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Structure of the nuclear encoded γ subunit of CF_0CF_1 of the diatom *Odontella sinensis* including its presequence

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Using a PCR-product as homologous probe for screening of a cDNA library of the diatom *Odontella sinensis* overlapping cDNA clones were obtained which showed homologies to *atpC*-genes of F_0F_1-ATPases from different sources. Comparison of the deduced amino acid sequence with the N-terminal sequence of the *Odontella* γ subunit obtained by protein sequencing, indicated that the complete 370 amino acid protein is processed to a mature protein of 315 amino acids. The 55 amino acids comprising the presequence consists of two segments, one resembling a signal sequence for cotranslational transport through ER membranes and one showing characteristics of a transit sequence for transport of proteins into chloroplasts of higher plants. This result is discussed with respect to the particular envelope structure of chromophytic plastids consisting of four membranes. The outer membrane contains ribosomes on its cytosolic surface. As in cyanobacterial γ subunits the regulatory sequence region, which is involved in thiol modulation of chloroplast ATPase of green algae and higher plants, is absent in the *Odontella* γ subunit.

F_0F_1-ATPase; γ Subunit; Protein import; Presequence; Diatom; *Odontella sinensis*

1. INTRODUCTION

F_0F_1-type ATPases couple transmembrane proton translocation with the formation of ATP from ADP and phosphate. In accordance with their significance as biological energy converters, the F_0F_1-ATPases of eubacteria including cyanobacteria, mitochondria and chloroplasts are very similar with respect to the catalytic mechanism, structure, subunit composition, subunit stoichiometry, and amino acid sequences. The ATPase consists of two subcomplexes, the membrane integral F_0 sector, which forms a proton channel and consists of three different subunits in eubacteria, four in chloroplasts and at least six in mitochondria, and the peripheral F_1 sector, which contains the catalytic centers. F_1 is composed of five different subunits [1].

During evolution of chloroplasts from endocytosis of cyanobacteria-like prokaryotic ancestors, genes were transferred from the plastid genome to the nucleus of the host cell [2]. With regard to the chloroplast ATPase of higher plants and green algae the genes coding for the CF_1 subunits γ, δ (*atpC, atpD*) and the CF_0 subunit II (*atpG*) have been shown to reside in the nucleus [3–5]. In the diatom *Odontella sinensis*, however, the genes *atpD* and *atpG* are still located on the plastid genome [6,7]. The gene *atpC* could not be detected in the *Odontella* plastid genome, suggesting that γ may be the only nuclear-encoded CF_0CF_1 subunit in this organism. Here we report on the isolation and structure of the nuclear *atpC* gene of *Odontella*, including the presequence of the deduced γ subunit. The presequence is subdivided into two consecutive segments. This bipartite structure may be connected with the mechanism of protein import through the complex chloroplast envelope of chromophytic algae. The deduced amino acid sequence of the mature protein shows homology to γ subunits from different sources. In green algae and higher plants a specific regulatory sequence is present in the γ subunit. This segment of nine amino acids comprises two cysteine residues which can form a disulfide bond. During so-called thiol modulation, the cysteine bridge is reversibly reduced via thioredoxin [8,9]. As in cyanobacterial γ this sequence motif is absent in the γ subunit of *Odontella*.

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Abbreviations: LHC, light harvesting complex; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, basepairs; SDS, sodium dodecylsulfate.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X70650.

2. MATERIALS AND METHODS

Odontella sinensis was grown and harvested as described in [10]. The cells were washed with sterile sodium chloride solution and frozen in liquid nitrogen. The frozen cells were crushed in a mortar using the guanidinium thiocyanate method as previously described [11]. After precipitation with isopropanol and redissolution of the nucleic acids the RNA was precipitated by 2 M LiCl (final concentration). For isolation of polyA$^+$-RNA the total RNA was separated on an oligo-(dT)-column. cDNA was prepared and cloned using the λZAP-cDNA

Synthesis kit and the Gigapack II Gold cloning kit (Stratagene, La Jolla). The synthesized cDNA was cloned unidirectionally into *Eco*RI/*Xho*I-digested λZAP vector. The recombinant DNAs were packaged in vitro and amplified in the hosts PLK-F′ and XL1-Blue. More than 10^6 independent clones were obtained. A gene probe of the *Odontella atpC* gene was prepared by the PCR technique. From two regions which are conserved in all known γ subunits from chloroplasts and cyanobacteria the following degenerated oligonucleotide primers were designed: 5′-CT(C/G)GT(G/C)GC(G/C/T)GCCGCTAA(A/G)GT(G/C)CGCCG-3′ (left primer) and 5′-CCGCA(C/A)A(G/A)ACC(G/C/A)CG(A/G)TCGCCGG-3′ (right primer). They comprised the amino acids Leu-25 to Arg-32 and Gly-84 to Cys-89, respectively (numbering according to the mature γ protein from spinach CF_1). PCR amplification of an aliquot of the cDNA library resulted in a 197 bp fragment of high homology to other *atpC* genes. This fragment was used to screen the cDNA library after random primed labeling with [α-^{32}P]dATP.

The isolated λZAP vectors were converted to pBluescript plasmids by in vivo excision using helper phage R408 (Stratagene, La Jolla), followed by creation of nested deletions to obtain overlapping clones. The deletions were performed by using exonuclease III/S1-nuclease enzymes from Boehringer (Mannheim). Klenow fill-in reactions prior to re-ligation enhanced the number of deleted clones. In this way sequencing could be carried out from both sides by employing the dideoxy chain termination method [12] using the T7-sequencing kit (Pharmacia, Freiburg). Regions forming secondary structures during sequencing reactions or during electrophoresis were additionally sequenced with the TAQuence kit from USB (Cleveland) using *Taq* polymerase both with and without 7-deaza-dGTP. For definite identification of the clones the derived amino acid sequence was compared with the N-terminal sequence of the γ subunit of CF_1 from *Odontella*. For DNA sequence analysis and translation we used the computer program NUCALN [13]. Multiple alignments were carried out using the program CLUSTAL [14]. Isolation of *Odontella* CF_1, separation of CF_1 subunits by SDS-PAGE and microsequencing was carried out as previously described [10].

3. RESULTS

CF_1 of *Odontella sinensis* was isolated and the subunits were separated by SDS gel electrophoresis [10]. Microsequencing of the γ polypeptide yielded an N-terminal amino acid sequence *GKANA-I*D*ITSVKNT. The asterisks mark amino acid positions that could not be determined with certainty.

Screening of the cDNA library of *Odontella sinensis* with the 197 bp PCR product resulted in four different positive clones, two of them (pOsγ821A and pOsγ711C) were completely sequenced. Three of the clones contained about 80% of the *atpC* gene, but lacked the 5′-end. The fourth clone (pOsγ821A), which was overlapping with the prior clones, contained the full N-terminal part of the gene including the presequence, but lacked the last 500 bp due to *Xho*I digestion during cDNA preparation. We found that the first three clones on the one hand and the fourth clone on the other hand differed in two base positions, suggesting errors of the reverse transcriptase reaction during cDNA preparation. Fortunately these two deviations were not relevant for the deduction of the amino acid sequence. From the overlapping clones we could conclude a length of the entire gene of 1,113 nucleotides corresponding to 370 amino acids. A 170 bp 3′-untranslated region downstream of the *atpC* gene is followed by a poly-A-tail. By comparison of the deduced amino acid sequence with the N-terminal protein sequence, the start of the structural *atpC* gene could be localized. Accordingly the presequence contains 165 nucleotides corresponding to 55 amino acids, and the structural gene consists of 945 nucleotides corresponding to 315 amino acids.

Although in the 5′-extended clone we could not find a termination codon upstream of the first ATG codon, we suppose this ATG to be the initiation codon because the sequence AAAAATGA found in *Odontella atpC* is characteristic of translation initiation sites in eukaryotes [15]. For example, in the *atpC* genes of spinach [16], *Chlamydomonas* [17] and *Arabidopsis* [18] the ATG initiation codons are preceded by 3, 4, and 3 A's, respectively, and followed by a purine. Moreover, alignment of the deduced amino acid presequence of *Odontella atpC* with those of precursor LHC proteins of the diatom *Phaeodactylum tricornutum* (FCP1, 2, and 3) [19] shows homology among the first 16 amino acids (Fig. 1). In accordance to the FCP-presequences this segment of the *Odontella* presequence exhibits features of signal sequences which serve to initiate transport of secretory proteins across the endoplasmatic reticulum membrane of eukaryotes [20]. Characteristically those signal se-

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                    -50                -30        -20        -10        +1
                     '        ⇩?        '          '          '         ↓
Odo atpC        MKFFCVAGLLASAAAF-QAQPAAFTTYSPAVGGATSNVFSESSSSPAHRNRRATIVMDGKAN---

                                                                  ↓
PhaeFCP1        MKFAVFAFLLASAAAFAPAWQSARTSVATNMAFE---
                                                                  ↓
PhaeFCP2        MKTAVIASLIAGAAAFAPAKNAARTSVATNMAFE---
                                                                  ↓
PhaeFCP3        MKFAVFASLLASAAAFAPAWQSARTSVATNMAFE---
                **.   * *.*.**** *   * *
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Fig. 1. Alignment of the presequences of the γ subunit of *Odontella* and the presequences of the FCP1, 2 and 3-proteins of *Phaeodactylum tricornutum* [37]. Cleavage sites as determined by protein sequencing of the mature protein are marked with filled arrows. The putative cleavage site of the signal sequence of the γ precursor determined according to the rules by von Heijne [22] is marked by an open arrow. Characteristic features of the putative transit sequence of γ are underlined, and written in bold letters, respectively. Numbering is according to the start amino acid of the mature γ protein. Asterisks mark amino acids that occur at the same position in all four sequences; dots indicate positions, where the amino acid of *atpC* is identical with at least one of the FCP amino acids.

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                1
cOdo            DGKANAIRDRITSVKNTKKITMAMKLVAAAKVRRAQDAVLATRPFSETLQSVFGGLIARM
cSpin            ANLRELRDRIGSVKNTQKITEAMKLVAAAKVRRAQEAVVNGRPFSETLVEVLYNMNEQL
cAra C1          ASLRELRDRIDSVKNTQKITEAMKLVAAAKVRRAQEAVVNGRPFSETLVEVLYNINEQL
cChla             GLKEVRDRIASVKNTQKITDAMKLVAAAKVRRAQEAVVNGRPFSENLVKVLYGVNQRV
Syn6803         MPNLKAIRDRIQSVKNTKKITEAMRLVAAAKVRRAQEQVLSTRPFADALAQVLYNLQNRL
Ana             MPNLKSIRDRIQSVKNTKKITEAMRLVAAARVRRAQEQVIATRPFADRLAQVLYGLQTRL
                       **** ***** *** ** ***** *****  *   ***   *  *

                61
cOdo            GGEALDLPLLTQREVSKVTLVVITGDRGLCGGYNSFMIKKAEARFNELKDQGVACDMVLI
cSpi            QTEDVDVPLTKIRTVKKVALMVVTGDRGLCGGFNNMLLKKAESRIAELKKLGVDYTIISI
cAra C1         QTDDVDVPLTKVRPVKKVALVVVTGDRGLCGGFNNFIIKKAEARIKELKGLGLEYTVISV
cChla           RQEDVDSPLCAVRPVKSVLLVVLTGDRGLCGGYNNFIIKKTEARYRELTAMGVKVNLVCV
Syn6803         SFAETELPLFEQREPKAVALLVVTGDRGLCGGYNVNAIKRAEQRAKELKNQGIAVKLVLV
Ana             RFEDVDLPLLKKREVKSVGLLVISGDRGLCGGYNTNVIRRAENRAKELKAEGLDYTFVIV
                       **   *    * * *  ******** *       * *  **   *

                121
cOdo            GKKGITYFQRRGYPIRKTF-ETGQNPDSKQALAISEELLNTYLSGESDAVELLYTKFISL
cSpi            GKKGNTYFIRRPEIPVDRYFDGTNLPTAKEAQAIADDVFSLFVSEEVDKVEMLYTKFVSL
cAra C1         GKKGNSYFLRRPYIPVDKYLEAGTLPTAKEAQAVADDVFSLFISEEVDKVELLYTKFVSL
cChla           GRKGAQYFARRKQYNIVKSFSLGAAPSTKEAQGIADEIFASFIAQESDKVELVFTKFISL
Syn6803         GSKAKQYFGRRDYDVAASYANLEQIPNASEAAQIADSLVALFVSETVDRVELIYTRFVSL
Ana             GRKAEQYFRRREQPIDASYTGLEQIPTADEANKIADELLSLFLSEKVDRIELVYTRFVSL
                * *   ** **              *    *                *  *   * * **

                181
cOdo            IASSPSARTLIPFSASE---------ITQQGDEVFQLTSSGGDFEVERTELEVAEPQDFP
cSpi            VKSDPVIHTLLPLSPKGEICDINGKCVDAAEDELFRLTTKEGKLTVERDMIKT-ETPAFS
cAra C1         VKSEPVIHTLLPLSPKGEICDINGTCVDAAEDEFFRLTTKEGKLTVERETFRT-PTADFS
cChla           INSNPTIQTLLPMTPMGELCDVDGKCVDAADDEIFKLTTKGGEFAVEREKTTI-ETEALD
Syn6803         ISSQPVVQTLFPLSPQG---------LEAPDDEIFRLITRGGKFQVEREKVEA-PVESFP
Ana             VSSRPVIQTLLPLDTQG---------LEAADDEIFRLTTRGGQFQVERQTVTS-QARPLP
                  * *   ** *                    ** * *    *   ***

                241
cOdo            NDMIFEQDPIQIINSILPLYLNGQILRTLQESVASELAARMQSMQSASDNAGDLAKRLST
cSpi            PILEFEQDPAQILDALLPLYLNSQILRALQESLASELAARMTAMSNATDNANELKKTLSI
cAra C1         PILQFEQDPVQILDALLPLYLNSQILRALQESLASELAARMSAMSSASDNASDLKKSLSM
cChla           PSLIFEQEPAQILDALLPLYMSSCLLRSLQEALASELAARMNAMNNASDNAKELKKGLTV
Syn6803         QDMIFEQDPVQILEALLPLYNTNQLLRALQESAASELAARMTAMSNASDNAGQLIGTLTL
Ana             RDSIFEQDPVQILDSLLPLYLSNQLLRALQESAASELAARMTAMSNASENAGELIKSLSL
                    *** * **    ****     ** ***  ********  *  *  **  *   *

                301
cOdo            EYNRARQAAVTQEILEIVSGASALE
cSpi            NYNRARQAKITGEILEIVAGANACV
cAra C1         VYNRKRQAKITGEILEIVAGANAQV
cChla           QYNKQRQAKITQELAEIVGGAAATSG
Syn6803         SYNKARQAAITQELLEVVAGANSL
Ana             SYNKARQAAITQELLEVVGGAEALT
                 **  ***  * *  * * **
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Fig. 2. Alignment of the protein sequences of the mature γ subunits of ATPases from chloroplasts of *Odontella*, spinach [6], *Arabidopsis* [18], and *Chlamydomonas* [17], and from γ subunits of the cyanobacteria *Synechocystis* 6803 [43] and *Anabaena* PCC 7120 [44]. Asterisks mark amino acids that occur at the same position in all organisms.

quences have one or two basic amino acids near their N-terminus, followed by a hydrophobic region and a largely variable region [21]. The presequence of *Odontella* γ subunit contains a Lys residue next to the N-terminus, followed by 14 mainly hydrophobic amino acids. Calculations of the secondary structure predict an α-helical shape of this strand. According to the rules elaborated by von Heijne [22], the most likely cleavage site of the signal sequence is between positions −41 and −40. The subsequent segment of the presequence, comprising 40 amino acids, has characteristic features of transit sequences found in nuclear-encoded chloroplast precursor proteins of higher plants [23]. In spite of large variability between transit sequences, some general features have been recognized [24,25] which can be detected in the second part of the *Odontella* presequence, too: a high content of hydroxylated amino acids (25%), a positive net charge and a block of basic amino acids (-HRNRR-) in position −10 to −6. This block together with the I- and V-residues at position −3 and −2 may form a β-strand structure which is thought to be an important element of the cleavage region [26].

Alignment of the amino acid sequence of the mature protein with γ sequences from other organisms (Fig. 2)

indicated homologies between 27 and 54% (Fig. 3). Highly conserved regions are at positions 5–48 and 82–97 (numbering according to the mature *Odontella* protein). These regions were considered to be essential in F_0F_1-assembly [27]. The cysteine residue at position 90 is conserved among γ sequences except that of the thermophilic bacterium PS3 [28] and of *Bacillus megaterium* [29]. In membrane-bound spinach CF_1 this cysteine was shown to be accessible to chemical modification only upon energization of the thylakoids by light ('light-site-cysteine') indicating conformational changes of γ during $\Delta\mu H^+$-dependent activation of CF_0CF_1 [30].

In γ subunits of CF_1 from higher plants and green algae, two more cysteyl residues are present in a 9 amino acids comprising segment. These cysteines, separated by 5 amino acids, form a disulfide bridge which can be reversibly reduced via thioredoxin. This so-called 'thiol modulation' changes the ΔpH profile for activation of the enzyme and is considered to play an important role in ATPase regulation of chloroplasts [31]. Most probably this regulatory sequence was acquired at a relatively late stage of evolution of photosynthesis as it is absent in the cyanobacterial γ subunits investigated so far [32,33]. Remarkably, this segment is missing in the γ subunit of *Odontella*, too.

4. DISCUSSION

The gene *atpC* is the only gene of the chloroplast ATPase of the diatom *Odontella sinensis* that is located in the nucleus. In a previous report we demonstrated that *atpD* and *atpG*, which likewise are nuclear genes in chlorophyll *a/b*-containing eukaryotic plants, reside in the plastid genome in *Odontella* [6,7]. This distribution of ATPase genes may be generalized for all chromophytic algae, since *atpD* and *atpG*, but not *atpC*, were found in the plastid genome of the brown alga *Dictyota dichotoma* (Kuhsel, M.G., Pancic, P.G. and Kowallik, K.V., unpublished). Recently the same arrangement of plastid ATPase genes was reported for the red alga *Antithamnion* sp., too [34]. In red algae *atpC* was not detected in the plastid genome, but its localization within the nucleus has not been established yet. If we suppose a monophyletic origin of plastids, these results indicate that the transfer of *atpC* from the genome of the endosymbiont to the genome of the host most likely has occurred before the two main algal lineages branched off from a common ancestral eukaryotic cell, whereas the transfer of *atpD* and *atpG* to the nucleus took place later and in the chlorophyll *a/b*-lineage only. Likewise, the insertion of the 9 amino acid block containing the two regulatory cysteine residues into γ subunit, may have occurred at a later stage in the chlorophyll *a/b*-lineage. So far, this segment was found only in *Chlamydomonas reinhardtii* and in higher plants.

Rather interesting is the structure of the presequence which we found to precede the mature γ polypeptide. This presequence can be subdivided into two segments. The first region (−55 to −40) strongly resembles signal sequences characteristic for cotranslational transport through ER membranes, whereas the second segment (−41 to −1) shows characteristics of transit sequences found for stromal or thylakoid membrane-attached proteins that are imported into the plastids of higher plants [25]. The bipartite structure of the γ-presequence allows some conclusions about the import of the precursor protein into the plastids of *Odontella*. Chromophyte plastids are surrounded by four membranes. The cytosolic side of the outer membrane contains ribosomes [35]. It is suggestive to assume that the precursor-γ is synthesized on these ribosomes and cotranslationally transferred across the membrane by means of its signal sequence segment. An intuitive model of protein import into chromophytic plastids by Gibbs [36] already pro-

	1	2	3	4	5	6	7	8	9	10	11
1 cOdo	100	52	52	49	53	54	34	30	34	35	27
2 cSpi		100	80	60	55	56	32	39	34	34	27
3 cAra C1			100	61	54	56	31	34	33	34	25
4 cChla				100	53	52	37	35	40	36	30
5 Syn6803					100	73	36	35	36	35	30
6 Ana						100	35	37	36	39	33
7 Rsp.rub.							100	32	38	36	38
8 PS3								100	38	75	32
9 E.coli									100	40	28
10 Bac.meg.										100	32
11 Bov. mit.											100

Fig. 3. Sequence similarity matrix of γ subunits from different sources. In addition to the sequences listed in Fig. 2 the γ sequences of *Rhodosprillum rubrum* [45], PS3 [28], *E. coli* [46], *Bacillus megaterium* [29], and bovine mitochondria [47] are included. Similarity is given in percentage identical amino acids.

posed cotranslational transport through the outer membrane, followed by a vesicular transport through the next two membranes. The inner membrane, phylogenetically resembling the inner membrane of higher plant chloroplasts, was thought to be traversed in the same way as in those organisms. The bipartite structure of the *Odontella* γ presequence may support this hypothesis. The function of the FCP-presequences of *Phaeodactylum* as signal sequences has been demonstrated by cotranslational import of these proteins in a heterologous microsomal membrane system [37]. However, the apparent lack of a transit segment in the FCP-presequences of *Phaeodactylum* suggests that the import of these proteins across the inner membrane may be different from that of the γ subunit.

Similar bipartite presequences have also been reported to precede the porphobilinogen deaminase [38] and the small subunit of Rubisco [39] in *Euglena*. Although plastids of *Euglena* contain only three envelope membranes with no ribosomes on its outer one, it was suggested that protein import is a two-step process, since the first segment of the presequences can be cleaved by a signal peptidase [40].

The occurrence of four envelope membranes is regarded to indicate that chromophytes have evolved by secondary endocytosis, i.e. by incorporation of a eukaryotic endosymbiont into a non-photosynthetic eukaryotic host [41,42]. As the nucleus of the eukaryotic endosymbiont has vanished during further evolution, its relevant genetic information must have been transferred to the nucleus of the final host cell. Since the *atpC* gene obviously had left the plastid genome before chromophytes arose (see above), we may conclude that the *atpC* gene has experienced two subsequent transfers, (i) from the plastid genome to the nucleus during establishment of primary endocytosis, and (ii) from the nucleus of the endosymbiont to the nucleus of the final host cell. The transit segment of the γ presequence was probably acquired as consequence of the first gene transfer, whereas the signal segment may have been added as a result of the final creation of the chromophytic cell.

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