Continuous Primary Sequence Requirements in the 18-Nucleotide Promoter of Dicot Plant Mitochondria*

(Received for publication, November 16, 1998, and in revised form, January 20, 1999)

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The nucleotide requirements of mitochondrial promoters of dicot plants were studied in detail in a pea in vitro transcription system. Deletions in the 5′ regions of three different transcription initiation sites from pea, soybean, and Oenothera identified a crucial AT-rich sequence element (AT-Box) comprising nucleotide positions −14 to −9 relative to the first transcribed nucleotide. Transversion of the AT-Box sequence to complementary nucleotide identities results in an almost complete loss of promoter activity, suggesting that primary structure rather than a simple accumulation of adenines and thymines in this region is essential for promoter activity. This promoter segment thus appears to be involved in sequence specific binding of a respective protein factor(s) rather than merely loosening and melting the DNA helix during or for an initiation event. Manipulation of nucleotide identities in the 3′ portion of the pea atp9 promoter and the respective 3′-flanking region revealed that essential sequences extend to positions +3/+4 beyond this transcription start site. Efficient transcription initiation at an 18-base pair promoter sequence ranging from nucleotide positions −14 to +4 integrated into different sequence contexts shows this element to be sufficient for autonomous promoter function independent of surrounding sequences.

In mitochondria of Saccharomyces cerevisiae a 9-base-pair-long highly conserved cis-element is found at each of the about 20 transcription initiation sites. This nonanucleotide motif autonomously supports efficient transcription initiation in vitro, with enhanced promoter activity depending on the presence of a purine at position +2 and a pyrimidine at +3. In Xenopus laevis mitochondria an eight-nucleotide-long cis-element drives bi-directional transcription initiation at two different locations. A 15-bp¹ consensus motif is sufficient for basal promoter activity in mammalian mitochondria, while maximal promoter function depends on additional upstream sequence elements (10, 11).

In vitro capping analyses have identified conserved sequence elements also at mitochondrial transcription initiation sites in plants. These investigations revealed the tetranucleotide 5′-CRTA-3′ to be the only common motif found at initiation sites of both monocot and dicot plant species (12–16). Functional studies of the maize atp1 promoter in a homologous in vitro transcription system confirmed the significance of some of the conserved sequence features (i.e. 5′-CRTA-3′), while other segments appear to be not required at least for basic promoter function in vitro (e.g. G(AT)₅₋₇; Refs. 16 and 17). Further investigations revealed also differences in the basic structures and requirements between individual promoters. While an A-rich upstream domain contributes significantly to the maize atp1 promoter function, such a domain appears not to be required for the cow3 promoter activity in the same species (18, 19).

Mitochondrial promoters in dicot plant species display a somewhat larger conserved sequence element compared with those found in monocots. Inspection of sequences adjacent to transcription initiation sites of several bona fide dicot promoters revealed a conserved nonanucleotide motif (5′-CGAGA²₋₃₋₃′, CNM), which covers the first two transcribed nucleotides (20). Functional studies, however, showed that this motif per se is not sufficient for the function of the pea atp9 promoter in vitro and that additional sequences are required (21). We have now determined these additional sequence requirements and report that essential promoter sequences require specific nucleotide identities within an 18-nucleotide region between positions −14 and +4 relative to the transcription initiation site.

EXPERIMENTAL PROCEDURES

Template Construction—5′ progressively deleted fragments of pea atp9, soybean atp9, and Oenothera rpl5 promoter region were amplified by PCR using Pfu polymerase in buffer supplied by the manufacturer (Stratagene). PCR conditions, primer pairs, and DNA templates were as follows. Pea atp9 promoter region (patp9): dp+355 and one of the oligonucleotides dp-16, dp-14, dp-12, dp-10, and dp-7 using patp9SK630 as template (21); soybean atp9 promoter region: Rev and one of the oligonucleotides GM-D-7, GM-D-10, GM-D-12, GM-D-14, and GM-D-16 using clone satp9XR482 as DNA template (21); Oenothera rpl5 promoter.

¹ The abbreviations used are: bp, base pair(s); CNM, conserved nonanucleotide motif; PCR, polymerase chain reaction; MP, mitochondrial promoter.

* This work was supported by grants from the Deutsche Forschungsgemeinschaft and a Landesforschungsschwerpunkt Baden-Württemberg. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 274, No. 15, Issue of April 9, pp. 10094–10099, 1999
Printed in U.S.A.
moter region: OL5–3 and one of oligonucleotides L5/dp-7, L5/dp-10, L5/dp-13, L5/dp-14, and L5/dp-7 using a BamHI/HindIII clone comprising the Oenothera rps5 gene and its 5'-flanking sequences. DNA fragments were amplified with 40 cycles of 1 min at 90 °C, 1 min at 50 °C (pea atp9), 40 °C (soybean atp9), and 45 °C (Oenothera rps5), respectively, and 1 min at 72 °C. PCR products were ethanol-precipitated and digested with the respective restriction enzymes and DpnI to remove the template DNA. After separation in agarose gels DNA fragments were cloned between BamHI and KpnI (patp9 and satp9) and BamHI and HindIII (orpl5) restriction sites of pBluescript vectors.

For substitutions in the 3'-flanking region of the pea atp9 conserved nonanucleotide motif inverse PCR was performed with oligonucleotides LS-5–1 and LS-3–1 for clone LS-1 and LS-5–2 and LS-5–2 for mutant LS-2 on patp9SK630 and patp9SK630 Δ-17 clones as templates, respectively. Partial digestion by FstI resulted in variations of the inserted sequences. Due to cloning artifacts during construction of LS-2 an XhoI site was used for linearization instead of KpnI with construct LS-1.

Primer pairs TVAT-u and atp9-decad (A/T TM), patp9–1u and patp9–1d (TM-1), patp9–2u and patp9–2d (TM-2), patp9–3u and patp9–3d (TM-3), and patp9–2uwt and patp9–2d (TM-2) were used in the inverse PCRs for transversion of nucleotide identities within the pea atp9 AT-Box, the CNM, and the 3'-flanking sequences, respectively. Inverse PCRs were exclusively performed with Klentaq polymerase (CLONTECH) in a buffer supplied by the manufacturer with 35 cycles under the following conditions: 1 min 94 °C, 1 min 46 °C (TM mutants) and 50 °C (LS mutants), respectively, and 4 min at 68 °C. After PCR the template DNA was removed by digestion with DpnI, the DNAs were separated on agarose gels and the respective fragments were ligated at 15 °C for at least 16 h.

All DNA templates used in the experiments described in this report are controlled by restriction digests and complete sequence analysis. Sequences of all oligonucleotides used are available on request.

About 18 pmol of each MP oligonucleotide (MP sense: 5'-ATAATAG-CATAAGGAGAAG-3' and the respective antisense oligonucleotide) were denatured for 30 s at 80 °C, incubated for 20 min at 28 °C, and slowly adapted to room temperature in a total volume of 10 μl. Prior to ligation into the Smal and EcoRV sites in pBluescript and the SmaI site of a clone containing the pea rps14/cob spacer region, 9 pmol of double-stranded MP oligonucleotides were concatenated in the presence of 12% (v/v) polyethylene glycol, 50 mmol ATP, 1 mmol dithiothreitol, and 10 units of T4 DNA ligase. 5 μl of the concatenated MP oligonucleotides were then cloned into pBluescript vectors and a rps14/cob clone in the presence of 0.05 mmol ATP, 1 mmol dithiothreitol, 11% (v/v) polyethylene glycol, and 10 units of T4 DNA ligase in a total volume of 10 μl.

In Vitro Transcription Assays—Enrichment of pea mitochondrial lysates and in vitro transcription reactions were performed as described previously (21). Between 23 and 47 μg of lysate were used in standard assays with 100 ng of template DNA quantified both photometrically and on agarose gels.

**RESULTS**

In Vitro Analysis of the Upstream Extent of the Pea atp9 Promoter—Initial studies of the mitochondrial pea atp9 promoter in a homologous in vitro transcription system had confirmed the functional importance of the nonanucleotide between nucleotides −7 and +2, which is conserved between several promoters. In addition the analysis of clones containing 5'-deleted promoter regions suggested that crucial sequence information for the function of this promoter is located between nucleotide positions −25 and −8 relative to the 5' end of the nascent transcript (21). To identify the functional requirements within this region, a series of deletion clones was constructed and assayed in the in vitro transcription system. While constructs with deletions upstream of positions −25, −21, and −16 contain full promoter function, the initiation activity is significantly reduced in construct Δ-12 and completely lost in mutant Δ-7 (data not shown). For further resolution of the nucleotide requirements in the comparatively AT-rich region between positions −16 and −7, the mitochondrial sequences were deleted in smaller steps (Fig. 1, upper part). While promoter function is not impaired in constructs Δ-16 and Δ-14, activity is entirely lost in deletion clones Δ-10 and Δ-7. Partial transcription initiation activity is again observed with clone Δ-12. Independent reruns of these experiments, which were calibrated by the addition of T7 RNA polymerase-generated transcripts to the in vitro transcription assays, confirmed that the reduced amount of run-off products originated from decreased intrinsic promoter activity of this template and not from a selective loss of in vitro generated RNAs during the assays (Fig. 1, lower part, T7(161 Nt)). These functional analyses demonstrate that sequences extending up to nucleotide position −14 are sufficient and necessary to support transcription initiation at the pea atp9 promoter in vitro.

Sequences Extending to Nucleotide Position −14 Are Generally Sufficient for Promoter Function in Vitro—To gain more information about the general significance of 5' sequences in

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between significant differences in transcription efficiencies were found. In the assays with soybean constructs no function of nucleotide position was required sequences are lost upon deletion upstream of respectively. In the assays with soybean constructs no function (data not shown). This observation suggests that sequences rich in adenosines and thymidines per se do not support promoter function and that this promoter segment contributes to the sequence-specific interaction(s) between the promoter and the transcriptional apparatus in dicot plant mitochondria.

Insertions of 1, 3, 5, or 11 guanosine nucleotides between the AT-Box and CNM also almost completely abolished promoter activity. The faint signal is only visible upon long exposures, which indicates the residual promoter function to be less than 1% of the wild type activity. This nucleotides per se or whether sequence specificity is important, adenosine and thymidine residues in this particular region of the pea atp9 promoter were transverted to the respective complementary nucleotides (Fig. 3, upper part). The melting temperatures of the mutated and the wild type promoter regions were calculated to differ by only 0.6 °C, and thus comparable initiation efficiencies are expected if the function of this segment is confined to access strand opening of the DNA. However, in vitro transcription assays with the mutated and wild type templates revealed that initiation is almost completely abolished by the transverted nucleotides (Fig. 3, lower part). Semi-quantitatively repeated assays confirmed the significant reduction of the promoter activity. The faint signal is only visible upon long exposures, which indicates the residual promoter function to be less than 1% of the wild type activity. This observation suggests that sequences rich in adenosines and thymidines per se do not support promoter function and that this promoter segment contributes to the sequence-specific interaction(s) between the promoter and the transcriptional apparatus in dicot plant mitochondria.

Insertions of 1, 3, 5, or 11 guanosine nucleotides between the AT-Box and CNM also almost completely abolished promoter function. Since promoter activity is not restored even when the distance between the two parts corresponds to a complete helix turn, the AT-Box and CNM appear to be interdependent in function (data not shown).

**Fig. 3.** Nucleotide identities within the AT-rich region are essential for promoter function. Adenosine and thymidine nucleotides in the AT-Box (−14 to −8) of the pea atp9 promoter were transverted to their complementary identities (boxed lowercase letters). Designations of the lanes correspond to those of the mutated (lane A/T TM) and wild type (wt) templates. Run-off products (355 Nt) are highlighted by an arrow on the right-hand side. The conserved nonnucleotide motif and the AT-Box are marked by gray and hatched boxes, respectively. Numbering of the sequence refers to the transcription start site (+1).

**Fig. 2.** The AT-Box is indispensable for promoter function in dicot plants. Deletions of mitochondrial sequences between nucleotide positions −16 and −7 relative to the transcription initiation sites of the soybean atp9 (A) and Oenothera rpl5 (B) genes, respectively, show the general significance of the AT-rich sequence element for promoter function in dicot plant species. Clones, lanes, and transcripts are marked analogously to Fig. 1.

other dicot promoters, analogous deletion studies were carried out with the soybean atp9 and the Oenothera rpl5 promoters, respectively. In the assays with soybean constructs no functionally required sequences are lost upon deletion upstream of position −14, but promoter activity is seriously reduced in clone −12 (Fig. 2). In Oenothera rpl5 deletion constructs no significant differences in transcription efficiencies were found between −17, −14, and −13. Promoter activity is, however, completely abolished in deletions −10 and −7 in both soybean atp9 and Oenothera rpl5 promoters. In summary these results support the conclusion that the promoter portions downstream of nucleotide position −14 are generally sufficient and also necessary to drive transcription at mitochondrial promoters of dicot plants. This region with a high content of adenosines and thymidines, which is already conspicuous in the sequence alignment of several dicot promoters, has been accordingly designated AT-Box (21).

**Transversion Mutagenesis of the AT-Box**—The high content in adenosines and thymidines in the above described 5’ regions between nucleotides −14 and −8 of the mitochondrial promoters in dicot plants suggests a function of these sequences in loosening or melting of the DNA strands during the initiation process. To determine whether this concentration of A and T nucleotides per se or whether sequence specificity is important, adenosine and thymidine residues in this particular region of the pea atp9 promoter were transverted to the respective complementary nucleotides (Fig. 3, upper part). The melting temperatures of the mutated and the wild type promoter regions were calculated to differ by only 0.6 °C, and thus comparable initiation efficiencies are expected if the function of this segment is confined to access strand opening of the DNA. However, in vitro transcription assays with the mutated and wild type templates revealed that initiation is almost completely abolished by the transverted nucleotides (Fig. 3, lower part). Semi-quantitatively repeated assays confirmed the significant reduction of the promoter activity. The faint signal is only visible upon long exposures, which indicates the residual promoter function to be less than 1% of the wild type activity. This observation suggests that sequences rich in adenosines and thymidines per se do not support promoter function and that this promoter segment contributes to the sequence-specific interaction(s) between the promoter and the transcriptional apparatus in dicot plant mitochondria.

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**Nucleotides Downstream of the Transcription Start Site Contribute to Promoter Function**—Analogous to the definition of the 5’ border of different dicot promoters, their 3’ extent was investigated with the pea atp9 transcription initiation site. Two 8-bp segments ranging from +3 to +10 (LS-1) and from +11 to +18 (LS-2) were replaced by 15- and 14-bp insertions of alien sequences, respectively. In vitro transcription assays with both templates indicate that the exchanged sequences are not important for promoter activity at least in vitro (data not shown). To extend this analysis further upstream into the conserved nonnucleotide motif, in three clones segments between nucleotides −3 to +2 (TM-1), +3 to +7 (TM-2), and +8 to +12 (TM-3) were transverted to the respective complementary sequences (Fig. 4A, upper part). The correct transcripts of 355 nucleotides obtained with construct TM-3 confirmed that the region downstream of position +8 does not participate in transcription initiation in vitro. The ability to initiate transcription is however completely lost in mutants TM-1 and TM-2 (Fig. 4A, lower part). While the loss of promoter activity in
Functional Analysis of Mitochondrial Promoters of Dicot Plants

18 Nucleotides Are Sufficient to Direct Transcription Initiation Irrespective of Surrounding Sequences—The above results of the functional analysis of the 5' and 3' regions of the pea atp9 promoter imply that transcription should be initiated at an 18-bp sequence segment (−14 to +4) irrespective of 5'- and 3'-flanking regions. A double-stranded oligonucleotide (MP) representing such a mitochondrial promoter was cloned into three different sequence contexts and tested for transcription initiation (Fig. 5). The MP oligonucleotides integrated into the Smal and EcoRV restriction sites of the pBluescript multiple cloning sites show the same full promoter activity as the respective control template with this sequence in its wild type context (pS and pE, Fig. 5, A and B).

Two 18-mer promoter sequences inserted in tandem into the EcoRV restriction site yield two transcription products differing by 18 nucleotides in length (pEx2, Fig. 5B). No specific transcription product is observed with the pBluescript vector alone clearly correlating transcription initiation with the integrated MP oligonucleotide(s). Insertion of the MP oligonucleotide into a SmaBI site within the pea rps14cob spacer region confers transcription initiation to this region where no promoter is observed in vivo and in vitro in the wild type sequence (data not shown). These results conclusively demonstrate that the sequence from −14 to +4 represents a fully functional dicot plant mitochondrial promoter autonomous of adjacent sequences.

DISCUSSION

The Mitochondrial Promoter in Dicot Plants Is a Single Entity of 18 Nucleotides—The functional analyses of several promoters by deletions and transversions 5' and 3' to the transcription start sites show that 18 nucleotides from −14 to +4 are essential and sufficient for transcription in vitro. Insertion of the 18mer DNA segment confirms its fully competent promoter function independent of 5'- and 3'-flanking sequences. This comprehensive analysis thus identifies extension and structure of such a promoter.

To evaluate the experimental results, the respective regions of eleven promoters from dicot plants were reinvestigated for conserved structures (Fig. 6). Only transcription initiation sites were included in this comparison, which have been unambiguously identified as such by primer extension (in vivo) and in vitro capping and/or in vitro transcription analysis (in vitro) (12–14, 24). Most striking features within this 18-mer promoter sequence are the CNM (−7 to +2) and the upstream AT-Box (−14 to −8). Although the apparently noncanonical nucleotide identities separate these two motifs, the in vitro analyses do not support individual functions of these different promoter portions. The promoter sequence rather appears to function as a single element, in which most nucleotides are involved in sequence-specific interaction with the RNA polymerase and/or additional transcription factor(s).

Requirement of the Primary Structure of the AT-Box for Promoter Function—Deletions in the 5' portion of three different promoters from three dicot plant species consistently show that functional sequences maximally extend to position −14. Transcription initiation at the Oenothera rpl5 construct Δ-13 occurs with an efficiency equal to clone Δ-14. This experimental result and the observation that the adenosine at position −14 is less conserved between different promoters (36%) suggest that this nucleotide position may not be crucial for the activity of all of
these promoters (Figs. 1, 2, and 6). Speculations that the AT-Box may only be necessary to facilitate melting of the DNA strands are rendered highly unlikely by the almost complete loss of activity in the transverted AT-Box mutant. This sequence dependence is reflected in the high conservation at positions $2^{14}$ (73% A), $2^{12}$ (82% A), $2^{11}$ (91% A), and $2^{10}$ (73% T) within this promoter segment. These observations strongly support this part of the promoter to be involved in sequence-specific interaction with the transcription apparatus, which does of course not exclude a parallel positive influence of these AT-rich sequences on melting of the DNA strands.

The increased promoter activity in constructs $D^{14}$ relative to $D^{16}$, which is reproducibly observed in independent experiments with both pea and soybean $atp9$ constructs, suggests that sequences upstream of position $2^{14}$ may somehow modulate promoter strength. Thus substitution of nucleotide identities with negative impact on promoter activity could result in a positive effect, a phenomenon, which has to be investigated in detail by site-directed mutagenesis at these particular nucleotides.

**Fig. 5.** An 18-nucleotide promoter sequence displays full promoter function in vitro. Oligonucleotide MP representing the wild type sequence of the $atp9$ promoter (wt) between nucleotides $-14$ and $+4$ was cloned into pBlue-script vectors. A, construct pS containing oligonucleotide MP in the Smal site was linearized with $NaeI$ and transcribed in vitro. The run-off transcripts of 372 nucleotides (pS) and 355 nucleotides (wt) are indicated. No specific transcript is initiated at a linearized pBlue-script template without the promoter oligonucleotide (MP) (C). Fragment sizes of a coelectrophoresed DNA marker (M) are given at the left-hand side. B, one (pE) and two MP oligonucleotides (pE $x2$) were cloned into the EcoRV site of pBlue-script. Run-off transcripts of 390 and 408 nucleotides, respectively, indicate correct transcription initiation at the inserted promoters. Marker (M) and control reactions (lanes C and wt) were identical to the ones in A.

**Fig. 6.** Conserved structure of mitochondrial promoters in dicot plants. Promoter sequences of 11 bona fide promoters of dicot plants are compared between nucleotide positions $-14$ and $+4$. The frequency with which each nucleotide identity (given at the left margin) is found in any position (top line) is given in percent. Predominant nucleotide identities are given in the consensus sequence. Character sizes in the consensus reflect the frequency with which the nucleotide identity is found at the given position. Promoters compared in this analysis are located upstream from the following genes: $atp9$, 18 S rRNA, and $rpl5$ genes from pea; $atp1$, $cox2$, $rpl5$, $trnF$, 18 S rRNA from Oenothera; $atp9$ and $rb / c$ from soybean; and the 18 S rRNA gene of potato (12–14, 21, 24).
Adenosines in positions -12 to -10 have been observed to also influence the activity of the maize atp1 promoter. Substitution of the -12 A by a T or a C considerably reduced the amount of run-off transcripts (between 30 and 50%) of the wild type level (18). These similarities between the 5' regions of the maize atp1 and the dicot promoters investigated here may indicate analogous promoter structures and recognition mechanisms. In the maize cox3 promoter the respective segment was, however, found to have only a minor role in transcription initiation in vitro suggesting major differences of sequence requirements between individual promoters in this plant species (19).

Nucleotides Downstream of the 5'-CRTA-3' Are Conserved and Essential for the Function of Promoters in Dicot Plants—Alignment and sequence comparisons of transcription initiation sites determined by in vitro capping studies identified the 5'-CRTA-3' tetranucleotide to be conserved between monocot and dicot plant species. Consequently this motif was assigned an important role in promoter function (15, 16, 25). Various in vitro promoter studies, however, showed that deviations are accepted in the first two positions of this tetranucleotide in both plant subdivisions (18, 26). The small size of the motif and the relaxed sequence constraints indicate that other nucleotides must be essential for promoter function. In addition to the importance of the upstream AT-Box, the nucleotides downstream of the 5'-CRTA-3' contribute significantly to promoter function. This is highlighted by the complete lack of initiation in the transversion mutant TM-1, in which the second half of the nonanucleotide sequence downstream of the 5' end is altered. The theoretical consensus deduced in the sequence comparison shows that this promoter segment contains three nucleotide positions at -2 (G), +1 (G), and +2 (A), which are 100% conserved in all compared dicot promoters. Furthermore, two adenosine residues are found at -3 and -1 with a frequency of 82%. This indicates that the 5'-CRTA-3' motif, although present in almost all mitochondrial promoters in seed plants, only represents a small fraction of the nucleotides contributing more or less equally to the promoter function.

Promoter Nucleotides Downstream of the Transcription Start Site Contribute to Promoter Function—The identification of the nonanucleotide motif had already shown that the conserved promoter core extends beyond the transcription start site. Nucleotide transversions now show that the transcribed promoter portion actually extends even further downstream, as far as nucleotide positions +3 and/or +4. Here the actual requirements on nucleotide identity may be relaxed, since not all substitutions downstream of position +2 interfere with successful transcription initiation (Fig. 5). The differing results obtained with constructs LS-1 and TM-2 may be due to the particular nucleotide identities inserted at positions +3 and +4, these being a guanosine at +3 in LS-1 and a thymidine residue in the respective position in TM-2. Although guanosine or thymidine residues are found in this position in some other promoters, at least one purine is found conserved at +3 or +4 in all wild type promoter sequences. Since TM-2 contains only pyrimidines in these positions, the loss of promoter activity of this construct could be due to the total absence of purine here. The precise requirements of nucleotide identities in these positions have to be determined by site-directed mutagenesis.

Implications of the Refined Mitochondrial Promoter Structure in Dicot Plant for the Interaction with the Transcription Apparatus—The analysis of the pea atp9 transcription initiation site now shows that a mitochondrial promoter in a dicot plant contains an 18-bp sequence, which corresponds to about two helix turns. This promoter appears to act in its entirety as recognition site for components of the transcription apparatus.

This may include one or two proteins, which could, similar to transcription factors in mammalian, yeast, and Xenopus mitochondria, direct a promoter-specific attachment of the RNA polymerase (10, 11, 27, 28). Two proteins that bind to this promoter have recently been enriched; however their direct participation in the transcription initiation process remains to be shown (29). All involved proteins must be in very close proximity, since contact is made with the 18-bp promoter sequence, which is rather short to accommodate attachment sites for several different proteins, particularly considering that the RNA polymerase itself already has a molecular mass of more than 100 kDa (7, 30).

The mode by which the transcriptional apparatus identifies a promoter sequence is at present unclear, but can be refined with the results presented here. The equal efficiencies with which transcription is initiated at the tandemly repeated promoters exclude an access of the proteins by scanning the DNA with a 5' to 3' processivity, since such an access would result in a preferential transcription initiation at the first promoter of this construct (Fig. 5). Thus, promoter recognition in plant mitochondria rather appears to involve a homing approach of one or more of the participating proteins.

The sequence requirements of mitochondrial promoters in dicot plants defined here now provide a base toward the identification of the components involved in transcription initiation. Furthermore, this refined promoter sequence will allow rapid identification of other mitochondrial promoters in this plant subdivision, e.g. in the complete sequence of the Arabidopsis mitochondrial genome (22, 31).

Acknowledgments—We thank Jutta Braune for excellent technical assistance and Axel Brennicke for his helpful comments on the manuscript.

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