Additional pathway to translate the downstream *ndhK* cistron in partially overlapping *ndhC-ndhK* mRNAs in chloroplasts

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The chloroplast *NAD(P)H dehydrogenase (NDH) C (ndhC)* and *ndhK* genes partially overlap and are cotranscribed in many plants. We previously reported that the tobacco *ndhC/K* genes are translationally coupled but produce NdhC and NdhK, subunits of the NDH complex, in similar amounts. Generally, translation of the downstream cistron in overlapping mRNAs is very low. Hence, these findings suggested that the *ndhK* cistron is translated not only from the *ndhC* 5' UTR but also by an additional pathway. Using an in vitro translation system from tobacco chloroplasts, we report here that free ribosomes enter, with formylmethionyl-tRNA^{fMet}, at an internal AUG start codon that is located in frame in the middle of the upstream *ndhC* cistron, translate the 3' half of the *ndhC* cistron, reach the *ndhK* start codon, and that, at that point, some ribosomes resume *ndhK* translation. We detected a peptide corresponding to a 57-amino-acid product encoded by the sequence from the internal AUG to the *ndhC* stop codon. We propose a model in which the internal initiation site AUG is not designed for synthesizing a functional isoform but for delivering additional ribosomes to the *ndhK* cistron to produce NdhK in the amount required for the assembly of the NDH complex. This pathway is a unique type of translation to produce protein in the needed amount with the cost of peptide synthesis.

overlapping gene | alternative initiation | Shine–Dalgarno sequence

Chloroplasts are photosynthetic organelles unique to plant cells, and they possess their own genomes. For example, the tobacco chloroplast genome contains genes encoding 80 different proteins and 35 different, stable RNA species (1, 2). As the genome is tightly packed, many genes are clustered and as many as four sets of protein-coding genes partially overlap. Clustered genes are transcribed as polycistronic mRNAs, which are then processed into multiple overlapping mRNAs including mono-, di-, and polycistronic mRNAs (3–5). The chloroplast is thought to have arisen from a photosynthetic prokaryote related to modern cyanobacteria. Hence, the translation apparatus of chloroplasts is related to that of eubacteria. Chloroplast gene expression is primarily regulated during translation (6–9). Similarly to eubacterial mRNAs, chloroplast mRNAs have no cap structure and no poly(A) tail. However, a striking feature is that over half of the tobacco chloroplast mRNAs have no Shine–Dalgarno (SD)-like sequence in a conserved position (10), and even the SD-like sequences are not always functional (11, 12). The situation is similar for chloroplast mRNAs from other plants and *Chlamydomonas reinhardtii* (8). Hence, many chloroplast mRNAs are likely to require specific *trans*-acting factors for translation (6, 7, 9, 13–16). Chloroplast ribosomes contain several unique proteins in addition to *Escherichia coli* homologs, and these unique proteins have been suggested to interact with such *trans*-acting factors (17).

The chloroplast contains the NAD(P)H dehydrogenase (NDH) complex involved in photosystem I cyclic electron transport and chlororespiration (18). This complex consists of over 25 subunits, of which 11 are encoded in the chloroplast genome. The *ndhC* and *ndhK* genes encode the NdhC and NdhK subunits, and these genes

overlap partially in the chloroplast DNAs of tobacco and many other plants (19–22). In tobacco chloroplasts, the two genes are cotranscribed with the downstream *ndhJ* gene to produce a major 2-kb transcript, indicating that the overlapping *ndhC* and *ndhK* cistrons are translated from the tricistronic mRNA (23). In overlapping cistrons, translation of a downstream cistron generally depends on that of its upstream cistron, and hence translation of the downstream cistron is thought to be lower than that of the upstream cistron (*Discussion*).

An in vitro translation system that we have developed using isolated tobacco chloroplasts is highly active, enabling estimation of the relative rate of translation via measurement of the fluorescence intensity of fused green fluorescent protein and of incorporated fluorescently labeled amino acids (24). Using our in vitro system, we previously reported that the initiation codon of the tobacco chloroplast *ndhK* cistron is located 4 nucleotides (nt) upstream from the *ndhC* stop codon (Fig. 1A) and that translation of the downstream *ndhK* cistron depends absolutely on translational termination of the upstream *ndhC* cistron, namely, translational coupling (25). This result can be explained by the downstream *ndhK* cistron being translated exclusively by the ribosomes that have completed translation of the *ndhC* cistron. In such cases, the ribosomes are thought to move bidirectionally at the termination site (26), and translation of downstream cistrons is very low because the majority of ribosomes coming from upstream cistrons are released at the stop codon and only a minority of ribosomes participate in translation of downstream cistrons (27). The Coomassie blue-stained polyacrylamide gel pattern of a tobacco functional chloroplast NDH complex showed that the amount of NdhK looks comparable to those of several other subunits (28). Based on the subunit composition of cyanobacterial NDH complexes (29, 30), the amount of NdhK is likely to be similar to, or more than, that of NdhC. Our previous in vitro translation assay of the *ndhC/K* mRNA showed that the *ndhK* cistron is translated as efficiently, or slightly more efficiently, than the preceding *ndhC* cistron and that removal of the 5' untranslated region (5'UTR) still supports substantial translation of the *ndhK* cistron (25). Therefore, in addition to translational coupling from the *ndhC* 5'UTR (pathway 1), the downstream *ndhK* cistron is likely translated by an additional mechanism (pathway 2), also in a termination-dependent manner (Fig. 1A).

Here, we report that an internal in-frame AUG codon in the middle of the upstream *ndhC* cistron is the entry site for free ribosomes in pathway 2. A translation product from the internal

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Deletion to around the middle of the *ndhC* cistron (from $\Delta 7$ to $\Delta 153$) still supported *ndhK* translation. The observed fluctuation in translation efficiencies could be due to differences in secondary structures of the test mRNAs. We previously reported that the *ndhC* stop codon UAG is essential for *ndhK* translation (25). Stop codons UAA or UGA also act as UAG (Fig. S1). Change of this UAG stop codon to UGG abolished translation of all test mRNAs (Fig. 1C, Right, mT1), indicating that translation from these deleted mRNAs is also termination-dependent. This mutation resulted in shifting the *ndhC* stop codon located 22 codons farther downstream and arrested translational coupling. Further deletions ($\Delta 202$ and $\Delta 245$) abolished *ndhK* translation. As mRNA $\Delta 153$ supported high translation but mRNA $\Delta 202$ lost translation activity, we deleted stepwise (7 nt each) from +153 to +202 and found that *ndhK* translation decreased with each deletion (Fig. 1D). These results indicate that pathway 2 requires the sequence after +153 (~200 nt) of the *ndhC* cistron (360 nt).

We then constructed a series of internal deletion mRNAs (each a 39-nt or a 13-codon deletion, which causes no frameshift) (Fig. 2A) from mRNA $\Delta 153$, as this mRNA exhibited high translation activity, and assayed *ndhK* translation. As shown in Fig. 2B, deletions including the sequence between +157 and +195 inhibited *ndhK* translation (lanes ID1 to 4). Translation was also impaired by deletions including another sequence between +313 and +351 (lanes ID5–ID8) immediately upstream of the *ndhK* start codon. These results indicate that the two regions (named element 1 and element 2) are essential for *ndhK* translation. The region between these two elements was apparently dispensable for *ndhK* translation (lanes ID9–ID11).

Fig. 2. Effect of internal deletions on *ndhK* translation by pathway 2. (A) Schematic of mRNA $\Delta 153$ (Fig. 1B). Test mRNAs with internal deletions (indicated as blanks, 39 nt each) are shown below. (B) Gel patterns of translation products as in Fig. 1C. Elements 1 and 2 are essential for *ndhK* translation by pathway 2. The determined efficiencies of *ndhK* translation are shown as bar graphs below ($\Delta 153$ value defined as 100%, $n = 3$, $\pm$ SEM).

Fig. 3. Effects of mutations of internal AUG codons on *ndhK* translation by pathway 2. (A) Schematics of the wild type (wt) and $\Delta 153$ mRNAs (Figs. 1B and 2A). mRNA $\Delta 153$ was used to eliminate pathway 1 translation. The AUG at +1 represents the *ndhC* start codon. The AUGs at +151, +190, and +250 are internal in-frame AUGs within the *ndhC* cistron. Changes of AUGs to "uac" are shown below the $\Delta 153$. (B) Gel patterns of translation products from mRNAs with mutated AUGs as in Fig. 1C. (Lower) The determined efficiencies of *ndhK* translation are shown as bar graphs ($\Delta 153$ value defined as 100%, $n = 3$, $\pm$ SEM).

One of Three Internal AUG Codons Is the Ribosome Entry Site. There are three in-frame internal AUG codons within the *ndhC* cistron (Fig. 3A). These AUGs are candidates for the entry site of free ribosomes. To eliminate translation from the *ndhC* 5'UTR, mRNA $\Delta 153$ was used for the following assay. The first internal AUG (AUG₁₅₁) is located just upstream of mRNA $\Delta 153$; hence, this AUG can be excluded as a candidate. The second AUG (AUG₁₉₀) and the third AUG (AUG₂₅₀) were each changed to "uac" (Fig. 3A) and translated in vitro. As shown in Fig. 3B, mutation of AUG₁₉₀ nearly abolished *ndhK* translation (lane m1), whereas mutation of AUG₂₅₀ showed no significant effect (lane m2). Inhibition was also observed by mutation of both AUGs simultaneously (lane m1/2). These results suggest strongly that AUG₁₉₀ functions as the entry site of free ribosomes for pathway 2.

Effect of Premature Stop Codons on *ndhK* Translation. To confirm the above result, we incorporated different mutations in the full-size *ndhC/K* mRNA (WT). We created premature stop codons in the *ndhC* cistron located either downstream or upstream from AUG₁₉₀ (Fig. 4A). Translational coupling requires a very short distance between stop and start codons, up to several nucleotides (27). When the distance increases, for example, by insertion of a premature stop codon in the upstream *ndhC* cistron, it abolishes translation of the downstream cistron (25). As shown in Fig. 4B, *ndhK* translation was hardly detectable when premature stop codons were introduced downstream (lanes mT2–mT4), whereas premature stop codons upstream caused partial inhibition of translation (lanes mT5 and mT6). These results indicate that both ribosomes from the 5'UTR and from AUG₁₉₀ are released at premature stop codons introduced after AUG₁₉₀ (mT2–mT4), leaving few ribosomes available for *ndhK* translation. On the other hand, premature stop codons before AUG₁₉₀ (mT5 and mT6) blocked only the ribosomes that migrated from the 5'UTR but not those that entered at AUG₁₉₀. These data support the idea that pathway 2 uses the ribosomes that entered at AUG₁₉₀.

Fig. 4. Effects of premature termination on *ndhK* translation by pathway 2. (A) Schematic of mRNA wt (Fig. 1B). AUGs are as in Fig. 3A. As a control, mT1 has a mutated Ugg from the authentic *ndhC* stop codon UAG, which causes shifting of its stop codon (UAG) 22 codons farther downstream and, hence, no *ndhK* translation (Fig. 1C). mT2–mT6 possess mutations of internal codons to stop codons, with the first position indicated above (+118 to +265). Horizontal lines with arrowheads indicate translation proceeding up to stop codons. (B) Gel patterns of translation products from mRNAs with premature stop codons as in Fig. 1C. (Lower) The determined efficiencies of *ndhK* translation are shown as bar graphs [wild-type (wt) mRNA value defined as 100%, $n = 3$, $\pm$ SEM].

Detection of Translation Products from the Internal AUG. The above result suggests that a ribosome that entered at AUG₁₉₀ starts to translate the remaining *ndhC*-coding region that comprises 57 codons of the entire *ndhC* sequence of 120 codons. To avoid translation from the *ndhC* 5'UTR, we used mRNA Δ 153 (Fig. 5A). Translation was performed in the presence of fluorescently labeled lysyl-tRNA, as the 3' half of the *ndhC* cistron includes one lysine codon. The translation products were resolved by denaturing PAGE, and the fluorescence from incorporated lysine residues was detected. As shown in Fig. 5B (Left), a band of the expected size (6.5 kDa, corresponding to 57 codons) was observed (Fig. 5B, lane Δ 153, C-NdhC, arrowheads). The band was faint, probably due to its small size (see magnified images below in Fig. 5B). To enhance translation of the sequence, we replaced the upstream sequence (+153 to +189) from AUG₁₉₀ with the 5'UTR (68 nt) of phage T7 *gene 10* mRNA, a highly active 5'UTR. This mRNA produced a large amount of a fluorescently labeled product of the same size, so that a clear band was detected even after loading one-fifth of the standard amount (Fig. 5B, lane T7). We designated this C-terminal half of the NdhC product as "C-NdhC." The gel pattern also showed NdhK bands of 28 kDa (Fig. 5B, arrowheads). Based on fluorescent intensity and the number of lysine residues (1 in C-NdhC and 20 in NdhK), the amount of C-NdhC was estimated to be several-fold more than that of NdhK.

We then used fluorescently labeled formylmethionyl-tRNA^{fMet} (fMet-tRNA) for translation of mRNA Δ 153. A translation product was clearly observed at the corresponding position (Fig. 5B, Right, Δ 153 and T7). This assay indicates that pathway 2 starts with fMet-tRNA. As fMet residues are incorporated once, we estimated the approximate ratio of C-NdhC and NdhK to be 2:1 based on their fluorescent intensities. Considering these observations together, we

demonstrated that free ribosomes enter at AUG₁₉₀, translate the 3' half of the *ndhC* cistron started by incorporation of fMet-tRNA, and reach the *ndhK* start codon and that then some of the ribosomes resume *ndhK* translation (pathway 2). As a result, pathway 2 produces a 6.5-kDa peptide, C-NdhC.

Discussion

In tobacco chloroplast *ndhC/K* genes, the *ndhK* start codon is located 4 nt upstream from the *ndhC* stop codon, and the expression of these two genes is translationally coupled. In addition, the *ndhK* cistron is translated by an additional pathway (Fig. 1A) to produce the necessary amount of NdhK. Here, we demonstrated that free ribosomes enter, with fMet-tRNA, at the second AUG codon (AUG₁₉₀) of the three internal in-frame AUGs within the *ndhC* cistron. We detected a translation product from AUG₁₉₀, C-NdhC.

One possibility for the role of C-NdhC is as a functional isoform of NdhC and a component of the chloroplast NDH complex. Based on a study on the bacterial complex (33), NdhC is thought to directly interact with NdhK via the long loop between two of its transmembrane domains. C-NdhC lacks this loop and

Fig. 5. Detection of translation products from AUG₁₉₀. (A) Schematics of test mRNAs. mRNA Δ 153 is in Fig. 2A. mRNA T7 has the T7 *gene 10* 5'UTR (68 nt) in front of AUG₁₉₀. (B) Gel patterns of translation products separated by denaturing PAGE. Translation reactions included fluorescently labeled lysyl-tRNA (left lanes) or fMet-tRNA (right lanes). Size markers were Coomassie-stained polypeptide standards (indicated at left) and FITC marker (center lane). (Lower) Magnified patterns around the 6.5-kDa regions.

hence could not be a functional isoform. Attempts to detect C-NdhC in chloroplasts were unsuccessful. Another possibility is that C-NdhC is a by-product and hence turns over in chloroplasts. We estimated that over 2/3 of NdhK is synthesized via pathway 2 (Fig. 4B, mT5 and mT6). Because a substantial portion of the ribosomes engaged in 3'-*ndhC* translation would presumably be released at the *ndhC* stop codon, C-NdhC should be synthesized at levels several-fold higher than NdhK (Fig. 5B). No such isoform has so far been reported in NDH complexes in chloroplasts (18) or in cyanobacteria (31). In addition, the *E. coli* complex comprises 14 subunits, and these subunits are sufficient to perform all bioenergetic functions (34). We surveyed several chloroplast proteomics databases (35) but have not yet found any related peptides. In vitro assays with a proteinase inhibitor probably allowed us to detect C-NdhC. Hence, C-NdhC is likely to be a necessary by-product to carry ribosomes entering at AUG₁₉₀ toward the downstream *ndhK* cistron.

Alternative or internal initiation occurs in a limited number of mRNAs. In eukaryotes, some exceptional mRNAs bypass the scanning mechanism from 5' cap structures and use internal ribosome entry sites (IRESs) to recruit ribosomes (36). Unlike our case, these IRESs are located in 5'UTRs or in intergenic regions and are several hundred nucleotides long and highly structured. Alternative AUG start codons are rarely found within a single mRNA that produces functional protein isoforms with different N-terminal lengths (37). The use of alternative start sites is probably due to leaky ribosome scanning, namely, ribosomes from a 5' cap bypass the first AUG and start translation from the next AUG. In prokaryotes, intracistronic secondary initiation sites have been reported in only a few cases (38). For example, the *E. coli* gene coding for a stationary-specific σ -factor produces the σ -factor of 330 amino acids (aa) and the N-terminal truncated σ -factor of 277 aa from the secondary initiation site; both are functional (39). Therefore, we propose a model in which the internal initiation site AUG₁₉₀ is not designed for synthesizing a functional isoform but instead for delivering additional ribosomes to the *ndhK* cistron to produce NdhK in the amount required for the assembly of the NDH complex. This pathway is a unique type of translation to produce protein in the needed amount with the cost of peptide synthesis.

Within the *ndhC* cistron, two regions (elements 1 and 2) are required for pathway 2 (Fig. 2). Element 1 is located in the middle of the cistron and includes AUG₁₉₀ at its 3' end. The 30-nt sequence upstream from AUG₁₉₀ is AU-rich (7 adenosines and 13 uridines) and hence is probably unstructured (its sequence is shown in Fig. S2). The 20-nt AU-rich region just upstream of the start codon is essential for *atpB* translation, and a 50-kDa protein (p50) that could bind to this region was detected in tobacco chloroplasts (40). Therefore, translation initiation from AUG₁₉₀ is likely to be promoted by a positive *trans*-acting factor(s).

Element 2 is located upstream of the *ndhK* start codon and is probably responsible for termination-dependent reinitiation (its sequence is shown in Fig. S3). An SD-like sequence, GGGGG, is present 8 nt upstream from the *ndhK* start codon, which is situated in the proper position (12). GGGGG is found in tomato, potato, and soybean, and GGAG is found in many other flowering plants (CpBase: <http://chloroplast.ocean.washington.edu>). Mutation of this motif to GaaaG abolished *ndhK* translation, whereas mutation to GGAAG, a typical SD sequence in *E. coli*,

led to an over twofold increase in *ndhK* translation (Fig. S3), indicating that the SD-like sequence is required for the translational reinitiation. SD sequences are known to be required for translational coupling in *E. coli* (41). The overlapping region probably forms secondary structures to which free ribosomes are inaccessible. We hypothesize that ribosomes that come from AUG₁ and from AUG₁₉₀ open the structure so that only the nearby ribosomes—those that have just moved away from the *ndhC* stop codon—can initiate translation of the *ndhK* cistron.

Among the three internal AUGs in *ndhC* cistrons, only AUG₁₉₀ is highly conserved from flowering plants to cyanobacteria (Fig. S4). Many of these species possess overlapping *ndhC/K* genes. Hence, pathway 2 might operate in these species. However, pathway 2 does not always participate in other overlapping genes of tobacco. We found that translation of overlapping tobacco *atpB/E* mRNAs is not coupled (42) whereas translation of overlapping tobacco *psbD/C* mRNAs is partially coupled (43). These downstream cistrons are fully or mostly independently translated. In addition, they have additional promoters within the upstream genes and produce monocistronic *atpE* and *psbC* mRNAs, which are actively translated in vitro (42, 44).

Materials and Methods

mRNA Synthesis. The plasmid used for synthesizing mRNA WT has been described (24, 25). 5'-Deletion constructs were prepared using PCR-amplified *ndhC/K* fragments from various positions to 18 nt after the *ndhK* AUG (Fig. 1B). Internal deletions were introduced using a KOD -Plus- Mutagenesis Kit (Toyobo). Mutation of internal ATG codons and introduction of premature stop codons were performed using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). The PCR-amplified fragment from the *ndhC* AUG₁₉₀ to the 3'UTR end was fused to the T7 5'UTR (Fig. 4, mRNA T7). Details are in Fig. S5, and primers are listed in Table S1. Constructs were cut with XhoI and purified using an illustra PCR DNA and gel-band purification kit (GE Healthcare). mRNAs were synthesized from linearized DNAs using an AmpliScribe T7-Flash transcription kit (Epicentre Biotechnologies) and purified using an SV Total RNA Isolation System (Promega).

Chloroplast Extracts and in Vitro Translation. Tobacco chloroplast S30 extracts were prepared as described (24). An RNase inhibitor (TaKaRa Bio) and the protease inhibitor PMSF were added during preparation. Translation was performed at 28 °C for 2 h in a 20- μ L reaction mixture including 4 pmol mRNA and 20 U/mL S30 fraction as described (24). Products were separated by 12.5% (wt/vol) native PAGE, and the fluorescence intensity of mGFP was quantified with a Typhoon 9400 imager using 488-nm actinic light and a 520BP40 filter (GE Healthcare). To detect C-NdhC, fluorescent Lys-tRNA (0.1 μ L FluoroTect Green_{Lys} in vitro translation labeling system; Promega) or fluorescent fMet-tRNA (0.1 μ L F-Detector tRNA solution; iNtron Biotechnology) was added. After incubation, products were precipitated with acetone and dissolved in a loading buffer [125 mM Tris-HCl, pH 6.8, 10% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, 2% (wt/vol) SDS, 0.02% (wt/vol) Bromophenol Blue]. Samples were denatured at 70 °C for 3 min to prevent mGFP autofluorescence and then were resolved by tricine-SDS/PAGE (45). A molecular mass marker fluorescently labeled with FITC (APRO Science) and polypeptide SDS/PAGE molecular weight standards (Bio-Rad) were used as standards. The fluorescence intensity was quantified as above. Gels were then fixed and stained with Bio-Safe Coomassie Stain (Bio-Rad) to visualize the polypeptide markers.

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