A single nuclear transcript encoding mitochondrial RPS14 and SDHB of rice is processed by alternative splicing: Common use of the same mitochondrial targeting signal for different proteins

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ABSTRACT The rice mitochondrial genome has a sequence homologous to the gene for ribosomal protein S14 (rps14), but the coding sequence is interrupted by internal stop codons. A functional rps14 gene was isolated from the rice nuclear genome, suggesting a gene-transfer event from the mitochondrion to the nucleus. The nuclear rps14 gene encodes a long N-terminal extension showing significant similarity to a part of mitochondrial succinate dehydrogenase subunit B (SDHB) protein from human and a malarial parasite (Plasmodium falciparum). Isolation of a functional rice sdhB cDNA and subsequent sequence comparison to the nuclear rps14 indicate that the S′ portions of the two cDNAs are identical. The sdhB genomic sequence shows that the SDHB-coding region is divided into two exons. Surprisingly, the RPS14-coding region is located between the two exons. DNA gel blot analysis indicates that both sdhB and rps14 are present at a single locus in the rice nucleus. These findings strongly suggest that the two gene transcripts result from a single mRNA precursor by alternative splicing. Protein blot analysis shows that the size of the mature RPS14 is 16.5 kDa, suggesting removal of the N-terminal 22.6-kDa peptide region. Considering that the rice mitochondrial genome lacks the sdhB gene but contains the rps14-related sequence, transfer of the sdhB gene seems to have occurred before the transfer of the rps14 gene. The migration of the mitochondrial rps14 sequence into the already existing sdhB gene could bestow the capacity for nuclear expression and mitochondrial targeting.

MATERIALS AND METHODS

Plant Material and Nucleic Acid Isolation. Etiolated seedlings of rice (Oryza sativa L., cv. Nipponbare) were used as plant material. Total DNA, mitochondrial DNA, total RNA, and mitochondrial RNA were prepared as described (10). Poly(A)^+ RNAs were enriched by oligo(dT) cellulose column chromatography (11).

Construction and Screening of Recombinant Library. Rice mitochondrial DNA, cDNA, and genomic DNA libraries were constructed as described (10). The liverwort rps14 gene (3) was kindly provided by K. Ohyama (Kyoto University). Preparation of DNA fragments, probe labeling, and hybridization were performed by using the enhanced-chemiluminescence direct nucleic acid-labeling system (Amersham Pharmacia) according to the manufacturer’s instructions. The condition of the final washing was 2× SSC (0.15 M sodium chloride/0.015 M sodium citrate, pH 7) and 0.1% SDS at 42°C.

Reverse Transcription–PCR Analysis. cDNA synthesis and subsequent PCR amplification were performed as described by Kubo et al. (12). Amplified cDNA was cloned into a pBlue-script SK II(+) vector (Stratagene) and sequenced.

Abbreviation: DDBJ, DNA Data Bank of Japan.

Data deposition: The nucleotide sequences reported in this paper have been deposited in the DDBJ [accession nos. AB017426 (mitochondrial rps5–rps14 genes), AB017427 (nuclear rps14 cDNA), AB017428 (sdhB cDNA), and AB017429 (sdhB–rps14 genomic sequence)].

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DNA Sequencing and Subsequent Sequence Analysis. DNA was sequenced by the dideoxy chain termination method by using fluorescent dye primer (Applied Biosystems). Nucleotide and deduced amino acid sequences were analyzed as described (10).

DNA and RNA Gel Blot Analyses. Total DNA (5 μg) and mitochondrial DNA (2 μg) were digested with EcoRI or XbaI, separated on a 0.7% agarose gel, and blotted onto Hybond-N+ membrane (Amersham Pharmacia) by the conventional capillary method (13). Poly(A)+ RNA (1 μg) and mitochondrial RNA (5 μg) were fractionated through a 1% agarose/formaldehyde gel and blotted onto Hybond-N+ membrane. Three specific 3P-labeled probes were used for hybridization. The probes included a 243-bp XhoI fragment specific for sdhB cDNA (probe 1), a 594-bp BamHI–EcoRV fragment containing a common region for sdhB and rps14 cDNAs (probe 2), and a 348-bp EcoRV–PstI fragment specific for rps14 cDNA (probe 3; see Fig. 3). Hybridization was carried out at 42°C overnight according to the standard protocol (13).

Protein Blot Analysis. Preparation of mitochondrial proteins and protein blot analysis were performed as described (14). Antibody raised against a malarial parasite (Plasmodium falciparum) SDHB SHP was kindly provided by K. Kita (University of Tokyo). RPS14 antibody was prepared as follows. An rps14-homologous region of the rice nuclear rps14 cDNA was amplified by PCR, ligated in frame into pGEX-4-T3 vector, and overexpressed in Escherichia coli as a fusion protein according to the manufacturer’s instructions (Amersham Pharmacia). The peptide was electrophoretically purified, lyophilized, and injected into a rabbit (15).

RESULTS

rps14-Related Sequence in Rice Mitochondrial Genome Is Not Functional. A mitochondrial DNA library of rice was screened by using the liverwort rps14 gene as a probe to determine the organization of the rps14 gene in rice. A clone was successfully obtained, and its DNA was sequenced. The nucleotide sequence indicated that an rps14-homologous sequence was located 1 nucleotide downstream of an rpl5 gene. The nucleotide sequence of the rice mitochondrial rpl5–rps14 genes is not shown but may be found in the DNA Data Bank of Japan (DDBJ) database under accession no. AB017426. The presence of rps14 gene in the mitochondrial genome has been reported from lower plants such as liverwort (3) and chloroplast algae (16). Among the higher plants, intact rps14 genes have been found in the mitochondrial genome of broadbean (17), Oenothera (18), and rapeseed (19), whereas rps14 is present as a pseudogene in Arabidopsis (20, 21) and potato mitochondria (22). The nucleotide and deduced amino acid sequence comparison with those from other plant species showed that the rice rpl5 gene retains an intact ORF, whereas the original reading frame of the rps14 is interrupted by nucleotide deletions at four positions. A similar gene disruption has been reported in rice nuclear rps14 (20, 21) and potato (22), but the nucleotide deletion sites are different among the three plants. RNA gel blot analysis proved that the rps14 sequence was transcribed (see Fig. 3). The reverse transcription–PCR and subsequent cDNA sequence analyses of the two genes indicated that rice rps15 had one RNA-editing site, whereas no editing event was observed in the rps14 sequence (DDBJ accession no. AB017426). Thus, mitochondrial rps14 cannot be functional.

Functional Mitochondrial rps14 Is Encoded in the Nuclear Genome. Because the mitochondrial-encoded rps14 sequence in rice is a pseudogene, a functional rps14 gene is likely to be encoded in the nuclear genome. A rice cDNA library made from poly(A)+ RNA was screened by using the rice mitochondrial rps14 pseudogene sequence as a probe, resulting in the identification of nine positive cDNA clones. The DNA sequence analysis enabled us to classify the nine cDNA clones into five groups based on the end of 5’ and/or 3’ flanking sequences, whereas the internal coding sequences of the cDNAs were identical. The largest cDNA clone includes an ORF capable of encoding 350 amino acids (Fig. L4). Comparison of the amino acid sequences deduced from the cDNAs isolated in this study with the rps14 genes from other plant mitochondria (3, 17–19) as well as with the rice mitochondrial pseudogene showed 62–73% amino acid identity (Fig. 1B). On the other hand, the amino acid sequences of the above cDNA clones share only 35% amino acid identity (data not shown) to rice chloroplast RPS14 (23). These results led us to conclude that the cDNAs isolated encode a mitochondrial RPS14.

Fig. 1. (A) cDNA and deduced amino acid sequence of the rice nuclear-encoded rps14 gene. The amino acid sequence comparison of the RPS14-homologous region is indicated by a bent arrow. A region homologous to SDHB is underlined. The intron site is marked by an arrowhead. (B) Amino acid sequence comparison of RPS14. Deduced amino acid sequence of rice nuclear rps14 gene (this study) is aligned with amino acid sequences of mitochondrial rps14 genes from rice (this study), broadbean (17), Oenothera (18), rapeseed (19), and liverwort (3). Amino acid residues identical to rice nuclear RPS14 are indicated by dots. Gaps are shown by dashes. An asterisk represents a translational stop codon that truncates an ORF of rice mitochondrial RPS14. Frameshift in the rps14-coding region of rice mitochondria was not considered here.
region of the rps14 gene does not include the entire sdhB gene, suggesting the presence of a functional sdhB gene in the rice nuclear genome. Complete sequence information of the sdhB gene from plants, including the lower plant species, is not still available, to our knowledge, except for reports of a partial sdhB sequence in Arabidopsis obtained by PCR amplification with degenerated primers (27) and in rice identified by random cDNA sequencing analysis (28). Therefore, we tried to isolate an intact sdhB gene from rice.

A rice cDNA library was screened by using the sdhB-homologous region of the nuclear rps14 cDNA clone as a probe. Four cDNA clones were isolated, and the largest cDNA encoded the whole region of an sdhB gene. The entire cDNA sequence of the rice sdhB gene is not shown in the text but may be found in the DDBJ under accession no. AB017428. The rice sdhB gene showed 58% and 57% amino acid identity to the human and P. falciparum SDHB, respectively (Fig. 2). Three cysteine clusters needed for the formation of an iron–sulfur cluster are highly conserved among the compared sdhB genes, but the nuclear rps14 gene contains only two cysteine clusters (Fig. 2).

It is surprising that the sdhB and the nuclear rps14 genes of rice have identical nucleotide sequences not only for their coding regions, but also for the 5′ and 3′ flanking sequences (data not shown). This finding leads us to two possible explanations. The first one could be that the sdhB and the nuclear rps14 genes are located at different genomic positions with identical nucleotide sequences because of a recent duplication and recombination event. The second one could be that both the sdhB and the nuclear rps14 genes are encoded by the same genomic sequence, transcribed as a single mRNA precursor, and two forms of mRNAs result from an alternative splicing event.

**Genomic Organization of the sdhB and the rps14 Genes.**

DNA gel blot analysis was carried out to examine the two possible explanations given above. Only one signal was observed for each of two restriction enzymes in rice total DNA (Fig. 3B) when a probe specific for the sdhB cDNA (Fig. 3A, probe 1) and a probe containing a common region for the sdhB and the nuclear rps14 cDNAs (Fig. 3A, probe 2) were used. No signal was detected in rice mitochondrial DNA. These results clearly indicate that the sdhB gene is present as a single copy in the rice nuclear genome. When a probe specific for the nuclear rps14 cDNA (Fig. 3A, probe 3) was used as a probe, three (18.0, 4.1, and 3.0 kb) or two (4.7 and 2.5 kb) bands were detected in EcoRI- or XbaI-digested total DNA, respectively (Fig. 3B). The 4.1-kb and 3.0-kb signals in EcoRI digest were derived from the nuclear DNA. These results clearly indicate that the RPS14-coding region belongs to a single locus, because signals of the same size were also detected in the mitochondrial DNA. Two bands were detected in EcoRI-digested mitochondrial DNA, because there are two copies of the rps15–rps14 gene cluster, having different 3′-flanking sequences in the mitochondrial genome. In conclusion, only one signal of 18.0 kb in EcoRI digest or 2.5 kb in XbaI digest is derived from the nuclear DNA. These results clearly indicate that the RPS14-coding region belongs to a single locus.

To confirm the DNA gel blot analysis, cloning of the sdhB genomic sequence was carried out by using the sdhB cDNA as a probe, and four clones were isolated. Physical mapping of the isolated genomic clones showed that they are derived from independent clones. Nucleotide sequence analysis and sequence comparison of the isolated genomic clones indicated that the sequence order is the 5′ end, the sdhB–rps14 common region, an intron, the RPS14-coding region, an intron, the C-terminal region specific for the sdhB gene, and the 3′ end (Fig. 4). The sdhB–rps14 genomic sequence is not shown in the text but may be found in the DDBJ under accession no. AB017429. According to this scheme, three exons are separated by two introns. Each of the four isolated clones has the same physical structure with the clone sequenced, confirming that the sdhB–rps14 genomic sequence represents a single locus.

**Expression of the sdhB and the Nuclear rps14 Genes.**

The expressions of the sdhB and the nuclear rps14 genes were examined by RNA gel blot analysis by using the three probes specified in Fig. 3A. A 1.2-kb band was detected with all of the three probes (Fig. 3C). Considering the size of the sdhB (1,132 nt) and the nuclear rps14 (1,233 nt) cDNAs, the size of 1.2-kb band is in good agreement with the sizes of the two cDNA clones. A primary transcript of 3.7 kb was identified by the RPS14-coding probe, suggesting the occurrence of splicing event. Splicing events of the two gene transcripts were also confirmed by reverse transcription–PCR (data not shown).

The deduced amino acid sequence of the nuclear rps14 gene has an N-terminal extension that seems to be a targeting signal to mitochondria. To clarify whether the targeting signal is cleaved off after protein import into mitochondria, an anti-rice RPS14 antibody was raised in a rabbit, and protein blot analysis was carried out by using antibodies against rice RPS14 and P. falciparum SDHB proteins. A 16.5-kDa peptide was detected when the anti-RPS14 antibody was used (Fig. 5B). The size of this peptide is smaller than that of the RPS14 peptide, as deduced from the nuclear rps14 cDNA sequence (39.1 kDa). The size of the 16.5-kDa peptide is larger than that of the predicted mitochondrial-encoded RPS14 peptide (∼12 kDa),
but it is suspected that most of the SDHB-homologous region has been removed.

A signal of 27.2 kDa was detected with the anti-\( P. falciparum \) SDHB antibody (Fig. 5D), implying translation and import of SDHB protein into mitochondria. The peptide size of 27.2 kDa is 3.9-kDa shorter than that of the SDHB peptide deduced from the \( sdhB \) cDNA sequence (31.1 kDa) and similar to that of eubacteria, suggesting processing of SDHB protein after protein import.

**DISCUSSION**

The data in this manuscript present an alternative splicing event, as well as a gene-transfer event from the mitochondrion to the nucleus. An \( rps14 \)-homologous sequence is located downstream of an \( rpl5 \) gene in the rice mitochondrial genome, which is transcribed but not functional. A nuclear copy of \( rps14 \) gene has been isolated by using this \( rps14 \)-sequence. The nuclear \( rps14 \) gene has a long N-terminal extension similar to that of \( sdhB \) genes found in other organisms. Because complete nucleotide information about \( sdhB \) gene has not been reported from plants, we isolated and analyzed the \( sdhB \) gene from the rice nucleus. The \( sdhB \) gene is absent from the mitochondrial genome of lower and higher plants, suggesting that transfer of the \( sdhB \) gene occurred before the evolutionary split of higher and lower plants. On the other hand, the gene transfer of the rice \( rps14 \) seems to have occurred relatively recently during the evolution of flowering plants, after the gene transfer of the \( sdhB \) gene, because a rice mitochondrial genome still retains the \( rps14 \)-related sequence. The rice \( rps14 \) sequence would have been integrated within an intron of the \( sdhB \) gene after the \( sdhB \) gene acquired all elements for functional expression (Fig. 6). Finally, the integrated \( rps14 \) sequence may have been recognized as an alternative exon by splicing factors. Although the nuclear \( rps14 \) gene seems to have transferred to the nucleus relatively recently, the sequence shows some dissimilarity to mitochondrial-encoded \( rps14 \) genes (Fig. 1B). This dissimilarity may be due to the higher mutation rate of the nucleus than of the mitochondria in plants (29, 30). In addition, the differences in codon usage between the two organelles may have accelerated nucleotide alteration in the transferred gene.
Materials and methods.

Many examples of alternative splicing events have been found in animals. Alternative splicing is thought to be a general mechanism for regulation of gene expression (31). Although alternative splicing events cause exon deletions resulting in production of protein isoforms with modified activities or tissue-specific expression, these events generally have been shown neither to modify protein function nor to generate totally different proteins, except for calcitonine and the calcitonine gene-related peptide (32). In this study, we have found that the alternative splicing generates two different proteins, RPS14 and SDHB. They are involved in protein synthesis and the respiratory chain in mitochondria, respectively. This fact implies that both RPS14 and SDHB are mitochondrial proteins but with completely different functions. Our results present a case in which a single targeting signal is used by two different mitochondrial proteins.

Fig. 6. Model for the gene transfer of sdhB and rps14. Rice nucleus and mitochondrion are shown by a square and an enclosure, respectively. Exons and introns are represented by boxes and broken lines, respectively. RPS14-homologous regions are shown by black boxes. sdhB-related regions are shown by boxed boxes. Black and hatched circles represent the products of rps14 and sdhB genes, respectively. Other symbols correspond to those in Fig. 4.

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