Characterization of Tbc2, a nucleus-encoded factor specifically required for translation of the chloroplast psbC mRNA in Chlamydomonas reinhardtii

Andrea H. Auchincloss,1 William Zerges,1 Karl Perron,1 Jacqueline Girard-Bascou,2 and Jean-David Rochaix1

1Department of Molecular Biology and Department of Plant Biology, University of Geneva, 1211 Geneva 4, Switzerland
2Institut de Biologie Physico-Chimique, F-75005 Paris, France

Genetic analysis has revealed that the three nucleus-encoded factors Tbc1, Tbc2, and Tbc3 are involved in the translation of the chloroplast psbC mRNA of the eukaryotic green alga Chlamydomonas reinhardtii. In this study we report the isolation and phenotypic characterization of two new tbc2 mutant alleles and their use for cloning and characterizing the Tbc2 gene by genomic complementation. TBC2 encodes a protein of 1,115 residues containing nine copies of a novel degenerate 38–40 amino acid repeat with a quasiconserved PPPEW motif near its COOH-terminal end. The middle part of the Tbc2 protein displays partial amino acid sequence identity with Crp1, a protein from Zea mays that is implicated in the processing and translation of the chloroplast petA and petD RNAs. The Tbc2 protein is enriched in chloroplast stromal subfractions and is associated with a 400-kD protein complex that appears to play a role in the translation of specifically the psbC mRNA.

Introduction

It is well established that the photosynthetic complexes of the thylakoid membrane have a dual genetic origin. Some subunits are encoded by chloroplast genes and translated on chloroplast ribosomes, whereas others are encoded by nuclear genes, translated as precursor proteins on cytosolic ribosomes, and imported into the chloroplast where they associate with their chloroplast-encoded partners, pigments, and cofactors to form functional complexes (Wollman et al., 1999). Thus, a coordinated expression of the nuclear and chloroplast genomes is essential for the proper biogenesis of the photosynthetic apparatus. Analysis of mutants of Chlamydomonas reinhardtii and higher plants deficient in photosynthetic activity has revealed that this coordination is complex and involves the functions encoded by a large number of nuclear loci (Goldschmidt-Clermont, 1998; Rochaix, 2001). Many of these are required for specific posttranscriptional steps of chloroplast gene expression such as RNA stability, RNA processing, splicing, and translation. Recently, several of these factors have been characterized at the molecular level (Barkan and Goldschmidt-Clermont, 2000). A common feature of some of these proteins is the presence of tandem arrays of degenerate 34 (TPR) or 35 (PPR) amino acid repeats. One class, the TPR repeats, is present in Nac2 and Mbb1, two nucleus-encoded proteins of C. reinhardtii that are specifically required for the stable accumulation of the psbD and psbB mRNAs, respectively (Boudreau et al., 2000; Vaistij et al., 2000). Both of these proteins are associated with high molecular weight RNA–protein complexes. The other class, PPR repeats, has been identified in Crp1, a protein of maize that is required for the processing and translation of the petA and petD mRNAs. PPR repeats appear to have a structure similar to the TPR repeats, consisting of two short α-helical regions (Fisk et al., 1999; Small and Peeters, 2000). Genes encoding proteins with the PPR motif are part of a large family in Arabidopsis thaliana (Small and Peeters, 2000). Clues to the biochemical functions of some nucleus-encoded factors have come from identification of motifs or regions of homology these proteins share with enzymes known to be involved in RNA metabolism and other processes. For example, the splicing factor Raa2 (formerly called Maa2) resembles pseudouridine synthase and is required for the transsplicing of the second psaA intron in the chloroplast of C. reinhardtii (Perron et al., 1999). The splicing factor Crs2 of maize, re-
required for the splicing of several plastid group II introns, is related to peptidyl-tRNA hydrolase enzymes (Jenkins and Barkan, 2001). Another splicing factor Raa3, which is involved in the splicing of the first psaA intron of C. reinhardtii, contains a short region of homology with pyridoxamine 5’ phosphate oxidase (Rivier et al., 2001). In contrast, other factors do not resemble any known protein in the database. These include the Chlamydomonas Ac115 protein which is implicated in the translation elongation of the psbD mRNA (Rattanachaikunsopon et al., 1999).

The control of expression of the chloroplast genes encoding the major core photosystem (PS)*II subunits D1, D2, P5, and P6 of C. reinhardtii has been studied intensively in recent years. Biochemical approaches have identified two nucleus-encoded proteins that are involved in the light activation of the translation of the psbA mRNA of C. reinhardtii (Danon and Mayfield, 1991): one is a 47-kD protein resembling polyA-binding proteins (Yohn et al., 1998), and the other is a protein disulfide isomerase that both appear to be under redox control (Kim and Mayfield, 1997; Fong et al., 2000; Trebitsh et al., 2000, 2001). Analysis of several nuclear mutants of C. reinhardtii deficient in PSII activity has revealed that each of these mutations identifies a nuclear locus that is required for the translation of the mRNA of one specific PSII subunit (Goldschmidt-Clermont, 1998). For example, translation of the psbC mRNA requires functions encoded by two nuclear loci, TBC1 and TBC2. The psbC mRNA has the largest 5’ untranslated region (UTR) of known chloroplast mRNAs in C. reinhardtii. It consists of 550 nucleotides, and acts as a target site for Tbc1 and Tbc2, strongly suggesting that these factors play a role in the initiation of translation (Zerges and Rochaix, 1994). A striking feature of the psbC 5’UTR is a large inverted repeat structure in its middle that is required for translation of the psbC mRNA. Mutations within this inverted repeat or deletion of the entire structure completely abrogate translation (Rochaix et al., 1989; Zerges and Rochaix, 1994; Zerges et al., 1997). A nuclear suppressor of these mutations has identified a third locus, TBC3, involved in the initiation of translation of psbC mRNA. This suppressor also reverses the translational defect caused by the tbc1, but not by the tbc2, mutation (Zerges et al., 1997). The two factors defined by TBC1 and TBC3 and the middle part of the psbC 5’UTR appear to interact functionally (Zerges et al., 1997). UV crosslinking studies with chloroplast extracts and the psbC 5’UTR have revealed an RNA binding activity in S100 fractions of a mutant strain carrying the tbc2-F64 mutation, but not in similar fractions prepared from wild-type strains (Zerges and Rochaix, 1994). To understand the exact role of these factors, and in particular their interactions with the psbC mRNA, it is important to examine their genes and the products that these genes encode. As a first step toward this goal, we have cloned the Tbc2 gene and characterized its product.

**Results**

**Isolation and characterization of two new TBC2 alleles**

The TBC1 and TBC2 loci encode factors that are specifically required for the translation of the psbC mRNA. As only single mutant alleles of TBC1 and TBC2 have been reported, a genetic screen for new mutant alleles of these loci was performed. Among a number of mutants affected in a variety of processes, two mutants affected specifically in psbC mRNA translation were obtained: G314 and B23. In vivo pulse labeling of thylakoid membrane proteins with [14C]acetate for 45 min revealed that both mutants fail to synthesize the P6

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*Abbreviations used in this paper: HA, hemagglutinin; PS, photosystem; UTR, untranslated region.*
polypeptide, which is encoded by psbC and is the homologue of CP43 in vascular plants (G314, Fig. 1 A; B23, unpublished data). These results were confirmed with a pulse-labeling time of 5 min using total cell proteins from F64 (Fig. 1 B) containing a mutation that is allelic to those of G314 and B23 (see below). Similar to the F34 and F64 mutants previously described (Rochaix et al., 1989), which carry the tbc1 and tbc2 mutations, respectively, G314 and B23 accumulate wild-type levels of the psbC mRNA, as determined by RNA blot analysis (unpublished data; Fig. 1 C). When crossed to a strain containing the wild-type TBC2 locus, G314 and B23 produced tetrads exhibiting a 2:2 segregation of PSII deficiency and wild-type photosynthesis, as determined by the analysis of fluorescence transients and tests for growth on minimal medium. More than 15 tetrads were examined for each cross, indicating that a single nuclear mutation in each strain is responsible for its PSII deficiency. However, we cannot exclude the occurrence of two tightly linked mutations (less than three map units apart).

To determine whether the new mutations abolish translation of the psbC mRNA or cause a rapid degradation of the P6 polypeptide, we asked whether either mutation affects the expression of the addA reporter gene driven from the psbC 5’ UTR in the chimeric gene psbC(WT)-addA (Zerges and Rochaix, 1994; Zerges et al., 1997). This chimeric gene was integrated into the chloroplast genome of the Fud50 (mt−) strain, giving rise to a photosynthetic strain that was crossed to G314 and B23. In each cross, six tetrads were examined. The chimeric gene was transmitted to all the progeny because the chloroplast genome is inherited uniparentally. Neither mutation affects the accumulation of the psbC(WT)-addA mRNA or the psbC mRNA, as demonstrated by RNA blot analysis of total RNA samples from a representative tetrad from each cross (Fig. 1 C). However, there is an enhanced accumulation of the psbC-addA transcript in the progeny bearing the tbc2-G314 allele (Fig. 1 C, lanes 3 and 4) and the tbc2-B23 allele (Fig. 1 C, lane 7). Because the promoter and 5’ UTRs are the same for the psbC and psbC-addA RNAs, these differences are due to the different coding sequences and 3’ UTRs. In contrast, the addA protein was not produced from the psbC 5’ UTR in any of the PSII deficient progeny; all were sensitive to spectinomycin (Fig. 1 D). The wild-type (photosynthetic) progeny expressed addA; all were resistant to the drug (Fig. 1 D).

The new mutations in G314 and B23 eliminate the expression of addA from the psbC 5’ UTR. As these effects occur in the absence of the P6 coding sequence and are mediated by the 5’ UTR, a sequence element that is involved in translational regulation of psbC (Zerges and Rochaix, 1994; Zerges et al., 1997), we conclude that both mutations abolish translation of the psbC mRNA. Therefore, G314 and B23 identify new mutations that specifically abolish translation of the psbC mRNA.

To determine whether these new TBC mutations are in either of the two known TBC loci, we asked whether they are allelic to tbc1-F34 or tbc2-F64. G314 and B23 were crossed to strains carrying tbc1-F34 or tbc2-F64, and the resulting zygotes were tested for photosynthetic activity by fluorescence transient analysis (Bennoun et al., 1980). As all of the parental strains are completely PSII deficient, the appearance of PSII activity in the zygotes would indicate complementation of the mutations being tested; i.e., that the respective mutations are nonallelic and, therefore, are probably in distinct genes. The zygotes obtained from the crosses of G314 or B23 to a strain carrying tbc1-F34 gave wild-type fluorescence transients, indicating that neither of the new mutations is allelic to tbc1-F34 (Table I). In addition, the recombination tests from the crosses of G314 and B23 to the strain carrying tbc2-F64 did not yield any photosynthetic progeny, indicating that the mutant alleles in these strains are linked (Table I). In contrast, crosses of G314 and B23 to the strain carrying tbc1-F34, which is not linked to TBC2, yielded many photosynthetic progeny (Table I). Therefore, the mutations in G314 and B23 are allelic and linked to tbc2-F64, and we have named them tbc2-G314 and tbc2-B23, respectively. The positive complementation between the three tbc2 alleles and tbc1-F34 indicate that all three tbc2 mutant alleles are recessive.

| tbc2-F64 | tbc2-G314 | tbc2-B23 | tbc1-F34 |
|---------|----------|----------|----------|
| tbc2-F64 | ≤10−6    | −        | −        | +        |
| tbc2-G314 | 0/62     | ≤4×10−6  | −        | +        |
| tbc2-B23 | 0/20     | 0/41     | ≤10−9    | +        |
| tbc1-F34 | 42/45    | 20/30    | 18/32    | ≤5×10−6  |

Results of the complementation test are shown above the diagonal. The young zygotes displayed either (PSII) (−) or wild-type phenotype (+). Results of the recombination tests are shown below the diagonal. The scores represent a1/a2, where a1 is the number of zygotes that germinated and gave rise to colonies on minimal medium, and a2 is the number of zygotes that germinated. Frequencies of spontaneous reversion of mutations are shown in diagonal (109 cells tested on minimal medium). The results of the same tests performed between the tbc2 mutants and the tbc1-F34 mutant strain are shown as controls.

Complementation and cloning of TBC2 and its cDNA

In order to clone the Tbc2 gene, pools of genomic cosmid clones from an indexed cosmids library (Zhang et al., 1994) were tested for their ability to complement the photosynthetic deficiency produced by tbc2-B23. One pool yielded five photoautotrophic colonies. From this pool we isolated a cosmid that complements all three mutant tbc2 alleles (see Materials and methods).

To subclone the rescuing cosmid DNA, restriction fragments obtained from single, double, and triple digests of the cosmid DNA were used to transform the three tbc2 mutant strains. A Bam HI fragment of ~9.5 kb was identified that was able to complement all three alleles. No subfragments of this Bam HI fragment were found to be able to complement the mutant phenotypes, suggesting that the DNA encodes a functional TBC2 gene with little extraneous sequence. This Bam HI fragment was cloned and partially sequenced. It was found to contain two Hind III sites and, upon digestion with Hind III, fragments of ~0.9, 3.8, and 4.8 kb were generated. The two larger fragments were used to screen 6×105 phage of a cDNA library. Initially, only one 4.1-kb cDNA was isolated, and it hybridized to both fragments. However, this cDNA was unable to rescue the mutants. It was cloned and sequenced in its entirety. Analysis of its coding capacity (Gene-mark 2.4) indicated a probable frame shift around residue 1200. Indeed, fusions between the 5’ genomic DNA (up to
Figure 2. Map of the TBC2 locus and its cDNA. (A) Region of the promoter region and 5' part of the Tbc2 gene. The first three introns are indicated in grey. HindIII sites (HII, at 273; 1,110; 1,574; and 2,596 bp), and the approximate location of the HindIII sites used to generate probes to clone the cDNA are shown (HdIII). The positions of probes I (1110–1574), II (2273–2767) on the genomic DNA are indicated. Dotted regions indicate unsequenced DNA (this section is not to scale). (B) The map of the longest Tbc2 cDNA (4,098 nucleotides without the polyA tail) is represented. The unique SacI (1771) and NotI (3108) sites as well as the start codon (ATG, 174–176) and stop codon (stop, 3519–3521) are also noted. The location of probes I (195–506), II (630–965), and III (892–1386) relative to the cDNA sequence are shown. The Δ indicates the region deleted in three from eight cDNAs examined (1107–1285). The PCR primers used to insert a HindIII site immediately upstream of the stop codon are indicated by arrows. The HA epitope was inserted into this HindIII site or at the SacI site. The locations of the first three introns are marked by wedges. The corresponding HindIII, HindIII, and SacI sites on the genomic and cDNA are shown by thin lines.

The SacI site and the cDNA downstream of the SacI site were able to complement the TBC2 mutations, indicating that the 3' half of the cDNA (downstream of the putative frameshift) encodes a functional protein (Fig. 2; unpublished data). Screening another 8 × 10^6 phage of the cDNA library yielded eight additional phage, two of which were able to complement. The 5' halves of the rescuing cDNAs were sequenced and found to contain an additional internal 179-bp DNA fragment relative to the first cDNA, also found in the genomic sequence, that restored the reading frame (nucleotides 1107–1285 of the cDNA). PCR analysis was performed on the other cDNAs unable to rescue the mutants. Three contain a similar deletion to that of the first cDNA, one is missing a different internal region, and the other two do not have any detectable rearrangements (unpublished data). It is not yet known whether the differences observed are due to alternative splicing or to cloning artifacts.

Molecular analysis of TBC2

To confirm that the cloned gene corresponds to TBC2, we examined restriction digestion patterns within the three alleles (Fig. 3). Genomic DNA was digested with HindIII, blotted, and probed with PCR-amplified probe III (Fig. 2). Size markers are indicated in kb on the left of the gel (Eurogentech Smart ladder). The 2-kb fragment in lane 7 is undetectable because of overexposure.

Figure 3. DNA blot analysis of the TBC2 locus in wild-type and the tbc2 mutants. 5 µg of the indicated genomic DNA was digested with HindIII, separated on a 0.7% agarose gel, transferred to Hybond-C extra, and probed with PCR-amplified probe III (Fig. 2). Size markers are indicated in kb on the left of the gel (Eurogentech Smart ladder). The 2-kb fragment in lane 7 is undetectable because of overexposure.

The 2-kb fragment in lane 7 is undetectable because of overexposure. Mutations from the wild-type Tbc2 gene, and the 1.9- and 1.2-kb fragments specific to tbc2-B23 (lane 7). Hybridization of the same blot with probe II (Fig. 2, a PstI fragment corresponding to cDNA nucleotides 630–960) detected the 1.2-kb fragment in tbc2-B23, and the 1.0-kb fragment in the other allelic mutants (unpublished data). Hybridization with probe I (a genomic HindIII fragment) corresponding to the 466-bp genomic fragment (Fig. 2) revealed no differences between the wild-type, tbc2-B23, and tbc2-G314. Thus, a DNA rearrangement has occurred near the HindIII site comprised within probe III. Taken together, these results strongly suggest that this DNA rearrangement in tbc2-B23 is the cause of the mutations in these alleles.

To test whether the TBC2 locus is linked to other nuclear loci involved in PSII biogenesis, the tbc2-F64 mutant was crossed with two PSII-deficient strains, ac-114 and ac-115, containing mutations that have been mapped on linkage groups III and I, respectively (Harris, 1989). Analysis of the progeny from 20 tetrads of the cross with ac-114, and 8 tetrads of the cross with ac-115, indicated that the TBC2 locus is not linked to these two loci (see Materials and methods).

Characterization of the predicted TBC2 amino acid sequence

The Tbc2 cDNA sequence predicts an ORF encoding a protein of 1,115 amino acids with a molecular mass of 114.8 kD (Fig. 4). The longest isolated cDNA has a 5' UTR of 174
bases, with an in-frame stop codon 60 nucleotides upstream of the presumed initiation codon. This ATG probably encodes the translation initiation codon because the next ATG codon is 369 nucleotides downstream and would yield a protein that is significantly smaller than the observed size (see below). In the NH₂ and COOH termini of the Tbc2 protein, there are stretches of the same amino acid repeated from 3 to 11 times (most often alanine, but also glutamine, threonine, and other residues; Fig. 4, A and B). The central section of the protein is free of these strings of repeated amino acids, and contains nine copies of a degenerate 38–40 amino acid repeat which is neither a TPR (Lamb et al., 1995) nor a PPR repeat (Small and Peeters, 2000) (Fig. 4 C). Five of the repeats are arranged in tandem, whereas the last two are also consecutive in the COOH-termiinal section of Tbc2. Whereas the NH₂-terminal part of the repeats is poorly conserved, sequence identity is more apparent in the COOH-terminal part, especially the five residue PPPEW motif with the first P and the W present in all repeats. This motif is not repeated in any protein in the SWISS-PROT database (Release 40.4, 23 November, 2001; Bairoch and Apweiler, 2000), nor is it a previously characterized motif. The region of Tbc2 corresponding to the 179-bp deletion in the cDNAs that were unable to rescue the \textit{tbc2} mutations is underlined in Fig. 4, A and B. These cDNAs would give rise to a polypeptide of 598 residues that lacks the PPPEW repeats and whose 227 COOH-terminal amino acids are read in a different phase relative to Tbc2. Whether such a protein exists and has any function is unknown.

BLAST searches did not indicate high sequence identity to any characterized proteins (March 2002). However, a Smith and Waterman–type search using GeneMatcher (http://www.ch.embnet.org/software/GMFDF_form.html) revealed several proteins with low sequence identity to Tbc2. The most interesting among these is the \textit{Zea mays} Crp1 protein (Fisk et al., 1999). This protein is also encoded by a nu-

Figure 4. Tbc2 protein. A. Schematic view of the Tbc2 protein. The nine 38–40 amino acid internal repeats and the region similar to the Crp1 protein of \textit{Zea mays} are indicated. The regions of Tbc2 containing the stretches of A, D, L, Q, S, and T residues are shaded. The region corresponding to \textit{Δ} in Fig. 2 B is marked with a box. The two sites used for HA epitope tagging are indicated. (B) Sequence of the Tbc2 protein. Stretches of A, D, L, P, Q, S, and T residues are highlighted. The region corresponding to \textit{Δ} in Fig. 2 B is underlined. (C) Alignment of the nine PPPEW repeats. Residues which appear at least four times amongst the nine repeats are highlighted and indicated in the consensus sequence (CONS). (D) Regions of partial sequence identity in Tbc2 and Crp1. The first seven PPPEW repeats are shown in rows and indicated by Arabic numerals, whereas the first seven PPR repeats of Crp1 are shown in separate rows and indicated by Roman numerals. Spacings were introduced to optimize the alignment with the first seven PPR repeats of Crp1. The last six residues of PPPEW repeats 2–6 are shown duplicated in two rows to facilitate the comparison of the PPPEW and PPR repeats. Residues of the repeats that conform to the consensus are shown in bold letters. Identical amino acids are marked with *, similar amino acids are indicated with +. The COOH-terminal consensus (consC) of the PPPEW repeats and the PPR consensus are shown in the lower part. Because of the differences in these two repeats, it is not possible to align them over their whole length simultaneously. These sequence data are available from Genbank/EMBL/DDBJ under accession no AJ427966.
clear gene and has been implicated in the processing and translation of the chloroplast petA and petD RNAs and contains PPR repeats (A. Barkan, personal communication; Small and Peeters, 2000). As can be seen in Fig. 4 A, the sequence similarity between Crp1 and Tbc2 extends over the central region of Tbc2 that contains the PPPEW repeats. Fig. 4 D shows that this region comprises the first seven PPPEW repeats of Tbc2 and the first seven PPR repeats of Crp1. In spite of this overlap, the PPR repeats of Crp1 are distinct from the PPPEW repeats of Tbc2, although they share three consensus residues, L, G, and P in the COOH-terminal part of the repeats (Fig. 4 D). However, the PPPEW motif is not present in the PPR repeats and the NH2-terminal parts of the two repeat families are unrelated. Secondary structure predictions indicate considerable amounts of α-helical structures in Tbc2 (PSIPRED, http://insulin.brunel.ac.uk/psipred/), but no evident folds. In addition, no known RNA-binding motifs can be detected.

To further characterize the Tbc2 protein, its gene was tagged with a triple hemagglutinin (HA) epitope in either the middle of the coding sequence, at codon 593, or immediately before the stop codon (Fig. 4 A). The constructs were used separately for transformation of a C. reinhardtii strain carrying the tbc2-F64 mutation by selecting for growth on minimal medium. Several independent colonies from each transformation were screened by immunoblotting for expression of the HA epitope–tagged Tbc2 protein. Although both constructs were able to rescue the mutant and expressed detectable HA-tagged protein of the same size, and are therefore functional, the signal from the construct with the epitope at the COOH terminus yielded a much stronger signal (unpublished data). It was used exclusively in the experiments that follow.

Characterization of the Tbc2 protein

The NH2 terminus of Tbc2 is enriched in basic and hydroxylated amino acids, but also contains a higher portion of acidic residues than expected for a typical chloroplast transit peptide. Analysis of this sequence with PSORT (http://psort.nibb.ac.jp/) or ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) predicts a mitochondrial rather than a plast transit peptides from chloroplast location. However, a particular feature of chloroplast transit peptides from the membranes before centrifugation (Zerges and Rochaix, 1998) did not alter the fractionation results obtained with Tbc2 (unpublished data). The protein is found in chloroplasts, and is detected mostly in the soluble fraction (Fig. 5, lane 9). In the experiment presented in Fig. 5, the chloroplast extracts were not treated with salt. However, a 0.5-M (NH4)2SO4 wash to remove loosely bound proteins from the membranes before centrifugation (Zerges and Rochaix, 1998) did not alter the fractionation results obtained with Tbc2 (unpublished data). As a control for the fractionation experiment, the distribution of a soluble chloroplast protein (ribulose-bis-phosphate carboxylase-oxygenase; Rubisco), D2, a PSII reaction center subunit in the thylakoid membrane, and thioredoxin-h, a cytosolic marker protein, were also determined (Fig. 5). Each of these proteins fractionated as expected, indicating that the chloroplasts used were not significantly contaminated with cytosolic proteins. Thus, despite the absence of an identifiable chloroplast transit peptide, the Tbc2 protein is targeted to the chloroplast. Similar results were obtained using cells complemented with Tbc2 HA-epitope tagged in the middle of the protein (in the Sac I site, Fig. 2 B; unpublished data). This substantiates the chloroplast localization of the protein.

We expected that Tbc2 might form a complex with other proteins and possibly also with RNA in order to exercise its function in psbC mRNA translation. To test this possibility, we fractionated total soluble cell extracts by size exclusion chromatography (Fig. 6). The column used fractionates proteins and complexes over the size range of 5–2,000 kD. In the presence of 10 mM MgCl2, 50 mM KCl and 1 mg/ml

![Figure 5. Accumulation and subcellular localization of Tbc2. Immunoblot analysis of Tbc2 in wild-type (lanes 1, 3, and 5) and tbc2-F64 rescued with the HA-tagged Tbc2 cDNA (lanes 2, 4, and 6–9); tot, total cell extract (lanes 1, 2); total insoluble fraction (lanes 3 and 4); total soluble fraction (lanes 5, 6); cp total, total chloroplast extract (lane 7), chloroplast insoluble fraction (lane 8), chloroplast soluble fraction (lane 9). After fractionation by SDS-PAGE and blotting, the immunoblots were reacted with antisera raised against HA, D2, SSU Rubisco, and thioredoxin-h (cytosolic protein). For details see Materials and methods.](image-url)
heparin, Tbc2 elutes as a complex with a peak at ~400 kD (fraction 19). Very little signal is detected in fraction 22, where a monomeric form of the protein would be expected to elute. Treatment of the whole cell lysate with RNase does not alter the migration of the complex on the column, indicating that the protein is probably not stably associated with RNA (α-HA, middle). As a control, the same filters were reprobed with an antibody against RB60, which is known to be part of an RNA–protein complex (Danon and Mayfield, 1991; Boudreau et al., 2000). In untreated samples the Tbc2:HA and RB60 proteins fractionate in a similar fashion. However, upon RNase treatment, the RB60 complex shifts to a lower molecular weight, indicating that Tbc2 and RB60 are not part of the same complex. The Tbc2 protein does not elute in the same fractions as Hsp70 and is thus not associated with this protein (unpublished data). When the whole-cell extract was prepared in the presence of 50 mM KCl, 10 mM EDTA and 1 mg/ml heparin, but without MgCl₂, the Tbc2 complex was observed to have a higher apparent molecular weight and was more spread out over the column. This may be indicative of some aggregation in the absence of divalent cations.

Because Tbc2 is involved in translation of petB mRNA we tested whether it is associated with polysomes. Polysomes were isolated from whole-cell extracts as rapidly as possible to prevent RNA degradation by centrifugation using two-step sucrose cushions. Immunoblot analysis of the supernatant and the polysome-containing pellet indicated that Tbc2:HA remains in the supernatant, and thus is not stably associated with polysomes (unpublished data). Also, if Tbc2 were associated with polysomes, one would expect a lower molecular mass without Mg²⁺, not a higher mass as was observed.

Discussion

Genetic analyses of mutants of *C. reinhardtii* deficient in photosynthetic activity have revealed several nuclear loci required for the expression of the chloroplast *psbC* gene. One nuclear locus is required for the stable accumulation of *psbC* mRNA (Sieburth et al., 1991) and at least three loci, *TBC1*, *TBC2*, and *TBC3* are involved in the translation of this mRNA, most likely at the level of initiation (Zerges and Rochaix, 1994; Zerges et al., 1997). This study represents a first step toward the identification and characterization of the transacting factors specified by these loci, in this case the *TBC2* locus. Because the *tbc2-F64* mutation undergoes phenotypic reversion at a frequency that is too high to select for complementation by pools of cosmids from the indexed library (see Materials and methods), we had to isolate more stable mutant alleles of the *TBC2* locus. One of these, *tbc2-B23*, was identified and used for the cloning of the *Tbc2* gene through genomic complementation with an indexed cosmid library.

Other loci involved in PSII biogenesis have been mapped previously. They include the *AC-114* locus on linkage group III and *AC-115* on linkage group I. Analysis of the progeny from crosses between the *tbc2-F64* mutant and the *ac-114* and *ac-115* mutants revealed that the *TBC2* locus is unlinked to these two loci. Thus, there is no evidence for the clustering of nuclear genes involved in posttranscriptional steps of the expression of plastid genes encoding PSII subunits.

Structural features of Tbc2

The *Tbc2* gene encodes a protein of 1,115 amino acids. A noticeable feature of this protein is that it contains nine degenerate repeats of 38–40 amino acids that extend from the middle to the COOH-terminal region. Although the Tbc2 protein is not obviously related to any protein in the databases, it is particularly interesting that the maize Crp1 protein is found among the proteins that displays low sequence identity with Tbc2. Crp1 is required for the processing and translation of the plastid *petA* and *petD* RNAs (Fisk et al., 1999). Crp1 contains PPR repeats (Small and Peeters, 2000) that are similar to TPR repeats, except that the repeat consists of 35 residues. The region of sequence similarity between Tbc2 and Crp1 includes the repeated motifs of these two proteins, although the PPR motifs are distinct from the internal repeats of Tbc2 aside from sharing three consensus residues in their COOH termini. The presence of TPR motifs has been found in a large set of proteins involved in many different activities such as cell cycle control, transcription, protein import into mitochondria, and chloroplast RNA metabolism (Blatch and Lasle, 1999). In particular, the Nac2 and Mbb1 proteins containing 9 to 10 TPR-like repeats are required for the stable accumulation of the chloroplast *psbD* and *psbB* mRNAs in *C. reinhardtii* (Boudreau et al., 2000; Vaistij et al., 2000). Changing one conserved residue of one of the Nac2 TPR repeats abolishes the activity of the protein, indicating that these repeats have an important functional role (Boudreau et al., 2000).

All of these proteins appear to belong to a large family of helical repeat proteins. The atomic structure of several representatives of these proteins has been determined. They in-
clude β catenin with its 12 Arm repeats of 42 amino acids (Huber et al., 1997), the A subunit of protein phosphatase 2A with its 15 HEAT repeats of 39 amino acids (Groves et al., 1999), Pumilio with its 8 Puf repeats of 36 amino acids (Edwards et al., 2001), and protein phosphatase 5 with its 3 TPR repeats of 34 amino acids (Das et al., 1998). These tandem helical repeats form an extended surface of the protein that is thought to be involved in protein–protein interactions. In the case of Pumilio, a translational regulator of the hunchback mRNA in Drosophila, this surface can also be used for recognizing RNA (Edwards et al., 2001). Although Tbc2 does not contain any known repeat, it is predicted to contain a considerable amount of α-helical structures and a novel repeated sequence. It is not yet known whether or not Tbc2 has RNA binding activity.

Further searches for sequence similarity revealed that Tbc2 shares a short stretch of 42 amino acids with selenophosphate synthase (52% sequence identity). Other unusual structural features of Tbc2 include the presence of long stretches of Ser, Ala, or Gln mostly in the NH2- and COOH-terminal regions that have also been noticed in other nucleus-encoded proteins from C. reinhardtii (Boudreau et al., 2000; Vaistij et al., 2000).

**Tbc2 is a chloroplast stromal protein**

The NH2-terminal region of Tbc2 does not resemble typical chloroplast transit peptides but is recognized as a mitochondriod presequence using PSORT or ChloroP subcellular localization programs. This finding is not necessarily incompatible with a chloroplast location, because the analysis of chloroplast transit peptides from C. reinhardtii has revealed that they share features with both mitochondrial and higher plant chloroplast presequences (Franzen et al., 1990). They contain both the potential amphiphilic α-helix of mitochondrial presequences and the amphiphilic β-strand of higher plant chloroplast transit peptides. It is also possible that the chloroplast targeting sequence is located elsewhere in the protein.

To determine the location of Tbc2, the protein was tagged with an HA epitope and was localized mainly in the stromal compartment of the chloroplast by cell fractionation and immunoblotting. It is interesting to note that among the nucleus-encoded factors known to be involved in chloroplast post-transcriptional events, some, like Tbc2, Crp1 (Fisk et al., 1999), Nac2 (Boudreau et al., 2000), Mbb1 (Vaistij et al., 2000), and Raa3 (Rivier et al., 2001), are found in the soluble chloroplast phase, whereas others, like RB47 and Raa2, are associated with a low-density membrane system (Zerges and Rochaix, 1998; Perron et al., 1999). RB60 appears to be partitioning both with the stroma (Boudreau et al., 2000) and thylakoids (Trebitsh et al., 2001). The functional significance of this different compartmentalization remains to be determined. It is possible that several of these factors interact only transiently with the chloroplast membrane during some of the steps leading to the integration of the newly synthesized polypeptides into the membranes. Although the HA-tagged Tbc2 protein is functional and rescues the tbc2 mutations, we cannot exclude that it may not behave exactly the same way as the authentic Tbc2 protein.

Tbc2 appears to be part of a high molecular weight complex of 400 kD. It could represent a homomeric complex, or Tbc2 could be associated with other factors. Possible candidates include Tbc1 and Tbc3, which are known to be involved in the initiation of translation of the psbC mRNA (Zerges et al., 1997). The Tbc2 complex does not appear to contain RNA based on the observation that its size is not altered by RNase treatment. In addition, as for Crp1, no stable association of Tbc2 with polysomes could be detected, suggesting that this factor interacts only transiently or indirectly with the translational machinery. As Tbc2 has a specific role in the translation of the psbC mRNA and its action is mediated through the psbC 5′UTR, it has to functionally interact with this region. Whether this interaction occurs directly or indirectly through other RNA-binding factors remains to be determined. Most factors involved in translation have been identified by biochemical approaches. This study, and others described here, reveal that genetic approaches can identify new translational regulators. The culmination of these approaches will be a comprehensive understanding of the molecular mechanisms underlying translation in vivo.

**Materials and methods**

**C. reinhardtii strains**

*C. reinhardtii* strains (wild-type 137c) were grown on TAP medium (Gorman and Levine, 1966), an enriched medium that allows for mixotrophic growth, under 50 μE/m²/s light unless otherwise indicated. Cells were grown on minimal medium (HSM; Harris, 1989) to select for photosynthetic growth. G314 and B23 were isolated from the wild-type strain 137C after treatment with 5-fluorodeoxyuridine as described (Bennoun et al., 1986), and a subsequent enrichment for photosynthetic mutants with metronidazole as described (Schmidt et al., 1977).

**Genetic analysis**

Mating, germination, and tetrad analysis were performed according to Harris (1989). Reversion and recombination tests were performed as described (Kuras et al., 1997), and complementation tests were done according to Goldschmidt-Clermont et al. (1990).

**DNA isolation**

Total Chlamydomonas DNA was prepared as described (Boudreau et al., 1997). Gels were transferred to Hybond N+ or Hybond C-extra (Amerham Pharmacia Biotech), and hybridized and washed as described (Church and Gilbert, 1984). PCRs were performed with a 5-ng/μl template and 0.5-μM primers in 50 mM Tris-Cl, pH 8.3, 1 mM MgCl₂, 250 μg/ml BSA, 200 μM dNTP, and 5% DMSO using Pfu polymerase (Stratagene).

**Pulse labeling of proteins**

Pulse labeling of cells with 35Cl]acetate was performed as described (Rochaix et al., 1989). At the end of the of the labeling period, 200 ml of culture was used for thylakoid membrane purification, and the extract was fractionated by electrophoresis on 7.5–15% SDS polyacrylamide gels (Chua and Bennour, 1975).

**Complementation and cloning of the Tbc2 gene and cDNA**

An indexed cosmid library (Zhang et al., 1994) of C. reinhardtii genomic DNA was used to transform tbc2-B23;cw15 essentially as described (Kinder, 1990). 2.5 μg of pooled DNA from each microtiter plate was used to transform 3 × 10⁶ cells in the presence of 100 μg/ml 20% PEG 8000 (in 5 mM Tris-Cl, pH 8.0) using sterile 0.4-mm glass beads (Thomas Scientific). Cells were plated on high-salt minimal medium and immediately placed under medium intensity light (60 μE/m²/s). Positive plates were generally detected after 14–18 d. Library plate number 64 yielded 0–2 colonies per plate after transformation. DNA was prepared from each column and row to identify the well containing the cosmid that complements the tbc2 mutation (F5). 1 μg of purified cosmid DNA yielded six colonies in tbc2-B2; cw15 (no DNA control yielded 0 colonies); 93 transformants in tbc2-F64; cw15 (no DNA control yielded 19 colonies); and 315 colonies in tbc2-G314;cw15 (no DNA control yielded two colonies per plate).

To identify which region of the cosmid rescued the tbc2 mutants, the cosmid DNA was digested with different restriction enzymes. The smallest
fragment that was able to complement was a 9.5-kb BamHI fragment. This fragment was cloned into pBluescriptKS- (Stratagene) in which the SacI, SalI, HindIII, and NcoI polylinker sites had been deleted, and was partially sequenced. This BamHI fragment contains two HindIII sites (Fig. 2). The two largest HindIII fragments (∼4.8 and 3.8 kb in size) were used separately to screen 6 × 10⁸ phage of a cDNA library constructed by H. Sommer and B. Planck (institute für Zuchtungsbiologie, Cologne, Germany). One cDNA that hybridized to both fragments was sequenced in its entirety by making exonuclease-deletions of the cDNA (Sambrook et al., 1989). A subsequent round of screening of 8 × 10⁸ phage using a 5′-terminal region of the cDNA (nucleotides 893–1208 of the first cDNA) identified another eight cDNA clones. The 5′-terminal ends of two were sequenced. These sequences were assembled using AssemblyLIGN software (Oxford Molecular Ltd.).

**Epitope tagging of Tbc2**

The triple HA epitope was inserted into the SacI site in the middle of the genomic coding sequence of Tbc2 (Fig. 2). The triple HA epitope was also inserted at the COOH-terminal end of the protein. A SalI-HindIII site was first introduced upstream of the stop codon of the genomic Tbc2 DNA to allow for the insertion of the tag. The HA epitope-containing DNAs were introduced into the chc2-F64/cw15 by selecting for restoration of photosynthe-
sis, and the transformants were screened for expression of the HA epitope by immunoblotting. HA epitope-tagged strains were maintained on HS medium to ensure continued expression of the tagged protein.

**Immunoblotting and cell fractionation**

Total protein extracts obtained from cell pellets of 10-ml cultures were re-
suspended in 250 μl HMK (Hepes-KOH 20 mM, pH 7.8, KC1 50 mM, MgCl2 10 mM) containing protease inhibitors: 5 mM ε-amino caproic acid, 1 mM benzamidine HCl, 25 μg/ml pepstatin A, and 10 μg/ml leupeptin, and then sonicated on ice. A portion of this extract was centrifuged at 100,000 g for 30 min at 4°C, giving rise to a pellet and supernatant fraction. The pellet was washed with 1 ml of STN buffer (0.4 M sucrose, 100 mM KCl, 10 mM EDTA) plus protease inhibitors (as above) in 10% saponin in H2O, incubated for 1 min on ice, and then centrifuged for 10 min at 100,000 g. The pellet was resuspended in lysis buffer (50 mM Tris, pH 8.0, 10 mM NaCl) and centrifuged again for 15 min at 50,000 g at 4°C to remove soluble contaminating proteins. The pellet was resuspended in lysis buffer (50 mM Tris, pH 6.8, 2% SDS, 10 mM EDTA, and protease inhibitors), incubated for 1 h at room temperature, and centrifuged for 5 min in a microfuge. The resulting supernatant was used as total insoluble protein extract.

Chloroplasts were isolated from a strain carrying the chc2-F64 and cw15 alleles, which had been transformed with Tbc2:HA. A 500 ml culture of cells grown to mid-log phase was centrifuged and resuspended in 10 ml of breaking buffer (300 mM sorbitol, 50 mM Hepes-KOH, pH 7.8, 5 mM MgCl2) with protease inhibitors. Saponin (S-4521; Sigma-Aldrich) was added at 2.5% (v/v) in breaking buffer (300 mM sorbitol, 50 mM Hepes-KOH, pH 7.8, 5 mM MgCl2) to disrupt chloroplast membranes and inhibit protease activity. The lysate was thawed on ice, KCl was added to 50 mM, MgCl2 to 2.5 mM, and NaCl to 100 mM, and the mixture was left to precipitate and pelletize at 4°C before loading on polyacrylamide gels. The resulting pellet was washed once in breaking buffer, and osmotically lysed in chloroplast lysis buffer (50 mM Tris, pH 6.8, 1.6% SDS, 80 mM DTT, 30% glycerol, 8 mM e-NH2 caproic acid, 1.6 mM benzamidine, 1.6 μg/ml leupeptin, 1.6 μM E64, 32 μg/ml pepstatin A, 2.6 μM PMFS, 1.6 μM 1,10-phenanthroline, and a 1:100 dilution of Sigma protease inhibitors P 8849 for 20 min at ambient temper-

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