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Transcription initiation sites for the potato mitochondrial gene coding for subunit 9 of ATP synthase (*atp9*)

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Abstract

The potato mitochondrial *atp9* gene has a simple expression pattern. To determine where transcription initiates, primary mitochondrial RNAs were labeled by in vitro capping and hybridized to the 5′ flanking sequences of the *atp9* gene. A single transcription initiation region was identified. Primer extension and nuclease S1 protection analyses were used to precisely map the transcript 5′ termini in this region. These results indicate that transcription initiates at 121–128 bp upstream of the *atp9* open reading frame, in a sequence which does not present any homology with proposed consensus sequences for plant mitochondrial promoters. Nuclease S1 protection were also used to map 3′ termini 67–71 nucleotides downstream of a putative single-stem loop structure.

Key words: Transcription initiation; ATP synthase subunit 9; Plant mitochondrion; Guanylyl transferase; *Solanum tuberosum*

1. Introduction

Higher plant mitochondrial genomes, which vary in size from 200 to 2500 kb, are much larger and more complex than those of animals and fungi [1]. Although many structural features of plant mtDNA are well established, much less is known about their transcription [2]. Promoters of mitochondrial genes differ in vertebrates, yeast and plants. The 16–17 kb vertebrate mtDNA is symetrically transcribed from one heavy-strand and one light-strand promoter [3]. By contrast, about 20 promoter sites characterized by a conserved nonanucleotide consensus sequence have been identified on the 75 kb yeast mtDNA [4]. Only recently have transcription initiation sites been identified for maize, wheat, soybean and *Oenothera* mitochondria, by capping in vitro the primary transcription products [5–10]. A loose consensus sequence has been defined by conserved nucleotide identities in the two monocots, and correct initiation at these sequences was confirmed by in vitro transcription [11–13]. From data on three initiation sites in *Oenothera* and two in soybean, a putative dicot promoter consensus sequence has been proposed that shows only a core of four nucleotides conserved with the monocot consensus [10]. Characterization of transcription initiation sites in additional species and genes is necessary to better define plant mitochondrial promoters.

Progress in understanding the transcription of plant mitochondrial genes has been hampered by transcript complexity. Numerous mRNA species have been detected for many plant mitochondrial genes, resulting from multiple transcription initiation sites and/or RNA processing [5,6,10]. One of the genes with several transcripts in different plant species is the *atp9* gene [14–19]. By contrast, Northern analysis has previously shown that potato *atp9* is expressed into one major transcript [20]. This transcript is extensively edited [20]. The simple *atp9* transcription pattern makes it an appropiate model to characterize the first transcription initiation site(s) in Solanaceae. In this paper, we have analyzed the potato *atp9* 5′ transcript termini and showed they map to a region where transcription initiates. This putative potato promoter does not show any homology with consensus promoters proposed for monocot or dicot mitochondria.

2. Materials and methods

2.1. Isolation of mitochondrial nucleic acids

Highly purified mitochondria were prepared from potato (*S. tuberosum* cv. Bintje) tubers according to Neuburger et al. [21]. Mitochondrial DNA and RNA were isolated from purified mitochondria as previously described [20].

2.2. Construction and screening of a potato mtDNA library

Potato mtDNA was digested with *Sal*I and ligated into vector pUC18. Recombinant clones containing the *atp9* gene were isolated by colony hybridization, using a 0.76 kb *Eco*RI restriction fragment from potato mtDNA as a probe [20]. Appropiate DNA fragments containing the *atp9* coding, upstream or downstream sequences were subcloned into Bluescribe or M13mp9 vectors, following standard protocols [22].

2.3. DNA sequencing

DNA sequencing of both strands was performed on single or double-stranded templates by the dideoxy chain termination method, using a T7 DNA polymerase sequencing kit (Pharmacia LKB).

2.4. Northern hybridization analysis

Total mtRNA (5–10 μg) was fractionated through agarose-formaldehyde gels, transferred to nylon membranes (Hybond-N, Amersham) and hybridized using probes labeled by nick-translation [22]. Size mark-

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Abbreviations: atp9, gene for ATP synthase subunit 9; bp, base pair; kb, kilobase; mt, mitochondrial; nt, nucleotide; RNase, ribonuclease.

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ers were RNA length standards (BRL) cofractionated in the agarose-formaldehyde gels.

2.5. In vitro capping

The in vitro capping reactions were carried out in a 20 μl volume as described by Binder and Brennicke [10], with 60 μg of mtRNA, 80 units of RNase inhibitor (Amersham), 250 μCi of [α-^{32}P]GTP (3000 Ci/mmol) and 15 units of guanylyl transferase (BRL). The capped mtRNA was purified and precipitated as described [10]. Southern blots were hybridized with 50 μg of labeled capped mtRNA following standard procedures [22]. After hybridization the blots were treated with 15 μg/ml RNase A and 2 units/ml RNase T1 for 1 h at 37°C.

2.6. Primer extension analyses

Potato mtRNA (100 μg) was treated with 50 units of RNase-free DNase I (BRL), extracted with phenol and precipitated with ethanol. Primer extension experiments were performed on 5 μg of mtRNA, with 30 units of avian myeloblastosis virus reverse transcriptase (BRL), and followed standard protocols [22]. Primers used in these experiments were (1): 5′-CGTCGATTCTTCCCCTCGTTC-3′ and (2): 5′-AGCAATTGTAGCAGCTCCTGC-3′. These primers were located as depicted in Fig. 2 and were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP [22].

2.7. Nuclease S1 protection analyses

Nuclease S1 protection experiments followed standard procedures [22]. Probes used to map 5′ termini were cloned in M13mp9, labeled with [α-^{32}P]dCTP and the large fragment of DNA polymerase I and obtained by digestion with restriction enzymes and electrophoresis through agarose gels. Probes used to map 3′ termini were purified DNA fragments 3′ end-labeled with the large fragment of DNA polymerase I and [α-^{32}P]dCTP. Heteroduplexes were formed with 50,000 cpm of labeled probe and 20 μg of mtRNA by denaturation at 80°C for 10 min and hybridization at 50°C for 14 h. For the nuclease S1 reaction, 1000 units/ml of the enzyme were added and incubated at 30°C for 1 h. Control reaction mixtures lacked nuclease S1 or mtRNA, and products were analyzed by electrophoresis through 5% polyacrilamide or sequencing gels.

3. Results

3.1. Transcription of the atp9 *gene*

Potato *atp9* gene has been previously identified on a 0.76 kb *Eco*RI restriction fragment of mtDNA which contains only 16 bp upstream of the *atp9* translation initiation codon [20]. This fragment was used to probe a potato mtDNA library and isolate a 14 kb *Sal*I clone which was characterized by restriction mapping (Fig. 1A). The *atp9* gene was localized on the restriction map by Southern hybridization and further sequence upstream of the *atp9* coding region was obtained (Fig. 2). The region 5′ to the coding region is homologous to the corresponding region in tobacco *atp9* and both petunia *atp9*-1 and *atp9*-2 genes for 200 and 165 nt upstream of the ATG start codon, respectively. Beyond these sites, the three sequences diverged.

The approximate location of potato *atp9* transcript termini was analyzed by hybridization of Northern blots with probes A–F (shown in Fig. 1A). Both the 0.76 kb *Eco*RI (probe E) and 0.32 kb *Rsa*I/*Eco*RI (probe D)

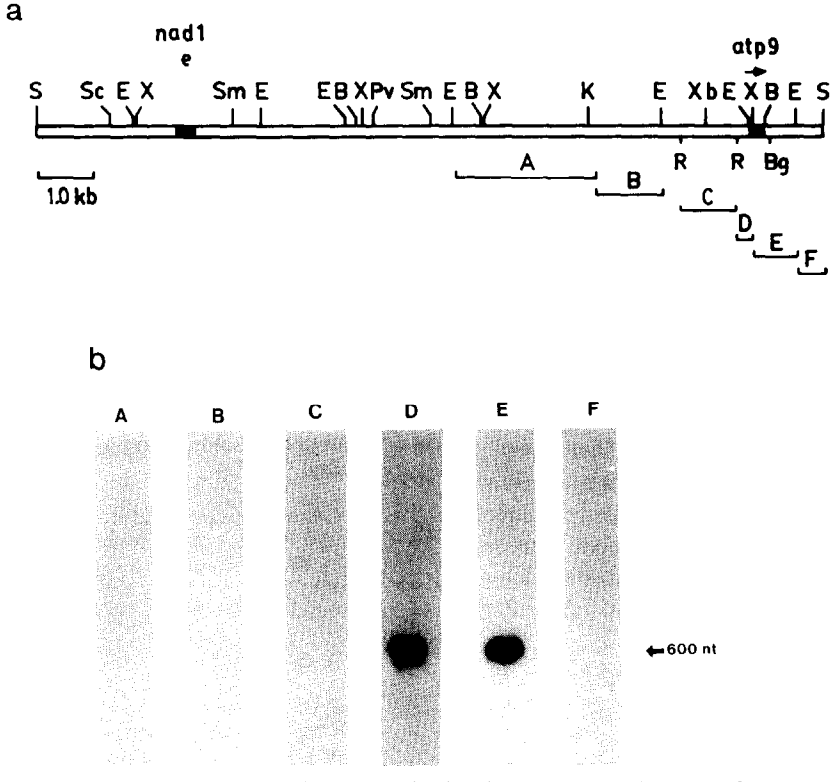

Fig. 1. Northern analysis of transcripts from the *atp9* locus in potato mitochondria (A). Restriction map of the potato mitochondrial *atp9* locus. The filled bar represents the *atp9* coding region. Direction of transcription is shown by an arrow. B = *Bam*HI; Bg = *Bgl*II; E = *Eco*RI; K = *Kpn*I; Pv = *Pvu*II; R = *Rsa*I; S = *Sal*I; Sc = *Sac*I; Sm = *Sma*I; X = *Xho*I; Xb = *Xba*I (all *Rsa*I and *Bgl*II sites are not indicated). A–F beneath the map represent the regions used for Northern hybridizations. (B) Each lane contains similar amounts (5 μg) of whole mtRNA. Blots were hybridized with probes A–F labeled by nick-translation.

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-451  GTGAGATCGT  TGTCCGATCT  TTATCGTCGA  ATCCCCAAAT  GTGCATCTTC  GAGTGTTTCA
-391  GCCGATCCCC  AATCTCCCAG  TATCAATATT  TCGGCTGCTC  AAGCCGGTAC  AGGTACAGAA
-331  GTGGCTACAA  CGGAGCAGCT  TACTCCTGAG  ATGGATTTAC  CGATTGAACT  AGATTTACCC
-271  GCCCTTTTCA  CCTTTTTTCT  TAATTATGGA  ATTGAAGTTA  TCTAAAAAAT  GAACTGACAA
-211  AGCGTCTGTT  CACGGAGATT  GTTGTAGTTT  CCGTAGTTGC  AAGAATTTCT  CACGATTGGG
-151  TTGGTGTGAA  GTCTACCGCC  TGTCTAGCCT  ATGCTTTGCA  TGAACATCTC  AATGTCCAAG
-  91  ATAAAAAGAA  CGAGGGGAAG  AATCGACGAG  GCGAGTGTTC  TCGAAGAGAA  AATCGTGATG
-  31  GAAAAAGCGT  GAGGAGAATT  CGAAACTCGA  G ATG TTA GAA GGT GCA AAA TCA ATG
+  25  GGT GCA GGA GCT GCT ACA ATT GCT TCA GCG GGA GCT GCT ATC GGT ATT GGA
      ------------------------------144  nt-------------------------TTC CAA GTT
+229  CGT TAG  TAATCGTTTA   CGGTGGGTGG  ATAAGCAGGA  AGGGATCCCT  GTGGTTAGAC
+285  TAACTGGCCG  AGAAGGCTAG  TGAGGTTCCT  GCTATGGTGA  AGTGAAAGAT  CTTTCACTAT
+345  AGTGGGAAGA  AGACAGGTGG  GAGCGAGCCG  AGCGAGAGCA  AAGCAAGTTT  CAGTGGTGGG
+405  CTGTCTTCGC  GGTCCCATTT  CATCGGCTTT  TTCTAGGCAA  GAGTCGCTCG  AATCGAAGTC
+465  AAGGTGAGGT  TTGGAACCCA  CATTGGTCTA  TCCCTTTTCT  TTGATAGGCG  TAGAAAGTAG
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Fig. 2. Potato *atp9* transcript termini. Pertinent sequence of the *atp9* 5′, coding and 3′ regions is presented, with sequence numbers in relation to the *atp9* ATG codon at position 1. Horizontal arrows below the sequence show the position of oligonucleotides used in primer extension analysis. Arrowheads above the sequence indicate 5′ and 3′ termini identified by nuclease S1 protection experiments, and arrowheads below the sequence identify the transcript 5′ ends determined by primer extension. Potential stem-loop forming sequences are underlined.

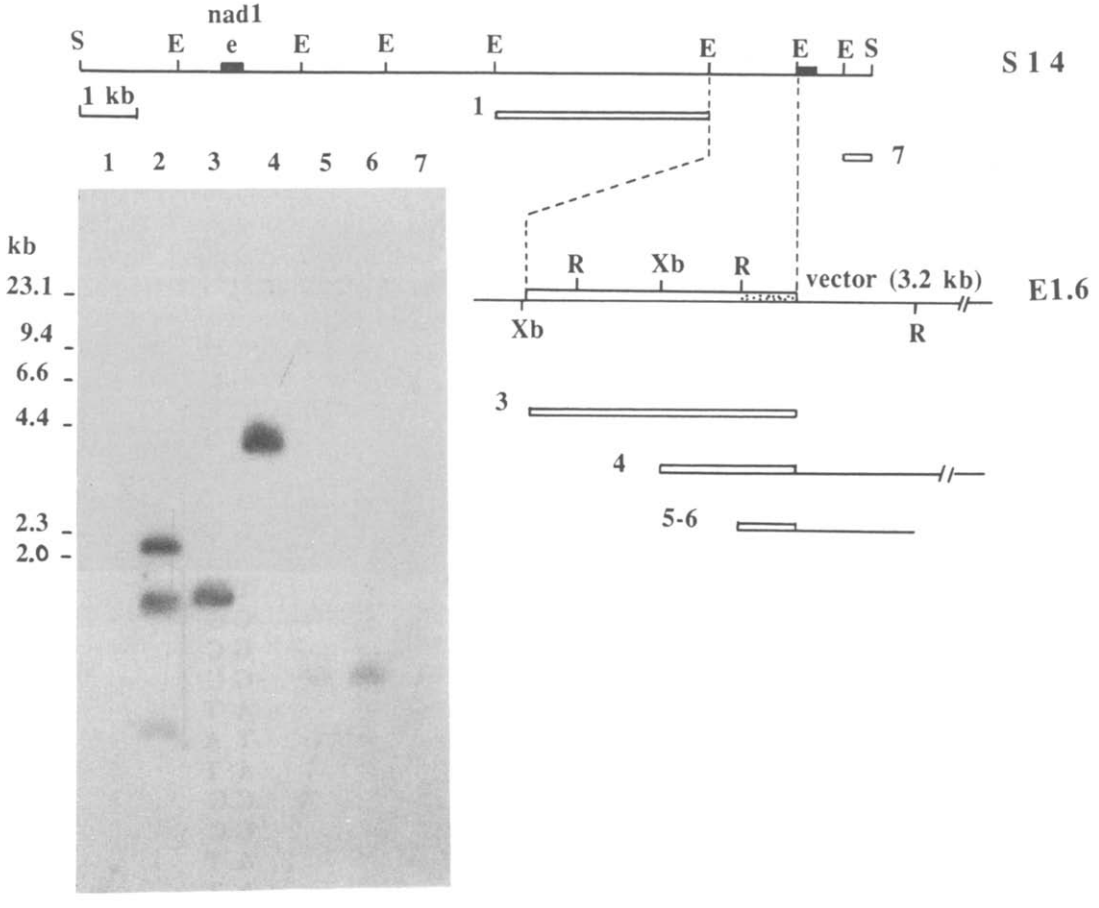

Fig. 3. Identification of the transcription initiation region for *atp9*. A Southern blot containing restriction fragments of the *atp9* coding region and adjacent sequences was hybridized with 50 μg of labeled capped mtRNA. The autoradiograph of the blot shows *Eco*RI digests of the genomic clone S14 (lane 2) and of subclones containing the 1.6 kb *Eco*RI (E1.6, lane 3), the 3.7 kb *Eco*RI (lane 1) and the 0.5 kb *Eco*RI/*Sal*I (lane 7) DNA fragments. Hybridization to digests of subclone E1.6 with *Xba*I (lane 4), *Xba*I/*Rsa*I (lane 5) and *Rsa*I (lane 6) are also shown. Sizes of marker DNA fragments are given on the left margin. Fragments hybridizing with the capped RNA in each lane are indicated under the restriction maps. Restriction sites are given for *Eco*RI (E), *Xba*I (Xb), *Rsa*I (R) and *Sal*I (S).

fragments hybridized to the same transcript of 0.5–0.6 kb (Fig. 1B). All other probes failed to hybridize to any transcript (Fig. 1B, probes A–C and F). Upon longer exposures, no additional hybridization signals appeared. These results confirmed the previously described simple *atp9* transcription pattern, and allow us to approximately map the 5′ and 3′ transcript termini to the 0.32 kb *Rsa*I/*Eco*RI and 0.76 kb *Eco*RI restriction fragments, respectively.

3.2. *Localization of transcription initiation site(s) for the* atp9 *gene*

Guanylyl transferase catalyzes the addition of cap structures in a reaction that is specific for di- or triphosphorylated transcripts. We have utilized the in vitro capping reaction to specifically label the 5′ end of primary, unprocessed transcripts of potato mitochondria. To identify the genomic region where primary transcript(s) of *atp9* originate, Southern blots of the coding and flanking regions of *atp9* were hybridized with capped mtRNA (Fig. 3). In *Eco*RI digests of the 14 kb *Sal*I clone (S14, top line in Fig. 3), only the fragments containing sequences upstream of *atp9* and exon e of *nad1* gave strong hybridization signals, while the *atp9* open reading frame itself hybridizes only weakly in some experiments (lane 2). Weak hybridizations probably result from incomplete digestion of unpaired capped ends with single-strand specific RNases A and T1. The *atp9* transcription initiation site(s) can be mapped to a 323 bp *Rsa*I/*Eco*RI fragment, indicated by a stippled bar in Fig. 3, by hybridization of capped mtRNA to digests of subclone E1.6 (Fig. 3, lanes 3–6). Further work is necessary to identify transcription initiation sites in the 2.2 kb *Eco*RI DNA fragment which contains exon e of *nad1* gene.

3.3. *Identification of 5′ transcript termini*

Precise identification of the 5′ termini of *atp9* transcripts was established by primer extension analysis. Two synthetic antisense oligonucleotides (primers 1 and 2, Fig. 2) were 5′ end-labeled and used in primer extension reactions (Fig. 4). The primer extension products show a series of cDNAs which terminate over approximately 8 nt. Comparison of these products with sequencing reaction products established 5′ transcript termini at positions −121 to −128 from the translation initiation codon (Figs. 4 and 2). Upstream 5′ mRNA termini were not observed even in longer exposures (not shown).

The 5′ termini of *atp9* transcripts were confirmed by nuclease S1 protection analyses, using three different probes (Fig. 5, probes *Xba*I/*Eco*RI, *Rsa*I/*Eco*RI and *Eco*RI/*Eco*RI). Larger protected products corresponded in size to 112–116 nt (Fig. 5A) and mapped 5′ transcript termini nearly identical to those identified by primer extension. The identification of the same transcript termini by these two methods indicate that these positions represent the only major termini within the 0.32 kb subclone where transcription initiates.

3.4. *Identification of 3′ transcript termini*

Nuclease S1 protection analyses were also used to map the 3′ end of *atp9* transcripts (Fig. 5B). 3′ termini were determined by hybridization of two probes (*Bam*HI/*Eco*RI or *Bgl*II/*Eco*RI DNA fragments, Fig. 5) with

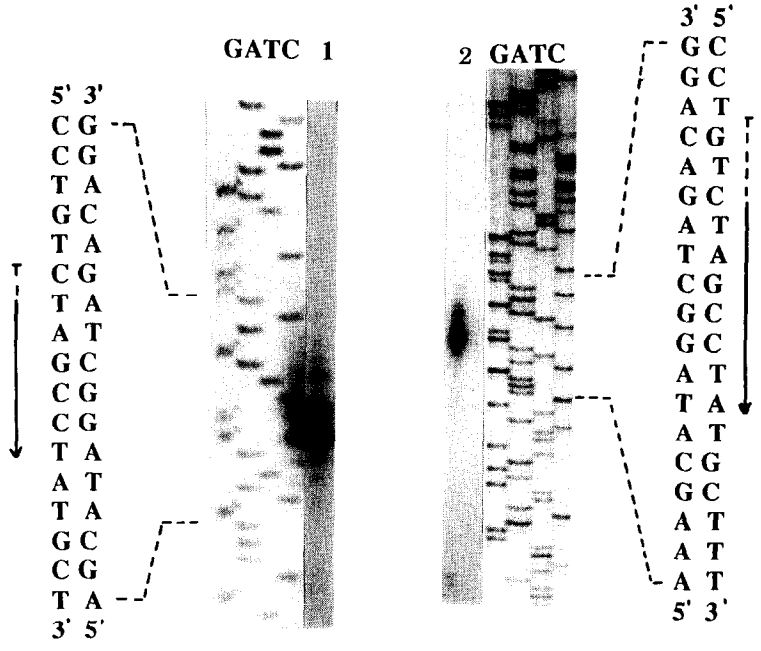

Fig. 4. Primer extension analysis of *atp9* transcripts. The sequencing gel shows primer-extended reaction products with oligonucleotides 1 (lane 1) and 2 (lane 2) (positions indicated in Fig. 2). Sequencing reactions were performed with the same primers (lanes GATC, antisense strand is read). Sequence is given (5′ to 3′, bottom-to-top) together with the reverse complement.

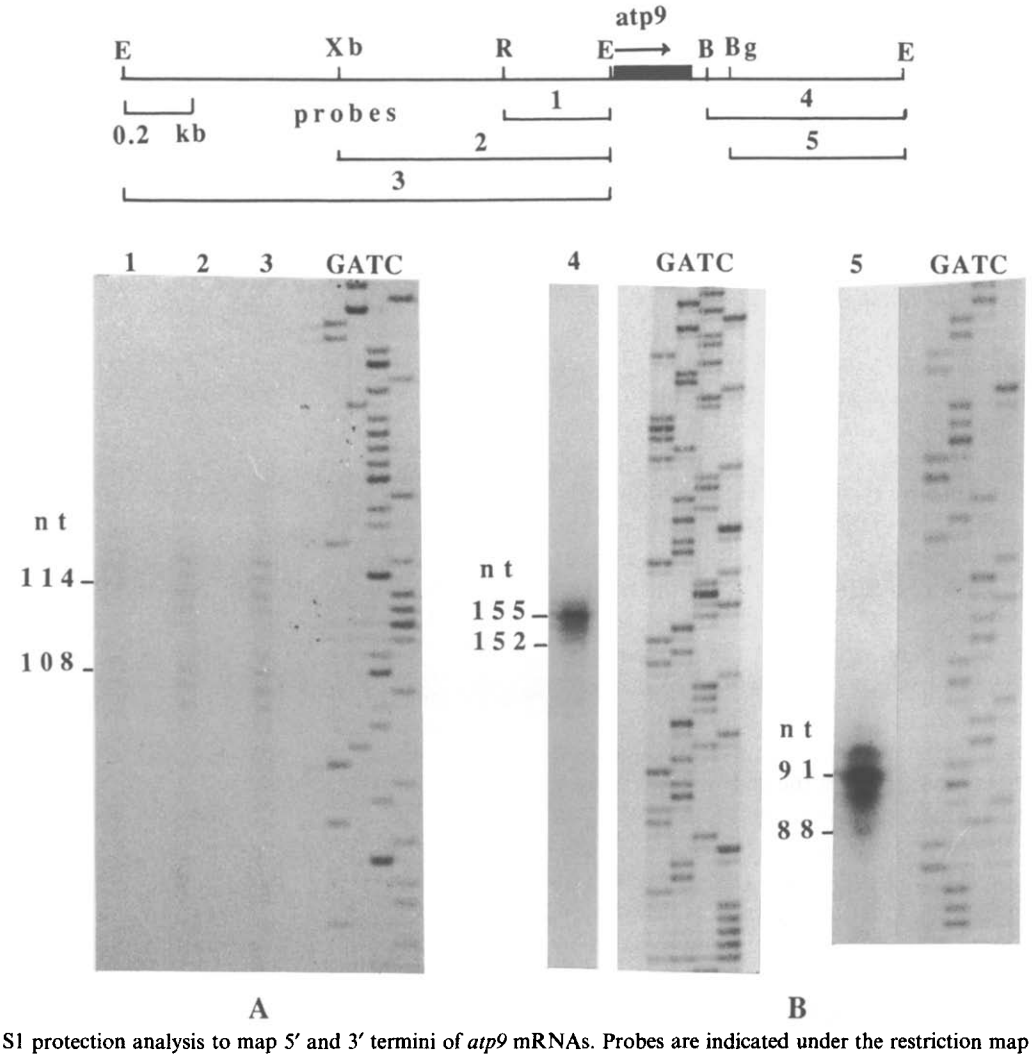

Fig. 5. Nuclease S1 protection analysis to map 5′ and 3′ termini of *atp9* mRNAs. Probes are indicated under the restriction map and were, for the 5′ ends (A), a 0.32 kb *RsaI*/*Eco*RI (lane 1), a 0.8 kb *XbaI*/*Eco*RI (lane 2) and a 1.6 kb *Eco*RI (lane 3) DNA fragments, and for the 3′ ends (B), a 0.48 kb *Bam*HI/*Eco*RI (lane 4) and a 0.42 kb *Bgl*II/*Eco*RI (lane 5) DNA fragments. Nuclease S1 protected fragments were electrophoresed next to a sequence ladder of a known standard as size marker.

mtRNA, followed by treatment with nuclease S1. Protected DNA fragments were 152–156 and 88–92 nt long with *Bam*HI/*Eco*RI and *Bgl*II/*Eco*RI probes, respectively, and located the 3′ termini at the same position, 194–198 nt downstream of the stop codon created by editing. These termini lie 67–71 nt downstream of a putative single stem-loop structure that spans positions + 310 to + 352 in the *atp9* 3′flanking region. The 3′ and 5′ ends obtained in these experiments define transcripts 540–551 nt long, which are consistent with the size of the transcript(s) detected on Northern blots.

4. Discussion

We have previously shown that the mitochondrial genome of *Solanum tuberosum* contains one functional gene for the proteolipid subunit of the ATP synthase complex [20]. This gene is organized as an independent transcriptional unit, being expressed into 540–551 nt long transcripts. The 5′ ends of these transcripts have been mapped precisely by primer extension and nuclease S1 protection experiments to a sequence located 121–128 nt upstream of the translation initiation codon. Hybridization of capped mtRNA to cloned restriction fragments identified the site(s) of transcription initiation in the same region, within a 320 bp *RsaI*/*Eco*RI DNA fragment upstream of the *atp9* open reading frame. The amount of capped transcripts obtained was not sufficient for RNAse protection experiments but the absence of any hybridization signal with capped RNA further upstream correlates with the absence of any other larger primer extension signals. These observations are considered strong evidence for these transcript termini to be derived from initiation of transcription.

A common characteristic of all mitochondrial promoters analyzed to date, including animals, fungi and plants, is a sequence element that covers the transcription initia-

tion site [4–6,8,12,23,24]. Since the corresponding elements in wheat and maize have been shown to promote correct transcription initiation in vitro, we consider the potato sequence described here as part of a putative mitochondrial promoter, although definitive identification is required by in vitro transcription assays. The sequence surrounding the potato initiation region shows no homology to proposed consensus promoter sequences for maize, wheat, *Oenothera* and soybean, or to the single initiation site described for soybean *atp9* [7–10]. However, plant mitochondrial promoters are poorly conserved across species, and considerably variability has been observed even within a single species and a single gene. Indeed, maize *atp9* has as many as 9 transcription initiation sites and the weak sites deviate significantly from the maize consensus [5,7]. Interestingly, the potato sequence 5′-CCTGTCTAGCCTA-3′ where 5′ ends map shares 11 out of 13 nucleotide homology with the weaker maize *atp9* initiation site [7]. These observations raised questions concerning the selectivity of plant mitochondrial transcription machinery. The putative promoter element identified in *S. tuberosum atp9* gene should allow the development of an in vitro transcription system for the dissection of this element and the characterization of the transcriptional apparatus in this dicot species.

Our primer extension and nuclease S1 protection experiments indicate that multiple transcript termini exist which differ by a single nucleotide; thus transcription may initiate at one of several adjacent nucleotides in potato mitochondria, although we cannot rule out that 5′ heterogeneity is created by nuclease activities in vivo or during our RNA preparation. Multiple clustered 5′ termini have been detected for both in vitro-generated and in vivo maize *atp1* transcripts, suggesting that they are derived from clustered initiation events and does not result from experimental artifacts [13]. We tentatively conclude that there is a cluster of transcription initiation sites within the potato *atp9* promoter.

Examination of 3′ flanking sequences in plant *atp9* genes had revealed homologies between potato, tobacco and one petunia gene [15,16,20]. This homology extends for 200 bp downstream of the stop codon, and includes a region of potential base pairing that could result in the formation of a single stem-loop secondary structure with a free energy change of −20.5 kJ/mol [15]. In tobacco it was suggested that it may be associated with transcript processing and/or stability rather than transcription termination, since *atp9* and downstream *rps13* are cotranscribed [15]. Similar inverted repeats have been shown to play a role in chloroplast transcript stability [25,26]. The 3′ ends of potato *atp9* transcripts have been mapped precisely by nuclease S1 protection experiments to a sequence located 67–71 nt downstream of this putative hairpin structure, at the same position where petunia *atp9*-1 transcript 3′ termini mapped [16]. Although we cannot rule out a role of such structure in transcription termination 70 nt downstream, it is more likely that the stem-loop structure may be an important determinant of transcript stability.

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