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Splicing and editing of *rps10* transcripts in potato mitochondria

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Abstract The structure and expression of the potato mitochondrial gene *rps10*, encoding ribosomal protein S10, has been characterized. The RPS10 polypeptide of 129 amino acids is encoded by two exons of 307 bp and 80 bp respectively, which are separated by a 774-bp class-II intron. Editing of the complete *rps10* coding region was studied by sequence analysis of spliced cDNAs. Four C residues are edited into U, resulting in the creation of a putative translational initiation codon, a new stop codon which eliminated ten carboxy-terminal residues, and two additional amino-acid alterations. All these changes increase the similarity between the potato and liverwort polypeptides. One additional C-to-U RNA editing event, observed in the intron sequence of unspliced cDNAs, improves the stability of the secondary structure in stem I (i) of domain I and may thus be required for the splicing reaction. All spliced cDNAs, and most unspliced cDNAs, were completely edited, suggesting that editing is an early step of *rps10* mRNA processing and precedes splicing. Earlier work on potato *rps10* (Zanlungo et al. 1994) is now known to comprise only a partial analysis of the gene, since the short downstream exon was not identified.

Key words Group-II intron editing · *rps10* transcript splicing · S10 ribosomal protein · *Solanum tuberosum* mitochondria

Introduction

The mitochondrial DNA (mtDNA) of flowering plants, with sizes between 200 and approximately 2500 kb, is much larger and more complex than that of animals and fungi (Levings and Brown 1989; Bonen 1991), and can thus potentially accomodate many more protein-coding genes. Contributing to the extended size of the genome are also large spacer sequences, promiscuous DNA of chloroplast and nuclear origin, numerous duplications, and sequences required for expression including introns. In flowering plants a number of mitochondrial genes are known to contain introns, all of which are group-II introns characterized by distinctive secondary structural features (Michel et al. 1989; Gray et al. 1992). In addition to splicing, RNA editing is required for maturation of mRNAs in plant mitochondria (Gray et al. 1992). In higher-plant mitochondria this modification occurs almost exclusively by C-to-U conversions and has so far been found in mRNAs of all conserved coding regions investigated. In most cases, editing leads to changes in the amino-acid sequence that result in a protein with a sequence better conserved when compared to the homologous protein in other organisms. Therefore, RNA editing seems to be essential to ensure the synthesis of functionally competent proteins.

Ribosomal protein genes comprise a significant fraction of the genes identified uniquely in plant mitochondria. While in vertebrates and many invertebrates these proteins are encoded by the nuclear genome, several ribosomal-protein genes have been identified in the mtDNA of higher plants, including *rps3*, *rps7*, *rps12*, *rps13*, *rps14*, *rps19*, *rpl5* and *rpl16* (Grohmann et al. 1993). Sixteen ribosomal-protein genes, including all of the above, have been identified in the complete sequence of the mitochondrial (mt) genome from the liverwort (Oda et al. 1992; Takemura et al. 1992).

Some of the ribosomal-protein genes that have been identified in the mtDNA of certain higher plants are incomplete in, or else absent from, the mt genome of other plants (Grohmann et al. 1993). Evolutionarily recent transfer events of functional genes to the nucleus, as shown for *cox2* in legumes and *rps12* in *Oenothera* (Nugent and Palmer 1991; Covello and Gray 1992; Grohmann et al. 1992), can explain these differences in the mt ribosomal-protein gene content between different plant lineages.

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We have recently reported the identification of an open reading frame (ORF) in the mitochondrial genome of *Solanum tuberosum* with significant similarity to ribosomal protein S10 genes (*rps10*) (Zanlungo et al. 1994). This *rps10* ORF is absent from wheat mtDNA. These results suggest a novel example of a rather recent gene-transfer event from the plant mitochondria to the nucleus. In this article we extend the results of our investigations on the organization and expression of the potato mitochondrial *rps10* gene and show that the *rps10* ORF is split by a group-II intron and undergoes RNA editing in both exons and the intron.

Materials and methods

Isolation of mitochondrial nucleic acids and cloning of the rps10 gene. Mitochondrial DNA and RNA were isolated from potato tubers (*S. tuberosum* cv Bintje) as previously described (Dell'Orto et al. 1993). A 5.1-kb *Eco*RI clone containing the potato *rps10* gene (located downstream from a truncated *cob* pseudogene) has been previously isolated from a potato mtDNA library (Zanlungo et al. 1994).

cDNA synthesis, PCR amplification and cDNA cloning. Potato mtRNA (50 μg) was treated with 40 units of RNase-free DNase I (BRL) in the present of 40 units of RNasin (BRL), extracted with phenol, and precipitated with ethanol. First-strand cDNA synthesis and PCR amplification of the cDNA were performed as previously described (Dell'Orto et al. 1993), using the following primers (located as depicted in Fig. 1 B):

1: 5′-CAAGGA*GGATCC*TATCCTAAAG-3′ (*Bam*HI site italicized)
2: 5′-AATTT*GGATCC*CCCTTTCCTCT-3′ (created *Bam*HI site italicized)
3: 5′-CCAGTCGGC*AAGCTT*TTTGGC-3′ (created *Hin*dIII site italicized)

PCR products were analysed by electrophoresis through agarose gels, digested with *Bam*HI or *Bam*HI+*Hin*dIII, and ligated to Bluescribe™ vectors (Stratagene).

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

Northern-hybridization analysis. Total mtRNA (5–10 μg) was fractionated through agarose-formaldehyde gels, transferred to nylon membranes and hybridized using standard procedures (Sambrook et al. 1989). The oligonucleotide probes used were oligo 4 (exon-1 probe, 5′-ACTATGCCTATCTTGGTGGTC-3′) and oligo 5 (intron probe, 5′-TACACTTCTTGAATTCTGTCCCAC-3′), and were labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP as described by Sambrook et al. (1989).

Results and discussion

Identification of two potato *rps10* exons

The potato *rps10* gene has been previously identified on a 5.1-kb *Eco*RI restriction fragment of potato mtDNA, downstream from a truncated *cob* pseudogene and upstream of *cox1* (Fig. 1 A). Sequence analysis identified an open reading frame (positions 1–324 in Fig. 1 B) whose translation product displays similarity to bacterial, chloroplast, and liverwort mitochondrial RPS10 sequences (Zanlungo et al. 1994). However, comparison of liverwort and potato amino-acid sequences revealed a 18 amino-acid C-terminal extension in the liverwort polypeptide. This apparent divergence les us to determine additional downstream sequences. A stretch of 89 bp (positions 1088 – 1176 in Fig. 1 B) could be aligned with the sequence coding for the 20 C-terminal amino acids of the liverwort polypeptide (and the 3′ flanking sequence). Analysis of the sequence immediately upstream of this 89-bp stretch identified conserved group-II-intron domains V and VI (discussed below), suggesting that a novel intron interrupts the *rps10* coding sequence in potato mitochondria, while in liverwort this gene has no introns (Oda et al. 1992). Either gain of the intron in potato or its loss in *Marchantia* must have occurred after the divergence of mosses and angiosperms.

The presence of the intron was confirmed by sequence analysis of spliced *rps10* cDNAs. Mitochondrial RNA was reverse transcribed using primer 2, and amplified by PCR between primers 2 and 1 (Fig. 1 B). The main PCR product of 0.5–0.6 kb leads to an estimation of 0.7–0.8 kb for the intron length (data not shown). The position of the *rps10* 5′ and 3′ splice sites was precisely determined by sequence analysis of several cloned PCR-amplified cDNAs (Fig. 2). The two exons are separated by an intron of 774 bp and are predicted to encode 102 and 27 amino acids respectively (Fig. 1 B), taking into account RNA editing events (discussed below).

In our previous work the potato *rps10* nucleotide sequence was aligned with a single-copy sequence located upstream of the pea *cox1* gene (Kemmerer et al. 1989; Zanlungo et al. 1994). The published pea sequence does not contain a complete uninterrupted open reading frame, since deletions and insertions disturb the coding sequence and introduce frameshifts and stop codons. However, in light of the fact that the published homologous region in pea mitochondria contains *rps10* exon-1/group-II intron/exon-2 sequences, these frameshifts might simply reflect sequencing errors in the early work by Kemmerer et al. (1989). This possibility has been considered by V. Knoop et al. (1995) who re-investigated this region in the pea mitochondrial genome and found a functional *rps10* gene similar to the potato gene.

Intron structure

Several features of the intron are clearly indicative of a group-II intron (Michel et al. 1989): a conserved block at the 5′ splice site (GUGCG), the helical domain-V structure, and the bulging A located 8 nt upstream of the 3′ intron-exon junction on the 3′ side of helix VI (this is the nucleotide which participates in "lariat" formation by covalent attachment to the 5′ intron end during attack of the 5′ splice site). The *rps10* intron can be folded into the conservative group-II structural model consisting of six domains (I through VI) radiating outward from a central core

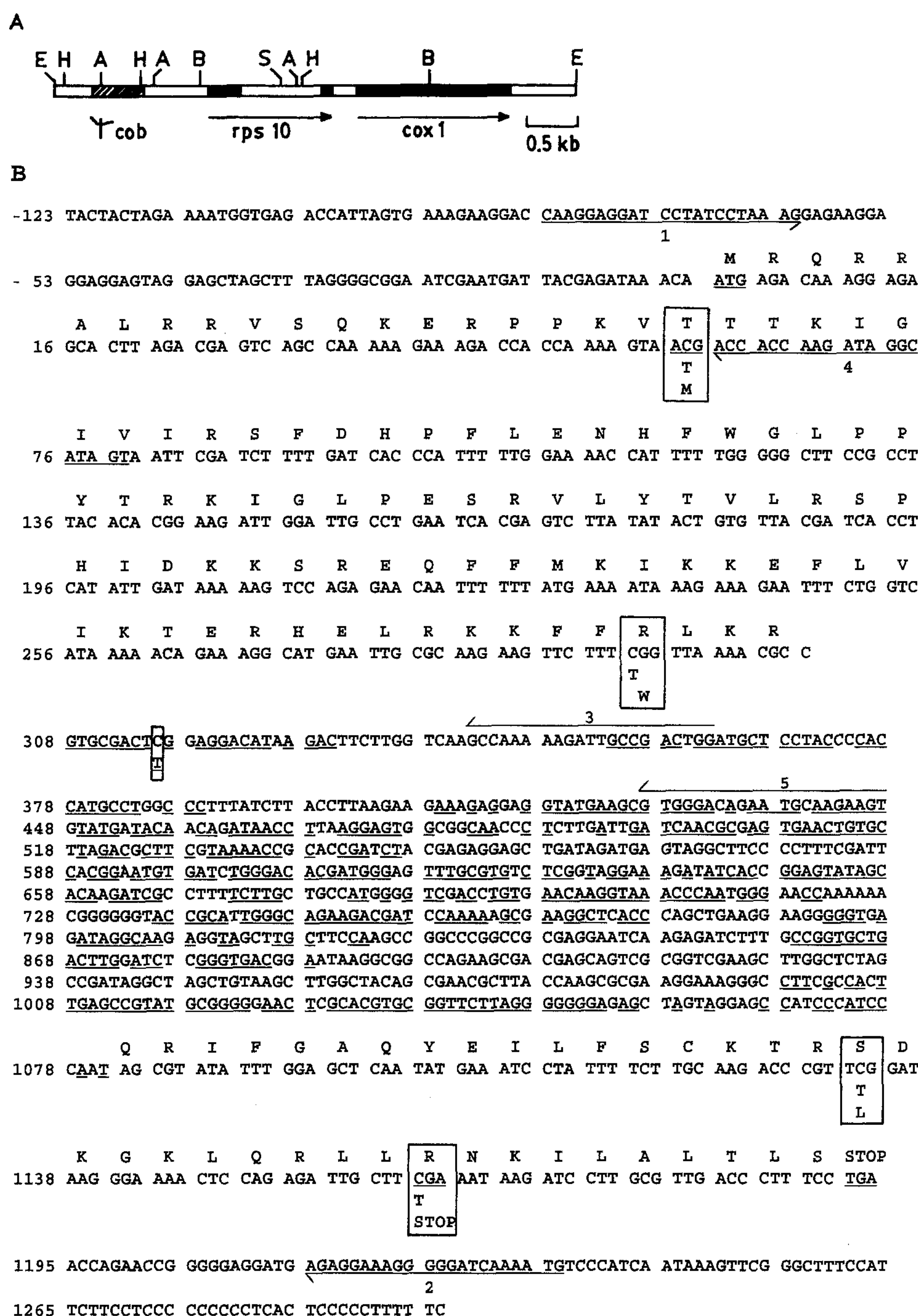

Fig. 1 A, B Nucleotide sequence of the potato *rps10* gene. **A** restriction map of the potato mitochondrial *pseudo-cob-rps10-cox1* locus. *Black bars* represent *rps10* exons and the *cox1* coding region, and the *hatched box* the truncated *cob* coding region. The direction of transcription is indicated by *arrows. A, Ava*I; *B, Bam*HI; *E, Eco*RI; *H, Hin*dIII; *S, Sal*I. **B** nucleotide sequence of *rps10* (EMBL Accession number X74826). The genomic sequence is presented, with the derived amino-acid sequence given above the triplets in the single letter code. Sequence numbers are in relation to the *rps10* genomic ATG codon at position 1. C-to-T conversions detected in *rps10* cDNAs are shown below the genomic sequence and the altered codons are translated underneath. Sequences identical in the liverwort *rrn26* intron are *underlined. Arrows* show the position of oligos (1–5) used in PCR amplifications and Northern analysis. The *cox1* initiation codon is created by editing (unpublished results) and is located 61 nt downstream from the sequence shown

(Fig. 3). The main features classifying this intron into subgroup IIB include the position of the bulging A at –8, a 4-nt C1(i)–C1(ii) internal loop with consensus sequence AARC, the intron 3′ end with the consensus sequence RAY, and the conserved nucleotides circled in Fig. 3 (Michel et al. 1989).

Two rather close relatives of the potato *rps10* intron were found by sequence comparisons in the liverwort mitochondrial genome (Oda et al. 1992). One of them is the intron in the *rrn26* gene (Fig. 1 B) and the other is intron 2 in the *cox3* gene (data not shown). The fact that these introns are so similar in primary sequence implies a common evolutionary origin and suggests that they may have been mobile from gene to gene in a common ancestor of bryophytes and angiosperms. Horizontal spreading of group-II introns has been postulated in fungi (Hardy and Clark-Walker 1991), *Marchantia* (Oda et al. 1992; Ohta et al. 1993), and *Oenothera* (Lippok et al. 1994). Most of the other introns of *Marchantia* show no homology in sequence or integration site wih higher-plant mitochondrial introns, suggesting different origins for these introns (Oda et al. 1992; Ohta et al. 1993).

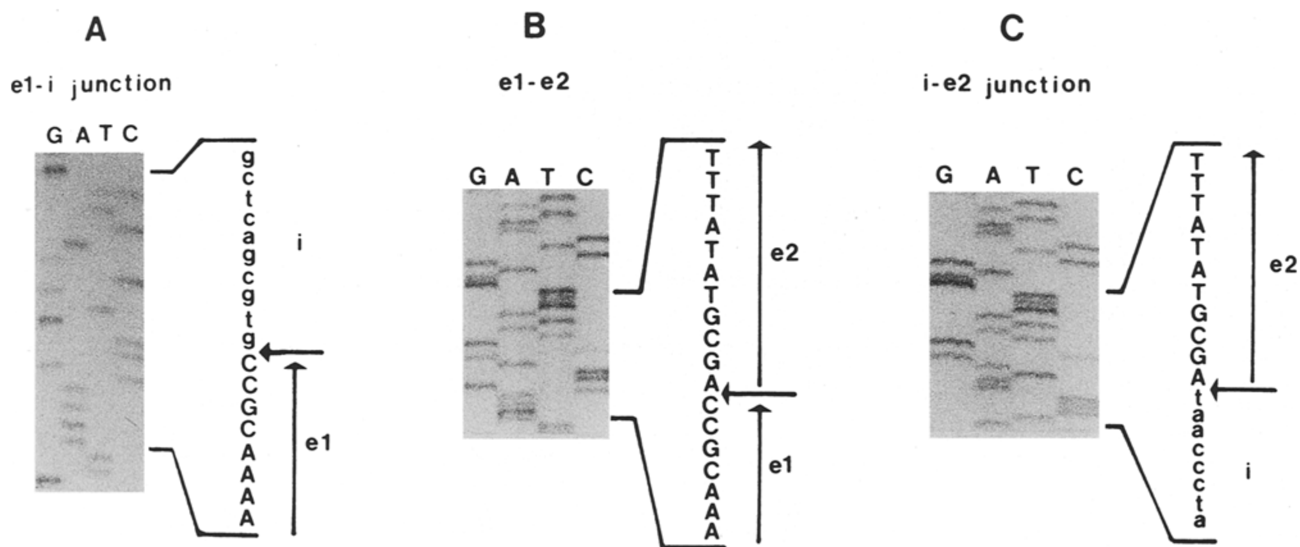

Fig. 2A–C Identification of splice sites between *rps10* exons. **A** DNA sequence of the 3′ end of exon 1 and the 5′ end of the *rps10* intron. **B** sequence of a spliced cDNA. **C** DNA sequence of the 3′ intron end and the 5′ end of exon 2. Exon and intron sequences are given in *uppercase and lowercase letters*, respectively. *Horizontal arrows* indicate exon-intron junctions in genomic sequences and the exon junction in the spliced cDNA. *Vertical arrows* indicate exon sequences

The *rps10* stop codon and a putative initiation codon are created by editing

Comparison of cDNA and genomic sequences revealed RNA editing events in both exons. Six independently derived spliced cDNAs were completely sequenced. All had in common four C-to-U modifications as compared to the genomic sequence (Fig. 1 B), with two editing sites in each exon. No partially edited sequences were observed, suggesting that these editing sites are altered efficiently in the mRNA. The first editing event in exon 1 changes a threonine ACG codon to an AUG methionine codon 20 amino acids downstream from a genomic-encoded in-frame AUG codon. The amino-acid sequence between the two potential initiation codons would extend the N-terminus of potato polypeptide in comparison to bacterial, chloroplast, and liverwort and pea mitochondrial RPS10 sequences, while the second AUG codon created by RNA editing coincides wih the liverwort and pea initiation codons (Fig. 4 and Knoop et al. 1995). These results suggest that translation initiates at the AUG codon created by editing in potato. C-to-U changes have been shown to produce an initiation codon in chloroplast *rpl2* mRNA of maize (Hoch et al. 1991) and *psbL* transcripts of tobacco (Kudla et al. 1992), as well as in the mitochondrial *nad1* mRNAs of wheat and probably also *Oenothera* (Chapdelaine and Bonen 1991; Wissinger et al. 1991). Isolation and direct sequencing of the N-terminus of potato RPS10 will answer this question. If the second methionine residue corresponds to the first translated codon, this RNA editing event can be assumed essential for protein synthesis to proceed.

mRNA editing in plant mitochondria can also create stop codons from arg or gln triplets (Gray et al. 1992). The last editing event in exon 2 modifies a CGA arginine triplet to a UGA termination codon, shortening the deduced polypeptide by ten amino acids. After editing the predicted C-terminus is more similar to that of the liverwort mt RPS10. However, both plant-deduced amino-acid sequences are extended at the carboxy-terminal end in comparison with bacterial and algal chloroplast RPS10 polypeptide sequences (Fig. 4). A potential specific effect of these carboxy-terminal extensions in the function of the plant RPS10 remains to be established. The two other editing events (amino acids 99 and 120) also improve similarity to the liverwort polypeptide but not to the bacterial and algal chloroplast proteins (editing at position 99 actually decreases this similarity). The highest similarity between the predicted amino-acid sequences encoded by the *rps10* genes of potato and other species is found with liverwort. After editing, potato and liverwort polypeptides show 60% identity in a 100 amino-acid overlap. The amino-acid sequence deduced for potato RPS10 corresponds to a basic polypeptide of 129 amino acids, with a predicted molecular weight of 15 798 Da.

Unspliced *rps10* transcripts are edited

The temporal relationship between RNA editing and intron splicing was examined by sequence analysis of cDNA clones of unspliced *rps10* transcripts. Unspliced cDNAs were obtained by reverse transcription of total mtRNA with oligo 3 and PCR-amplification of exon-1 and intron sequences between oligos 1 and 3 (Fig. 1 B). A RT-PCR product size with a length of 0.4–0.5 kb was as expected from the genomic sequence. Of the nine clones completely sequenced (Fig. 5), seven showed three C-to-U modifications with respect to the genomic sequence. These include

Fig. 3 Secondary structural model of the *rps10* intron. The secondary structure model of six domains (I to VI) is characteristic for group-II introns (Michel et al. 1989). The RNA editing event in domain I(i) is indicated by an *arrow* (C-to-U). The position of the bulging A to which the 5′ end of the intron is expected to join during splicing is indicated by an *asterisk*. EBS and IBS are candidates for exon- and intron-binding sites, respectively. The γ tertiary interaction in the central hub is indicated. *Arrows* point to exon-intron borders

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                                  T
potato (mt) 1 MRQRRALRRV SQKERPPKVM TTKIGIVIRS FDHPFLENHF WGLPPYTRK-
liverwort (mt)                    M :A::C:::K: :------::QR S::LLN:::-
Cryptomonas (cp)                 MQ KQ::R:RLKG YETNL:LDSC NKIIETATRT
Cyanophora                    MASNQ QL::R:QL:: Y:SSL:::SC EQIIEAAKRT
E.coli                           MQ NQR:R:RLKA :::RLIDQAT AEIVETAKRT
Mycoplasma                       MA EN:MR:KLKG Y::AIVDQSI TKIIQAAEGT
                                       .. * ... ..    . .     .   . .

potato     50 -------IGL PESRVLYTVL RSPHIDKKSR EQFFMKIKKE FLVIKTERHE
liverwort     -------::: :KKQT::::: :::::::::: :::E:R:H:Q L:::E::T:K
Cryptomonas   NSVTAGP:P: :TR:KI:C:: ::::VN:D:: :H:ETR:H:R VID:YEPNST
Cyanophora    DATAVGP:P: :TKKKI:C:: ::::V::D:: :H:EIRVHRR IIDLYLPSSK
E.coli        GAQVRGP:P: :TRKERF::: I:::VN:DA: D:YEIRTHLR LVD:VEPTEK
Mycoplasma    GAKVRGP:P: :TEKQVI:I: :AV:KY:D:: :::E:RTH:R L:E:LNPTAA
                     * * *  .    .* ...*  * .* .... ....    . .

                    R                      S          R
potato     93 LRKKFFWLKR QRIFGAQYEI LFSCKTRLDK GKLQRLL* (110 or 129)
liverwort     ::E:LNW::L HDLL:V:VK: I:YYQ::L:: VCKS*     (102)
Cryptomonas   TIDSLMQ:DL PAGIDIDIK: *                    (102)
Cyanophora    TIDTLTR:DL PAGVDVEVKL *                    (105)
E.coli        TVDALMR:DL AAGVDV:ISL G*                   (103)
Mycoplasma    TMDVLKRVQL PSGVDIEIKL *                    (102)
              . . .  . .   .  ..  .
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Fig. 4 Comparison of RPS10 amino-acid sequences deduced from potato cDNA and genomic nucleotide sequences. Amino-acid sequence comparison between RPS10 polypeptides specified in potato mitochondria and by genomic sequences from liverwort mitochondria (Takemura et al. 1992). *Cryptomonas phi* chloroplast (EMBL SWISS-PROT Database Accession P19460), *Cyanophora paradoxa* cyanelle (Accession P17009), *Escherichia coli* (Accession P02364) and *Mycoplasma capricolum* (Accession P10129). Only amino acids altered by RNA editing are given in the top lane for the potato genomic-encoded sequence. Gaps, indicated by *dashes*, were introduced to maximize the alignment. A *colon* (:) indicates identity with the respective potato residue. *Asterisks* below the amino acids indicate positions conserved in all species compared, and *dots* indicate positions conserved in 5 out of 6 proteins or involving only conservative amino-acid changes. *Numbering* refers to the potato protein and total numbers of amino acids are given in *parentheses*

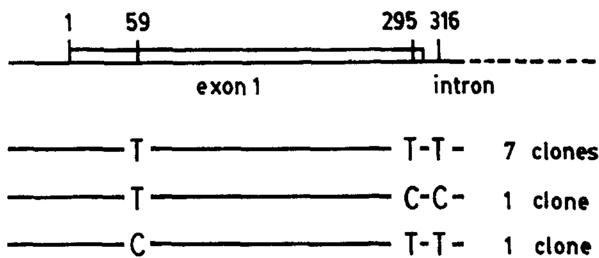

Fig. 5 RNA editing sites vary between individual unspliced *rps10* cDNA clones. Shown on the first line is a representation of *rps10* first exon (*open bar*) and intron (*thin line*). Line drawings below it represent the sequenced region of unspliced cDNAs. Edited or unedited nucleotides at the editing sites (nucleotides 59, 295 and 316) are indicated by *T* or *C*, respectively. Numbering is in relation to the *rps10* genomic ATG codon at position 1

the two previously identified editing sites in exon 1 and an additional editing event in the intron sequence.

A low level of partially edited unspliced precursors was found. Two out of the nine cDNA clones were not fully edited, one clone lacking the first editing event in exon 1 and the other being edited only at this position. The presence of differentially edited cDNA clones confirms that the individual cDNA clones analyzed are derived from separate cloning events of the mRNA population. Fully edited *atp9* mRNAs and low levels of partially edited *cob* mRNAs were previously observed in potato mitochondia (Dell'Orto et al. 1993; Zanlungo et al. 1993). Differentially edited mRNAs have also been observed in other plant mitochondrial genes and unselected translation of all these transcripts would direct the synthesis of variant polypeptides. However, partially edited transcripts appear to be, at least in some cases, intermediates in editing, since processed transcripts and polysomal mRNAs are more heavily edited than precursor or pooled total mtRNAs (Gualberto et al. 1991; Sutton et al. 1991; Yang et al. 1991). Consistent with this hypothesis, spliced *rps10* transcripts are found fully edited while a low level of partially edited *rps10* transcipts is detected in the unspliced fraction. On the other hand, RNA editing presumably precedes splicing in the maturation of *rps10* transcripts, since most unspliced cDNA clones are already completely edited. Recent work has shown that chloroplast editing is also an early processing event (Freyer et al. 1993; Ruf et al. 1994).

The intron RNA editing event eliminates an A/C mismatch of the genomic sequence in stem I(i) of domain I, and allows an additional A-U base pairing presumably essential for stable folding of the stem structure (Fig. 3). This stem is usually well conserved in its secondary structure and thus presumably required for correct intron excision. This editing event may thus be necessary for splicing of the intervening sequence, an hypothesis further substantiated by the fact that it is altered with high efficiency, i.e. in 8 out of 9 cDNA clones. However, editing at the intron site detected here cannot be the sole rate-limiting step of splicing, since most of the immature molecules are already edited at this site.

Although editing takes place predominantly within the coding regions, editing sites have also been observed inside of intron sequences, in *nad1* introns of *Oenothera* and petunia and in *nad2* and *nad5* introns of *Oenothera* (Knoop et al. 1991; Sutton et al. 1991; Wissinger et al. 1991; Binder et al. 1992). Some of these editing events improve base pairing in secondary structure models and may thus be required for correct processing of these introns. The editing event observed in the *rps10* intron stem I(i) of domain I is similar to an editing event observed in the *Oenothera nad2* intron 2 (Binder et al. 1992). A possible role of RNA editing in regulation splicing of *rps10* transcripts needs to be investigated. Further editing within the intron may be required for splicing. A good candidate for such an editing site is found in the helical stem of domain IV which contains an A/C mismatch that could be eliminated by a C-to-U change, thus correcting the base pairing and stabilizing the structure (Fig. 3).

Transcription of the *rps10* gene

Northern analysis previously showed that potato *rps10* is expressed as multiple transcripts, ranging in size from 0.8 to 3.3 kb (Zanlungo et al. 1994). Although *rps10* could be potentially co-transcribed with the upstream *cob* pseudogene, its complex set of transcripts differs from the hybridization pattern obtained with a *cob* probe. Intron and exon probes (probes 4 and 5 respectively, Fig. 1 B) reveal similar transcript patterns of mRNAs larger than 1400 nt which thus probably represent unspliced precursors (Fig. 6). The relative abundance of these mRNAs is similar except for a 1.7 – 1.8 kb transcript detected by the intron probe that may represent a splicing intermediate (i.e. a intron-exon-2 lariat) or an anomalous migrating free intron. mRNA species with sizes of about 800–1000 nt, sufficient to encode the entire *rps10* ORF, are specifically detected by the exon probe and thus might represent mature spliced *rps10*

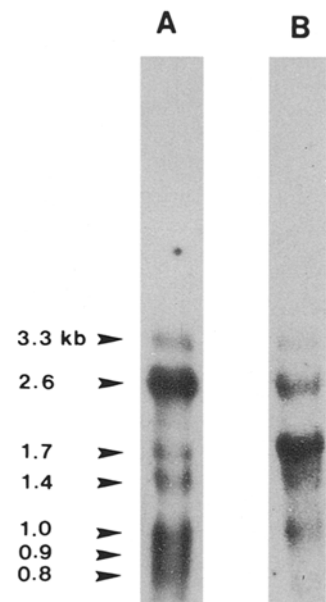

Fig. 6 Northern analysis of transcripts from the *rps10* locus in potato mitochondria. Blots were hybridized with *A* an exon probe (oligo 4, Fig. 1 B), and *B* an intron probe (oligo 5, Fig. 1 B). Size markers are RNA length standards (BRL RNA ladders) co-fractionated in the agarose-formaldehyde gel

mRNAs. Accumulation of multiple unspliced intermediates that probably include immature molecules with different 5′- and 3′-extensions suggests that the splicing reaction may proceed more slowly than the other processing steps (i.e. the processing reactions at the 5′ and 3′ ends). On the other hand, different 5′ ends may be generated by multiple promoters between the *cob* pseudogene and *rps10*. It will be necessary to analyse RNA transcription in greater detail to discover the nature and origin of these multiple mRNAs.

Potato *rps10* is likely to be a functional gene since its transcripts have been detected and shown to undergo processing in the form of editing and splicing. Evidence for the synthesis of ribosomal proteins in plant mitochondria includes the observation of Hunt and Newton (1991) that the NCS-3 phenotype in maize results from disruption of the *rps3/rpl16* locus, and the reaction of antibodies against RPS1A with proteins in wheat mitochondrial ribosomal fractions (González et al. 1993).

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