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Genetics

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

NAKAO KUBO*†, KYUYA HARADA*, ATSUSHI HIRAI‡, AND KOH-ICHI KADOWAKI†§

*Faculty of Horticulture, Chiba University, Matsudo 648, Matsudo, Chiba 271-0092, Japan; †National Institute of Agrobiological Resources, Department of Molecular Genetics, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan; and ‡Graduate School of Agricultural and Life Sciences, University of Tokyo, Yayoi 1-1-1, Bunkyo, Tokyo 113-0032, Japan

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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 5′ 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.**

The endosymbiont hypothesis for mitochondrial origin has generally been accepted (1). Recent studies show that the gene content of the plant mitochondrial genome is much larger than that of animals, insects, etc. (2). A striking example exists in liverwort and *Arabidopsis* in which 16 and 9 kinds of ribosomal protein genes have been identified, respectively, whereas none of these genes are encoded in mammals and yeast (3, 4). Several plant mitochondrial genomes have been shown to contain pseudogenes, and the total gene content of the mitochondrial genome is not always the same among higher plant species, including even evolutionarily related species. These observations suggest that gene-transfer events from the mitochondrion to the nucleus are carried out by an active process in plants (2). The existence of such a process has been established recently by isolation of mitochondrial genes transferred to the nuclear genome, such as *coxII* genes from cowpea and soybean (5, 6), the *rps12* gene from *Oenothera* (7), *rps10* and *rps19* genes from *Arabidopsis* (8, 9), and the *rps11* gene from rice (10). These findings added to the understanding of gene-transfer events such as RNA-mediated gene transfer (5–9), acquisition of mitochondrial targeting signal from a preexisting mitochondrial gene (10), and functional replacement of a missing *rps13* gene by another ribosomal protein gene (9). However, knowledge of gene transfer followed by a gene activation process is still very limited.

In this study, we report a gene-transfer event and a process of acquisition for a targeting signal as well as a nuclear expression. The rice mitochondrial genome contains a sequence homologous to the *rps14* gene, but the reading frame is disrupted. A functional *rps14* gene is encoded in the nuclear genome. Interestingly, the nuclear *rps14* gene encodes a long N-terminal extension showing similarity to mitochondrial succinate dehydrogenase subunit B (*sdhB*) genes. Sequence comparison between the *sdhB* and *rps14* genes, subsequent isolation of a functional *sdhB* gene, and genomic analysis of the *sdhB* gene strongly suggest that the two nuclear genes are encoded within the same genomic environment and that the mRNAs of the two genes result from alternative splicing. Here, we report that an alternative splicing event supplies the same targeting signal to different proteins and activates a newly transferred mitochondrial protein gene in the nucleus.

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 pBluescript SK II(+) vector (Stratagene) and sequenced.

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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 *rpl5–ψrps14* genes), AB017427 (nuclear *rps14* cDNA), AB017428 (*sdhB* cDNA), and AB017429 (*sdhB–rps14* genomic sequence)].

§To whom reprint requests should be addressed. E-mail: kadowaki@abr.affrc.go.jp.

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 *Eco*RI or *Xba*I, 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 ^{32}P-labeled probes were used for hybridization. The probes included a 243-bp *Xho*I fragment specific for *sdhB* cDNA (probe 1), a 594-bp *Bam*HI–*Eco*RV fragment containing a common region for *sdhB* and *rps14* cDNAs (probe 2), and a 348-bp *Eco*RV–*Pst*I 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 protein 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 chlorophyte alga (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 *Arabidopsis* (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 *rpl5* 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. 1*A*). 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. 1*B*). 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.

A Nuclear-Encoded *rps14* Gene Carries a Long N-Terminal Extension Homologous to a Part of the *sdhB* Gene. The deduced amino acid sequence of the nuclear *rps14* gene has an additional 250 amino acid residues at the N-terminal region compared with the mitochondrial *rps14* genes from other plants (Fig. 1*B*). Interestingly, a protein database search indicated that the extended portion (positions 49–225) showed a significant amino acid sequence similarity to mitochondrial SDHB, which is a component of complex II in the respiratory chain, from human (24, 25), yeast (26), and a malarial parasite (*P. falciparum*; DDBJ accession no. D86574). The N-terminal

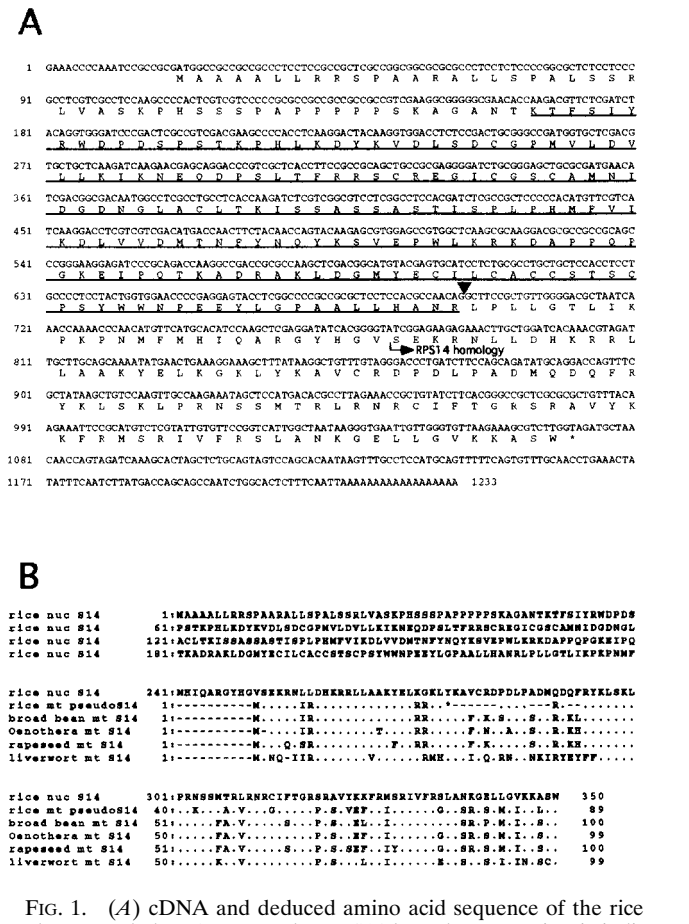

FIG. 1. (*A*) cDNA and deduced amino acid sequence of the rice nuclear-encoded *rps14* gene. The RPS14-homologous region is indicated by a bent arrow. A region homologous to SDHB is underlined. The intron site is marked by an arrowhead. (*B*) Amino acid sequence comparison of RPS14. Deduced amino acid 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* sequence. 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 still not 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′ 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. 3*B*) when a probe specific for the *sdhB* cDNA (Fig. 3*A*, probe 1) and a probe containing a common region for the *sdhB* and the nuclear *rps14* cDNAs (Fig. 3*A*, 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. 3*A*, 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 *Eco*RI- or *Xba*I-digested total DNA, respectively (Fig. 3*B*). The 4.1-kb and 3.0-kb signals in *Eco*RI digest and the 4.7-kb signal in *Xba*I digest seem to come from the mitochondrial DNA, because signals of the same size were also detected in the mitochondrial DNA. Two bands were detected in *Eco*RI-digested mitochondrial DNA, because there are two copies of the *rpl5–ψrps14* gene cluster, having different 3′-flanking sequences in the mitochondrial genome. In conclusion, only one signal of 18.0 kb in *Eco*RI digest or 2.5 kb in *Xba*I 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. 3*A*. A 1.2-kb band was detected with all of the three probes (Fig. 3*C*). 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. 5*B*). 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),

FIG. 2. Alignment of deduced amino acid sequences of *sdhB* genes from rice (this study), human (24, 25), malarial parasite (*P. falciparum*; DDBJ accession no. D86574), and nuclear *rps14* from rice (this study). Amino acid residues identical to the rice SDHB are highlighted by reverse contrast. The three thick bars above the sequence represent three cysteine-rich clusters, which are highly conserved among *sdhB* genes. An RPS14-homologous region in the rice *rps14* gene is indicated by a bent arrow. Arrowheads indicate the intron position of rice *sdhB* and *rps14* genes.

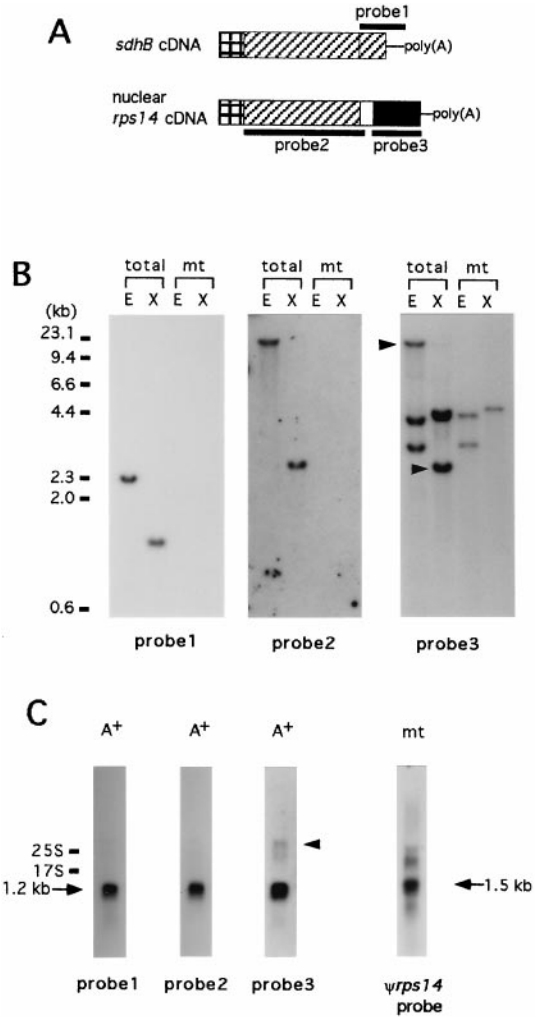

FIG. 3. DNA and RNA gel blot analyses of *sdhB* and nuclear *rps14* genes in rice. (*A*) Schematic representation of *sdhB* and nuclear *rps14* genes. The RPS14-homologous region is shown by a black box. *sdhB*-related regions are shown by hatched boxes. Thick lines indicate the DNA probes used for DNA and RNA gel blot analyses. (*B*) Rice total DNA (total) and mitochondrial DNA (mt) were digested with *Eco*RI (E) or *Xba*I (X). (*Right*; probe 3), the bands derived from nuclear DNA are indicated by arrowheads. A molecular length standard is shown at left. (*C*) Rice poly(A)$^+$ RNA and mitochondrial RNA are represented by (A$^+$) and (mt), respectively. The size of 25S and 17S ribosomal RNAs are indicated at left. The sizes of the transcripts are shown in the figure. A possible primary transcript is indicated by an arrowhead.

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. 5*D*), 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 *ψrps14* sequence. The

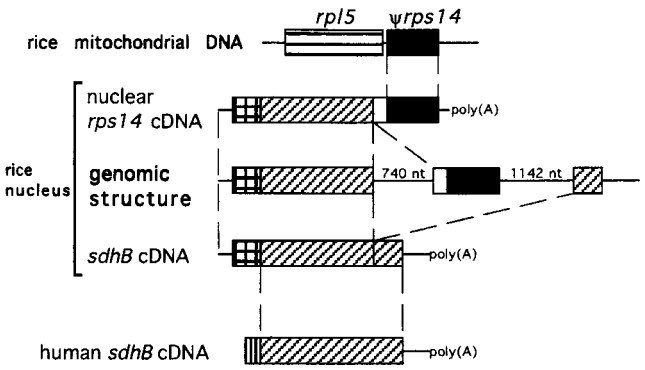

FIG. 4. Schematic representation of rice nuclear *rps14* and *sdhB* genes. Boxes and thin lines represent protein coding sequence and nontranslated regions, respectively. The box with horizontal stripes represents the *rpl5* gene. *rps14*-homologous regions are shown by black boxes. *sdhB*-related regions are shown by hatched boxes. Putative mitochondrial targeting signals of *sdhB* genes from rice and human are indicated by boxes with both horizontal and vertical lines and the box with vertical stripes, respectively.

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. 1*B*). 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.

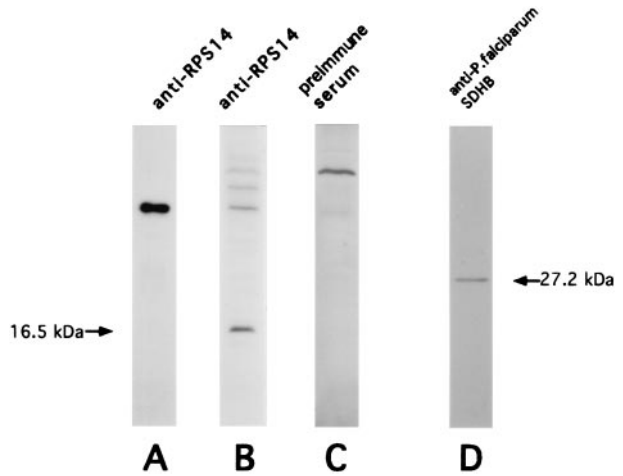

FIG. 5. Protein blot analysis of rice nuclear RPS14 and SDHB proteins. (*A*; positive control) Total *E. coli* protein in which GST–RPS14 fusion protein was overexpressed. (*B–D*; rice mitochondrial protein) The sera used in the protein analysis and the sizes of the peptides are indicated.

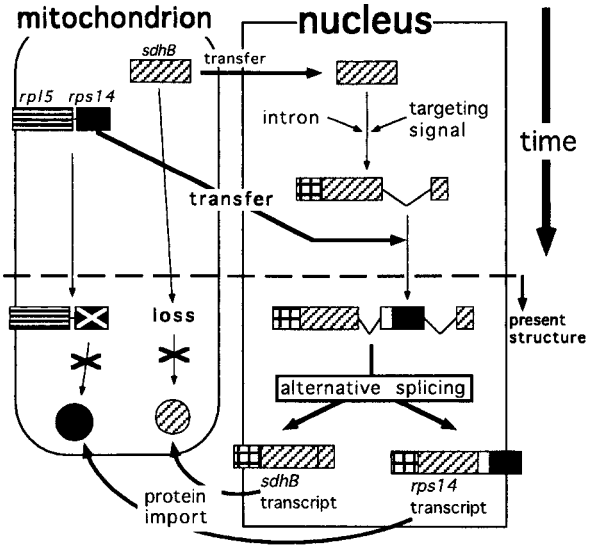

FIG. 6. Model for the gene transfer of *sdhB* and *rps14*. Rice nucleus and mitochondrion are shown by a square and an enclosure, respectively. Exons and introns are represented by boxes and broken lines, respectively. RPS14-homologous regions are shown by black boxes. *sdhB*-related regions are shown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

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.

A mitochondrial sequence that has migrated into a nucleus needs to acquire many sequence elements (e.g., promoter, mitochondrial targeting signal for protein import, and polyadenylation signal) for its functional expression because of the difference of gene expression systems between nucleus and mitochondrion. Several mechanisms for acquisition of a mitochondrial targeting signal have been proposed so far (10, 33, 34). The alternative splicing event found in this study is an example of the acquisition of a targeting signal. In short, a transferred mitochondrial sequence could acquire both a nuclear expression system and a mitochondrial targeting signal through its integration with an already existing mitochondrial protein gene in the nucleus. This example shows the diversity of a gene expression system. Further studies are required to identify the number of mechanisms involved in gene translocation from the mitochondrion to the nucleus and the subsequent activation during endosymbiosis.

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