ends of in vitro run-off transcripts, which are scattered over several nucleotides, and of in vivo pea atp9 mRNAs, where a single G is detected as a 5' terminal nucleotide, are derived through intrinsic problems of the primer extension experiments (Fig. 5A). The in vitro synthesized pea atp9 transcripts, which are recovered from the gel and used as RNA templates in the extension analysis, are always detected as a single sharp signal as seen for example in Fig. 1B. A series of transcripts with different 5' ends ranging over 10 nucleotides, as indicated by the primer extension analysis, should rather appear as an expanded signal separated in a 5% polyacrylamide gel (Fig. 1B).

Because a single signal consistent with the result obtained by Morikami and Nakamura (1993) (indicated by a bent arrow in Fig. 5A) is always detected in primer extension experiments with isolated in vivo pea mtRNA (data not shown), we conclude that the artificial length and composition of the in vitro run-off transcripts might be responsible for the scattered termini in the primer extension experiments most likely by a disadvantageous back folding.

Competence of the Pea in Vitro Transcription System for Promoters of Various Dicot Plants—Beyond the competent transcription of homologous templates and templates from another legume, soybean, the pea system initiates correctly also at promoters from the more distantly related dicot Oenothera (Onagracae). This includes, in addition to the initiation sites of cox2 and atp1, the promoter for a tRNA\(^{\text{Phe}}\) gene. The correct recognition of these promoters now provides experimental evidence for a more general conservation of promoter structures for at least two types of RNAs in mitochondria of dicot plant species. The dissemination of the conserved nonanucleotide motif at 5' ends of transcripts in other dicot species extends this broad significance, although evidence for genuine promoters in these species is still lacking (Moon et al., 1985; Young et al., 1986; Rothenberg and Hanson, 1987). However, in dicot plant mitochondria, transcription seems to be somehow additionally discriminatory, because the Oenothera 18 S rRNA promoter, although identical with respect to the conserved nonanucleotide, is not recognized by the pea in vitro system (data not shown).

The Conserved Nonanucleotide Motif Is a Core Element of the Dicot Plant Mitochondrial Promoters—Almost all DNA templates carrying the conserved nonanucleotide motif (5' AAGAGA(\(-2\),-3)') were recognized in vitro even on heterologous template DNA. Because this motif constitutes the only sequence element conserved in the active promoter regions, the in vitro transcription analysis strongly supports the conserved nonanucleotide motif to represent the core element of a mitochondrial promoter structure in various dicot plant species. This is confirmed by the deletion studies, in which only deletion of sequences immediately upstream of the transcription start point (i.e. 25 nucleotides preceding the transcription start site) significantly reduces initiation activity. Although deletion of the conserved nonanucleotide abolishes initiation completely (Fig. 6B, \(\Delta\)atp9 +2), a small amount of transcriptional activity remains in a construct with sequences up to position -7 (Fig. 6B, \(\Delta\)atp9 -7, detectable on the original x-ray film). The strongly reduced activity suggests an extended promoter structure, where the conserved nonanucleotide motif functions as a core element indispensable for transcription.
initiation and additional upstream sequences amplify initiation efficiency.

A comparison of sequences located between nucleotide positions −25 and −8 reveals a high content of adenosine-thymidine base pairs (67–83%) with almost 100% of the AT base pairs concentrated between nucleotide positions −13 to −9. This AT-box and a conserved adenosine nucleotide at position −1 are the most remarkable features within the critical upstream region (Fig. 7). The presence of the AT-rich box is found to be required to raise transcription initiation to significant levels.

Do Other Types of Promoters Exist in Mitochondria of Dicot Plants?—In addition to the conserved nonanucleotide promoters, several transcription initiation sites have been described that show almost no similarity to this motif or to each other. An example is the 26 S rRNA gene, whose transcription remains controversial in dicot plant mitochondria (Binder et al., 1994). Although transcription of this gene in potato is initiated at the mature 5' end of the RNA, the transcription initiation site of the Oenothera 26 S rRNA gene remains unclear. A test of both potato and Oenothera 26 S rRNA templates in the pea in vitro system failed to show any significant specific transcription activity. In the pea in vitro system, only promoters containing the conserved nonanucleotide appear to be transcribed. The transcription start site for RNA in soybean does not contain the nonanucleotide motif and is not recognized in vitro. Although located on the same template as the promoter for RNA b/c (Fig. 3A, KM2F2EHE), only RNAs with sizes that indicate initiation at the RNA b/c promoters were detected in these assays (Fig. 3B). Additional slightly smaller RNAs observed with the KpnI-linearized template (Fig. 3B, lane 1) are most likely degradation products, because these shorter transcripts are not present in the reaction with the HindIII-cleaved template. This observation suggests that mitochondria of dicot plants exploit different modes of transcription initiation using alternative promoter structures.

Only a few of these alternative promoters have been identified and so far no similarity could be detected between their primary structures. These nonconserved promoters represent probably gene- and/or species-specific single copy promoters. Because in animal and yeast mitochondria replication is primed by short RNAs initiated at promoter sequences, a sim-
ilar function might be attributed to some of these alternative promoters in plants (Schmitt and Clayton, 1993; Xu and Clayton, 1995). Much more data about these promoters in a single plant species are necessary to see whether different types or classes of promoters are indeed present in plant mitochondria and how they are recognized.

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