Termination codon-dependent translation of partially overlapping *ndhC-ndhK* transcripts in chloroplasts

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The chloroplast NAD(P)H dehydrogenase complex, a homologue of mitochondrial complex I, consists of >15 subunits, of which 11 are encoded by the chloroplast genome (*ndhA-K*). The *ndhC* and *ndhK* genes are partially overlapped and cotranscribed in many land plants. The downstream *ndhK* mRNA possesses 4 possible AUG initiation codons in many dicot plants. By using an efficient in vitro translation system from tobacco chloroplasts, we defined that the major initiation site of tobacco *ndhK* mRNAs is the third AUG that is located 4 nt upstream from the *ndhC* stop codon. Mutation of the *ndhC* stop codon (UAG) arrested translation of the *ndhK* cistron. Frameshift of the *ndhC* coding strand inhibited also translation of the distal cistron. The results indicated that *ndhK* translation depends on termination of the preceding cistron, namely translational coupling. Surprisingly, removal of the *ndhC* 5′-UTR and its AUG still supported substantial translation of the *ndhK* cistron. This translation was abolished again by removing the *ndhC* stop codon. Although translation of the downstream cistron of an overlapping mRNA is generally very low, we found that the *ndhC/K* mRNA produces NdhK and NdhC in similar amounts. Based on subunit compositions of the bacterial complex I, the stoichiometry of NdhK and NdhC is suggested to be 1:1 in chloroplasts. To meet this stoichiometry, the *ndhC/K* mRNA is translated not only by a translational coupling event but also by a termination codon-dependent pathway.

mRNA | cistron | initiation codon | translational coupling

Chloroplasts are photosynthetic organelles that contain their own genetic system. The chloroplast genome of higher plants contains ≈80 protein-coding genes (1). Many of these genes are transcribed as polycistronic pre-mRNAs by multiple RNA polymerases (2). These pre-mRNAs are generally processed into complex sets of overlapping transcripts including mono-, di-, and poly-cistronic mRNAs (3). Many nuclear-encoded proteins are involved in the mRNA processing as well as translation and mRNA stability (4–6). Translational control is the major step of chloroplast gene expression, and it is especially important for the stoichiometric production of individual subunits in photosynthetic complexes (7, 8). The existence of a NAD(P)H dehydrogenase (NDH) complex was first assumed based on chloroplast DNA sequences whose predicted amino acid sequences resemble those of human mitochondrial complex I subunits (9, 10). The chloroplast NDH complex consists of >15 subunits (11, 12). Eleven *ndh* genes (*A* to *K*) that encode subunits of the NDH complex are found in the chloroplast genome of most land plants except pines (13) and some parasitic plants (14). These genes are clustered and organized in 4 transcription units: *ndhC/K/J* in the large single-copy region, *ndhB* in the inverted repeat, and *ndhH/A/I/G/E/D* and *ndhF* in the small single-copy region (15). The *ndhC* and *ndhK* genes overlap in part in the chloroplast DNAs of many higher plants (www.ncbi.nlm.nih.gov/genomes/ORGANELLES/plastids_tax.html), but are separated by a spacer in some legumes (16). The *ndhC/K* overlapping genes are cotranscribed with the downstream *ndhJ* gene, and their major transcripts are long enough to include all 3 cistrons (15), suggesting that the overlapping *ndhC* and *ndhK* are translated by polycistronic mRNAs but not by monocistronic mRNAs.

The initiation process represents a crucial point for the synthesis of correct proteins. There are often multiple possible initiation codons in chloroplast mRNAs (17, 18). A striking case is the *ndhK* mRNA from tobacco and many other dicot plants, and the mRNA contains 4 possible AUG codons (15). Three in-frame AUG triplets are present in many monocot plants (19). There are several techniques to determine which is the real start codon. The N termini of proteins are generally determined, but this method requires protein isolation and is not always conclusive when nascent products are processed at the N terminus. The site-specific disruption of candidate codons by chloroplast transformation was successfully applied to study some of the *Chlamydomonas* chloroplast genes (20, 21). However, this method cannot be applied to genes essential with viability. In contrast, in vitro translation systems that support accurate translation initiation allow us to determine in principle the initiation site of any mRNAs. By using our chloroplast in vitro translation system, we found that translation of tobacco *ndhD* mRNAs starts only at the edited AUG from ACG but not at the upstream in-frame AUG and GUG (22). We recently improved the original in vitro system, and the refined system is highly active enough to measure the relative rate of translation by means of the fluorescence intensity of fused green fluorescent protein (23). We used the modified (m)GFP, in which 3 amino acids were replaced to enhance fluorescence (23), so that one can detect minor translation products that are undetectable by using ^{35}S-Met. This system has allowed us to analyze the effect of mRNA processing on translation (23), the translation efficiency of several synonymous codons (24), and the translation initiation site of *psbC* mRNAs (18).

Based on in vitro translation analyses, we here report that the major initiation site of the tobacco chloroplast *ndhK* cistron is the third AUG within the upstream *ndhC* cistron. We then show that the *ndhK* translation depends exclusively on translational termination codon (UAG) of the *ndhC* cistron. This result indicates that the ribosome translated from the *ndhC* cistron moves upstream after translation and reinitiates at the third AUG, translational coupling. Surprisingly, removal of the 5′-UTR and its following AUG from the *ndhC/K* mRNA still supported substantial translation of the *ndhK* cistron, which is also termination codon-dependent. Although translation of the downstream cistron of overlapping genes is generally very low, NdhK is produced as efficiently as NdhC from *ndhC/K* mRNAs. Therefore, in addition to translational coupling, the downstream *ndhK* cistron should be translated by an additional mechanism, also in a termination codon-dependent manner.

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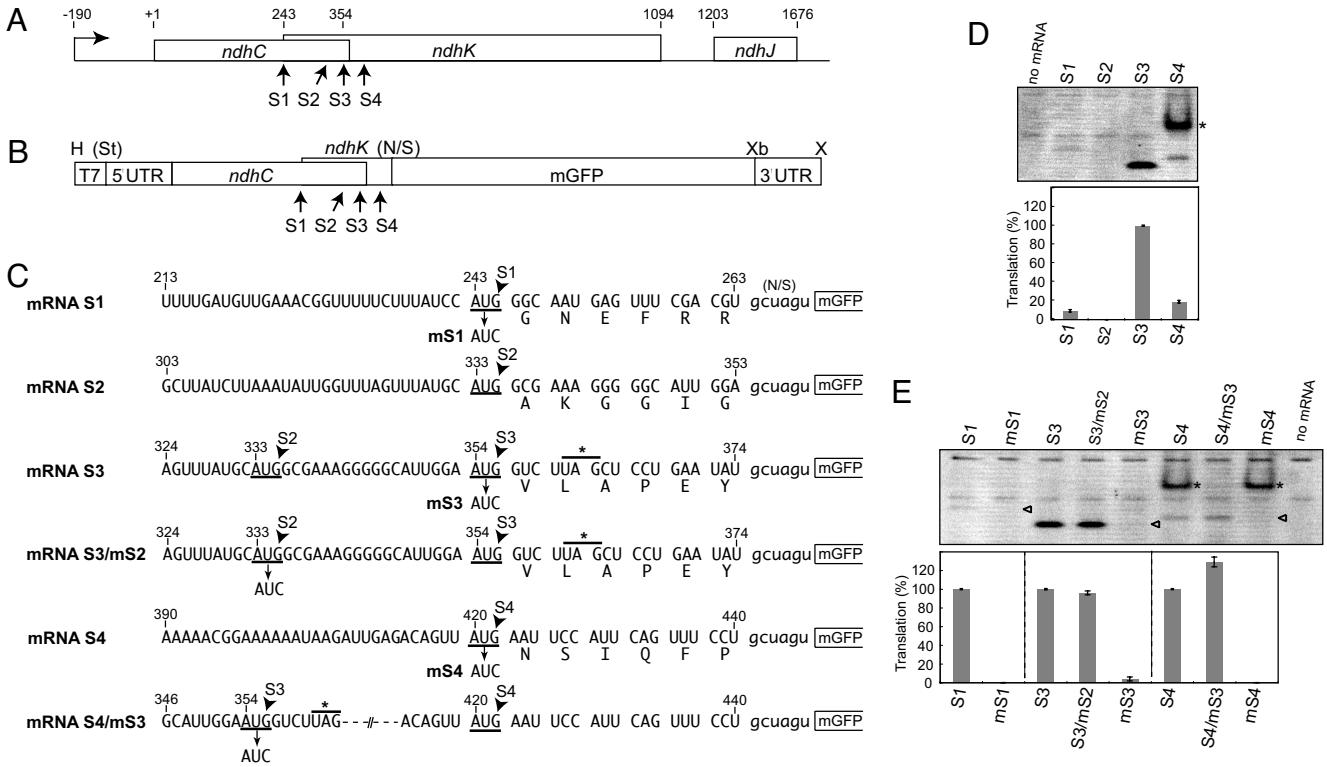

Fig. 1. Determination of the translation initiation site of *ndhK* mRNAs from the tobacco chloroplast *ndhC/K/J* cluster. (*A*) Schematic representation of the *ndhC/K/J*. Positions relative to the *ndhC* ATG start codon (A as +1) are shown above. Bent-arrow indicates the transcription initiation site. S1 to S4 indicate potential ATG start sites for *ndhK* translation. (*B*) Schematic representation of the plasmid construct, which contains the T7 promoter (19 bp, T7), the *ndhC* 5′UTR (190 bp), and its coding region (360 bp), a 5′ *ndhK* part (21–198 bp), the mGFP coding region (no ATG, 714 bp), and the *rps16* 3′UTR (199 bp). Restriction sites are H (HindIII), Xb (XbaI), and X (XhoI). St shows the StuI site removed during plasmid construction. N/S indicates the junction to mGFP (''gctagt'' derived from ligation of NheI- and SpeI- cut fragments). The construct in a pUC18 derivative was linearized with XhoI and transcribed with T7 RNA polymerase. (*C*) Parts of test mRNA sequences (mRNAs S1 to S4). Positions are as in *A*; mRNA S1 has S1, mRNA S2 has S1 and S2, mRNA S3 has S1 to S3, and mRNA S4 has S1 to S4. The middle AUG in sequences is 1 of the 4 AUGs (S1 to S4, underlined). The *ndhC* stop codon UAG is lined with an asterisk. Each AUG is replaced with AUC (mS1 to mS4). Predicted amino acid sequences are shown below. (*D*) Gel pattern of translation products (mGFP fluorescence). Translation efficiencies are shown relative to mRNA S3 as 100%. SEs were obtained from 3 independent assays. Asterisk indicates the translation from S3. (*E*) Gel pattern of products from mutated mRNAs mS1 mS3, S3/mS2, mS4 and S4/mS3. Translation efficiencies are relative to each of the mRNAs S1 to S4 as 100%. Triangles indicate expected positions of products.

Results

Translation Initiation Sites of Tobacco *ndhK* mRNAs. Fig. 1*A* shows the *ndhC/K/J* gene cluster from tobacco chloroplasts (25). In the *ndhK* coding frame, there are 4 ATG triplets (named S1 to S4), of which S1 to S3 are within the *ndhC* coding region. To determine which is the translation initiation codon, we performed in vitro translation assays. Transcription of the tobacco *ndhC/K/J* cluster starts at position −190 relative to the *ndhC* ATG (A as +1) initiation codon (34). We constructed chimeric mRNAs consisting of the full 5′ UTR, *ndhC* and *ndhK* coding parts, the mGFP coding region, and the 3′ UTR from tobacco chloroplast *rps16* mRNA as described (23). Because sequences downstream of the initiation codon were reported to be important for translation efficiency (26), we fused the mGFP coding region at 6 codons after each of the 4 AUGs, so that mGFP is in-frame with *ndhK*, but not with *ndhC* (mRNAs S1 to S4 in Fig. 1*C*). Translation in vitro of the chimeric mRNAs was performed by the standard procedure, and translation products were separated on native gels and monitored by mGFP fluorescence. Because the fluorescent signal was very low, we first reexamined reaction conditions to improve translation activity. The optimized reaction mixture included 40 mM KOAc, 8 mM MgOAc, 24 A_{280} units per mL S30, and 200 fmol/mL mRNA, which led to >2-fold increase in activity (data not shown). Next, we increased the reaction volume from 20 μL (standard mixtures) to 60 μL, and native gels 2-mm thick instead of 1-mm thick were used, resulting in ≈10-fold enhancement.

Fig. 1*D* shows a native gel pattern of mGFP products synthesized from the 4 test mRNAs. Translation of mRNA S3 was clearly observed, whereas that of mRNA S2 was not detected. A faint signal was observed from mRNA S1 and from mRNA S4 (the upper dense band was from the S3 site, indicated by an asterisk). The size of translation products was slightly different, probably due to differences in amino acid compositions of N-terminal extensions in mGFP products. Translation of mRNA S3 increased linearly for 1 h and gradually up to at least 3 h, whereas that of the other mRNA was too low to calibrate (data not shown). We then exchanged each of the 4 AUGs for AUC (mS1 to mS4, Fig. 1*C*). This alteration arrested translation completely from S1, S3, and S4 sites (Fig. 1*E*, indicated by arrowheads). Mutation at S2 did not affect translation from S3. Change of S3 in mRNA S4 slightly enhanced translation from S4. These results indicate that the major site of translation initiation of tobacco *ndhK* cistron is S3.

The *ndhC* Termination Codon Is Essential for Translation of the *ndhK* Cistron. The start codon S3 of tobacco *ndhK* cistron is located 4 nt upstream from the *ndhC* UAG termination codon (Fig. 2*A*, WT). To examine whether the *ndhC* termination codon affects translation of the *ndhK* cistron, we first altered the UAG to UGG, which resulted in no amino acid change in NdhK (UUA

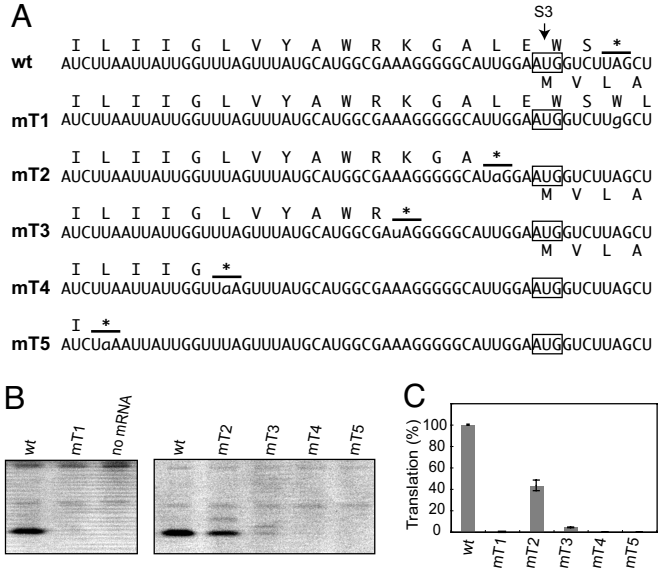

Fig. 2. Effect of removing the *ndhC* termination codon and introducing premature termination codons on *ndhK* translation. (*A*) Test mRNA sequences around S3. Predicted amino acid sequences from *ndhC* and *ndhK* are shown above and below, respectively. Lowercase nt represents altered nt. Asterisks with lines indicate the *ndhC* termination codons. The termination codon in mT1 is located 22 codons further downstream (data not shown). (*B*) Gel patterns of products. (*C*) Translation efficiencies are shown as in Fig. 1*D*.

to UUG, both coding for Leu) but in shifting the *ndhC* termination codon located 22 codons further downstream (Fig. 2*A*, mT1). Surprisingly, no translation of the *ndhK* cistron was observed with mRNA mT1 (Fig. 2*B*). This observation strongly suggests that the presence of the *ndhC* termination codon is crucial for translation of the downstream *ndhK* cistron, namely translational coupling. We then created premature termination codons, located upstream from S3. As shown in Fig. 2*B* (lines mT2–5), translation of the mRNA with a termination codon 2 nt upstream (mT2) decreased, whereas slight or no translation was observed from the mRNAs with termination codons further upstream (mT3–5). These results indicate that translation of the *ndhK* cistron requires the *ndhC* termination codon located in close vicinity of the start codon. Unexpectedly, *ndhC* translation was higher when the *ndhC* termination codon was situated after S3 (WT) than before S3 (mT2).

As the above mRNAs contain point mutations close to S3, these mutations may affect intrinsic translation of the *ndhK* cistron. Therefore, translation assays were performed by using frameshift mRNAs. We deleted 1 to 3 nt in the immediate downstream of S1, 107 nt upstream from S3, so that sequences surrounding S3 are identical with the wild-type mRNA (Fig. 3*A*). Deletion of 1 nt (Δ1) creates a premature stop codon (UGA) 4 nt after S1 in the *ndhC* cistron, 2 nt deletion (Δ2) leads *ndhC* and *ndhK* into 1 long reading frame, and 3 nt deletion (Δ3) results in 1 codon (GCA for Ala) shorter than the wild-type *ndhC* cistron. As shown in Fig. 3*B*, translation of the *ndhK* cistron was markedly reduced by mRNA Δ1, and that from mRNA Δ2 was hardly detected. However, mRNA Δ3 produced its *ndhK* product similar in amount to that from the original mRNA. The results obtained from mRNAs Δ2 and Δ3 further confirmed that translation of the *ndhK* cistron depends on translational termination of the *ndhC* cistron. However, translation from mRNA Δ1 was clearly observed (≈30% of WT) suggesting that there is an unknown mechanism to translate in part the *ndhK* cistron (see the next section).

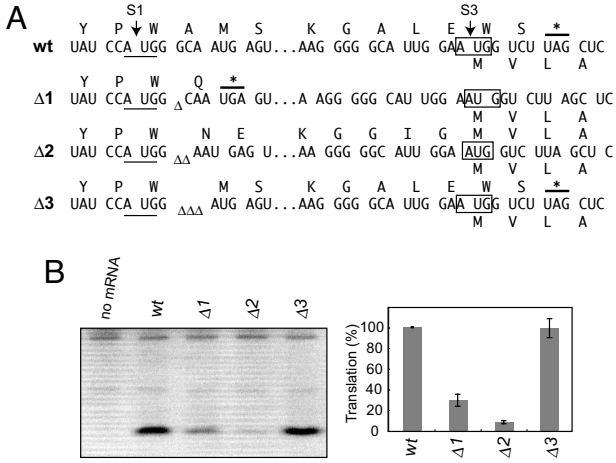

Fig. 3. Effect of frameshift of the *ndhC* coding strand on *ndhK* translation. (*A*) Test mRNA sequences around S1 and S3. Triplets are in-frame with the *ndhC* start codon. Asterisks with lines indicate termination codons. Triangles indicate deleted nt. Predicted amino acid sequences from *ndhC* and *ndhK* are shown above and below, respectively. (*B*) Gel pattern of products. Translation efficiencies are shown as in Fig. 1*D*.

Effect of 5′UTRs on Translation of the *ndhK* Cistron. As described, translation activity of the 5′UTR from the *ndhC/K* cluster was very low. A preliminary assay showed that translation from the *ndhC* 5′UTR-mGFP mRNA is <1/10th of that from the *psbA* 5′UTR counterpart (data not shown). To examine the effect of 5′UTRs on translation of the *ndhK* cistron, we replaced the authentic 5′UTR with that of *psbB* or *psbA* mRNA (Fig. 4*A*), because these 5′UTRs have high activity in vitro (23). The both chimeric mRNAs produced ≈4-fold more mGFPs from the *ndhK* cistron than the original *ndhC* mRNA (Fig. 4*B*). Also,

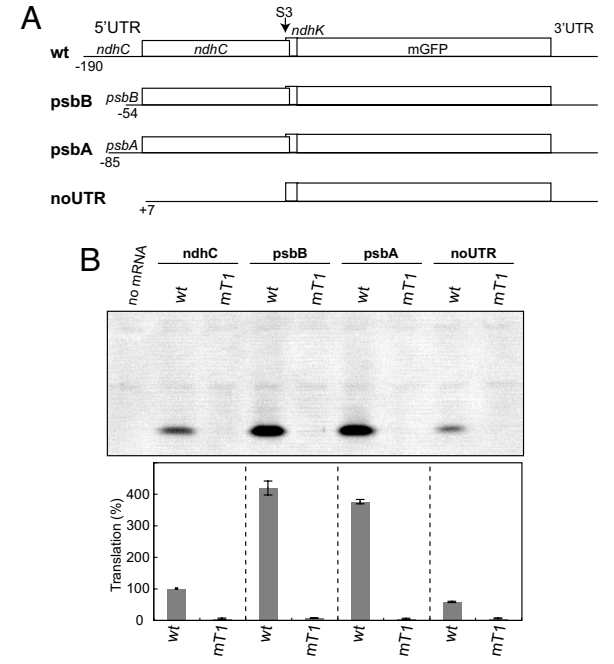

Fig. 4. Effect of replacing the *ndhC* 5′UTR on *ndhK* translation. (*A*) Schematic representation of test mRNAs. As a negative control mRNA, the 5′UTR and the following AUG and UUG codons were removed (noUTR). The *ndhC* termination codon of respective test mRNAs was removed as mT1 in Fig. 2*A*. (*B*) Gel pattern of products. Translation efficiencies are shown as in Fig. 1*D*.

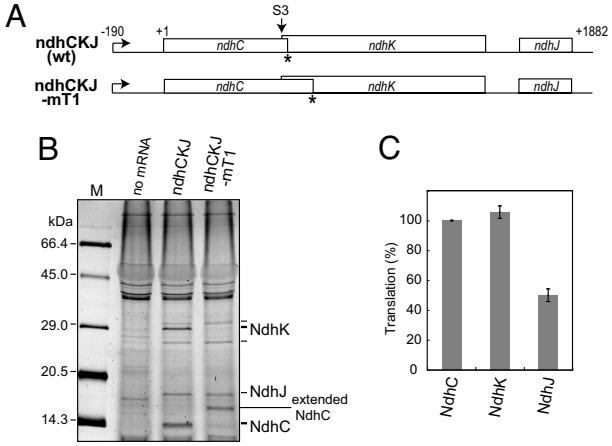

Fig. 5. Translation of full-length *ndhC/K/J* mRNAs. (*A*) Schematic representation of the tricistronic mRNAs. Asterisks indicate *ndhC* termination sites. (*B*) Gel pattern of products. Translation was performed with fluorescent fMet-labeled $tRNA^{fmet}$. Products were separated on denatured gels. The calculated kDa of NdhC, NdhK (S3), and NdhJ are 13.9, 28.0, and 18.6, respectively. Faint bands above and below NdhK are those from S1 (32.3 kDa) and S4 (25.5 kDa), respectively. A band between NdhJ and NdhC in lane mT1 is an extended NdhC (16.4 kDa). Size markers are fluorescent (FITC)-labeled molecular mass marker (APRO Life Science Institute). (*C*) Translation efficiencies are as in Fig. 1*D*. Only the major NdhK from S3 was used.

translation of the *ndhK* cistron again arrested when the *ndhC* termination codon was removed (UAG to UGG as before, lanes mT1s).

In the above assays, we used the test mRNA lacking the 5′UTR and the following AUG codon (noUTR) as a negative control, with which no translation was expected. Quite unexpectedly, substantial translation (≈58% of WT) was observed from the control mRNA (lane noUTR, WT). However, removing the *ndhC* termination codon abolished this translation (lane noUTR, mT1). These observations demonstrated the existence of an additional mechanism to translate the *ndhK* cistron. Translation from mRNA Δ1 (see Fig. 3*B*) was due probably to the additional pathway.

Efficient Translation of the Downstream *ndhK* Cistron. Then, translation efficiencies were compared between *ndhC* and *ndhK* cistrons. Because the tobacco *ndhC/K/J* cluster was cotranscribed to produce a major 2 kb mRNA (15), we prepared its tricistronic mRNA of 2,097 nt (Fig. 5*A*). We translated the tricistonic mRNA in the presence of fluorescent-labeled fMet-$tRNA^{fmet}$. Reaction mixtures included RNase and proteinase inhibitors from S30, and mRNA templates were stable during 2-h incubation and translation products were stable after 12-h incubation (23). The products from *ndhC/K/J* mRNAs were resolved by denatured gel and the fluorescence intensity from fMet (or Met) incorporated at the N terminus was detected and quantified. As shown in Fig. 5*B*, the major NdhK band of the expected size from S3 (28.0 kDa) was clearly observed together with faint upper and lower bands that correspond to translation products from S1 and S4, respectively, based on their sizes. The translation product from *ndhC* was also detected as a band of the expected size (13.9 kDa). Translation of the *ndhK* (S3) cistron occurred as efficiently as, or slightly higher than, that of the preceding *ndhC* cistron (Fig. 5*C*). By contrast, the last *ndhJ* cistron was translated less than the upstream 2 cistrons (≈50% of NdhC).

We then replaced the *ndhC* termination codon UAG with UGG as mT1 in Fig. 2*A*, which shifts its termination 20 codon further downstream (Fig. 5*A*). Removal of the authentic *ndhC* stop codon arrested translation from the *ndhK* S3 site, but hardly affected translation from the *ndhK* S1 and S4 sites and that from the *ndhC* cistron (to produce an extended NdhC of the expected size of 16.4 kDa). Using mRNA noUTR (see Fig. 4*A*) and fluorescent-fMet $tRNA^{fmet}$, no NdhC was detected, as expected, whereas translation of the *ndhK* -mGFP cistron was observed (data not shown). These results support that an additional pathway operates to translate the *ndhK* cistron.

Discussion

The chloroplast genome in higher plants is tightly packed, and protein-coding genes sometimes overlap each other to increase the number of proteins encoded by the size-limited genome. The *ndhC/K* cluster is such an example. Our studies showed that the major translation initiation site of *ndhK* mRNAs is the third AUG among 4 in-frame AUGs, which is situated 4 nt upstream from the *ndhC* stop codon. We then demonstrated that location of the *ndhC* termination codon has a crucial role in directing translation of the *ndhK* cistron, namely translational coupling or coupled translation reinitiation.

In eukaryotes, cellular mRNAs are generally monocistronic, and their translation initiation depends on the 5′cap structure (27). However, many virus transcripts are bicistronic or polycistronic. Translational initiation of a downstream cistron requires specialized mechanisms. Translation reinitiation, leaky scanning, ribosome jumping, and internal ribosome entry are possible mechanisms for translation of downstream cistrons (27, 28). For example, the M2 mRNA of human respiratory syncytial virus contains 2 ORFs that overlap partially. Translation of the second ORF initiates at 1 of the 3 AUGs located upstream of the termination codon for the first ORF, and translation of these AUGs requires the termination of the first ORF translation (29). However, unlike the case observed in *ndhK* translation, all of the 3 AUGs can function as initiators, although different in efficiency, and the distance between the AUG and the UGA in the virus mRNA is much longer (up to 26 nt) than that in the *ndhC/K* mRNAs (4 nt). Also, the frequency of such translation reinitiation was suggested to be very low. This indication would result in low levels of expression of the second ORF protein in viruses.

In prokaryotes, many genes are organized in operons and hence cotranscribed as polycistronic mRNAs. The start codon of each cistron is usually distinguished by its own Shine–Dalgarno sequence that pairs with the 3′ end of 16S rRNA, and translation can occur at multiple sites, in principle, independently throughout a polycistronic mRNA (27). However, translational coupling has been reported for some dicistronic mRNAs through mainly an overlapping termination and initiation codon (27, 28, 30). For example, expression of the upstream coat and downstream lysis genes in *Escherichia coli* RNA phage GA is translationally coupled by means of an overlapping termination and initiation codon, UAAUG (30). In this case, only 25–30% of the ribosome participated in translating the downstream cistron and the major portion of the ribosome was released at the UAAUG. Translational coupling was also reported for the maize chloroplast *atpB/E* gene cluster, which has overlapping translation initiation and termination codons (AUGA), introduced into heterologous systems *E. coli* and *Synecochocystis* sp. PCC6803 (31). The chloroplast ATP synthase includes 3 β subunits and 1 ε subunit, suggesting that translation efficiency of the downstream *atpE* cistron is lower than that of its upstream *atpB* cistron. Together, if the *ndhC/K* mRNA is translated only by means of translational coupling, translation efficiency of the *ndhK* cistron should be low.

We demonstrated that translation efficiency of the downstream *ndhK* cistron is similar to, or slightly higher than, that of the upstream *ndhC* cistron (see Fig. 5*C*). Current models of translational coupling cannot explain this finding. Although the stoichiometry of the chloroplast NDH complex is not elucidated yet due to very low amounts and its low functional stability,

coomassie blue-stained SDS/PAGE of a tobacco chloroplast NDH complex showed that NdhK is not a minor component but its amount looks comparable with several other subunits (NdhC could not be detected) (11). Based on the subunit compositions of cyanobacterial NDH-1 complexes (32), the stoichiometry of NdhK and NdhC in chloroplasts is likely to be 1:1. Because *ndhC* and *ndhK* are single copies in the chloroplast genome, the *ndhK* cistron should be translated at least as efficiently as the upstream cistron from the overlapping mRNA. If the ribosome participated in *ndhC* translation, moves 4 nt upstream after termination and resumes translation from the *ndhK* cistron, synthesis of NdhK should be no more than that of NdhC. It was suggested that the ribosome could undergo bidirectional diffusion after termination but reinitiation was very inefficient (33).

To meet the proper stoichiometry, translation of the *ndhK* cistron would need an additional pathway besides translational coupling. Surprisingly, removal of the *ndhC* 5′UTR and its AUG start codon still supported substantial translation of the *ndhK* cistron. This pathway is also termination codon-dependent. We favor the hypothesis that free ribosomes are loaded on somewhere within the *ndhC* coding region, migrate to the *ndhC* stop codon and start to translate the *ndhK* cistron. In conclusion, we propose that the downstream *ndhK* cistron is translated by 2 ways, a translational coupling event and a termination codon-dependent mechanism.

Materials and Methods

Plasmids. The plasmids (S1-S4) for synthesizing mRNAs S1-S4 were constructed as described (23) by using fragments from the transcription start site of *ndhCKJ* to 18 nt after each of the 4 *ndhK* AUGs. Site-directed mutants of the 4 *ndhK* AUGs and the *ndhC* stop codon were prepared by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). To construct deletion mutants, fragments were amplified from plasmid S3 by PCR by using 1 of the upstream primers including the NcoI site adjacent to 1–3 nt deletion and the downstream primer including the NdeI site, and then the sequence between the NcoI and NdeI sites in plasmid S3 was replaced with 1 of these fragments. Plasmids psbA and psbB were made by replacing the *ndhC* 5′UTR of plasmid S3 with the *psbA* or *psbB* 5′UTR. Plasmid noUTR was constructed as plasmid S3 except the sequence from the third codon (+7) of *ndhC* cistron was used as the upstream primer. Plasmid ndhCKJ was prepared by replacing the StuI/*Eco*RV region of pHK309 with the PCR-amplified fragments from the transcription start site to a predicted termination site of *ndhCKJ* (203 nt downstream from the *ndhJ* termination codon). All constructs were verified by sequence analysis.

Messenger RNA Templates and in Vitro Translation. Messenger RNA templates and tobacco chloroplast S30 extracts were prepared as described (23). Translation reaction was carried out at 28 °C for 2 h in a 60-μL reaction mixture with modifications as described in *Results*. Products were separated by 12.5% native-PAGE and the fluorescence intensity of mGFP was quantified as described (23). For fluorescent-fMet $tRNA^{fmet}$ labeling, 0.5-μL F-Detector tRNA solution (iNtRON Biotechnology) was added to 20-μL reaction mixture. After incubation, products were resolved by 15% denatured SDS/PAGE (23), and the fluorescent intensity was quantifies by a Typhoon9400 with 488-nm light and a 520BP40 filter (GE Healthcare).

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