

ISOLATION OF ALGAL GENES BY FUNCTIONAL COMPLEMENTATION OF YEAST¹

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Algal cDNAs were isolated and characterized by functional complementation of yeast auxotrophs. Two cDNA libraries, one derived from the diatom *Phaeodactylum tricorutum* Bohlin and the other from the dinoflagellate *Cryptocodinium cohnii* Biecheler, were constructed using the *Saccharomyces cerevisiae* expression vector pFL-61. These libraries were used for functional complementation of auxotrophic markers in two yeast strains. Yeast tryptophan auxotrophs, complemented by the *P. tricorutum* library, contained a plasmid that encoded a two-domain protein associated with tryptophan synthesis, indole-3-glycerol phosphate synthase-*N*-(5'-phosphoribosyl) anthranilate isomerase. Another cDNA originating from the *C. cohnii* library rescued *S. cerevisiae* from a defect in adenine biosynthesis. This cDNA encoded a fusion of phosphoribosylamidoimidazole-succinocarboxamide synthetase and phosphoribosylaminoimidazole carboxylase, which correspond to the yeast *ade1* and *ade2* genes, respectively. These results demonstrate that heterologous functional complementation can be used to identify algal genes and may provide advantages over other gene discovery methods.

Key index words: *Phaeodactylum*; *cryptocodinium*; tryptophan; adenine; biosynthesis.

Abbreviations: AIRC, phosphoribosylaminoimidazole carboxylase; IGPS, indole-3-glycerol phosphate synthetase; PRAI, phosphoribosyl anthranilate isomerase protein; SAICAR, phosphoribosylaminoimidazole-succinocarboxamide

Several techniques have been developed for isolating novel genes. Two of the most common are degenerate-oligonucleotide-primed PCR and mass sequencing of genomes or cDNAs. Both of these techniques rely on characterizing putative coding regions by sequence similarity to previously known genes. Thus, assignments of function can only be made indirectly. In this regard, heterologous functional complementation of auxotrophic yeast and bacterial mutants as a gene discovery method is advantageous because the very process by which a given gene is isolated provides clues to function, independent of its nucleotide sequence. The procedure restores a prototrophic phenotype in an auxotrophic organism by transformation with a gene or gene library derived from another or-

ganism. Using mutants of *Saccharomyces cerevisiae*, this technique has been applied to the discovery of genes from organisms as diverse as *Homo sapiens* (Michaeli et al. 1993), *Drosophila melanogaster* (Olsen et al. 1998), and *Arabidopsis thaliana* (Minet et al. 1992, Nasr et al. 1994, Collinge and Walker 1994). To study more specialized functions such as circadian rhythms or light harvesting, gene deletions have been complemented in *Neurospora crassa* (Bell-Pedersen et al. 1996) and *Chlamydomonas reinhardtii*, respectively (Lardans et al. 1997). Besides *Chlamydomonas*, *Volvox* is the only other eukaryotic alga for which functional complementation strategies have been used (Fabry et al. 1995). It is unknown whether this technique is practical for the isolation of cDNAs from other nongreen algal groups such as diatoms (Bacillariophyta) and dinoflagellates (Dinophyceae).

The study of diatoms at the molecular level has been limited when compared with that of fungi, plants, and animals. However, the recent development of a versatile genetic transformation system and the availability of useful reporter genes have provided new opportunities to study the molecular mechanisms of diatoms in general (Apt et al. 1996, Zaslavskaja et al. 2000). In particular, transgenic diatoms have been created to study intracellular Ca²⁺ flux in response to environmental changes (Falcitore et al. 2000). A few diatom genes have been cloned, most encoding proteins responsible for light harvesting (Apt et al. 1994, Eppard and Rhiel 2000) and silica transport and deposition (Hildebrand et al. 1998, Kröger et al. 1999).

The molecular biology of dinoflagellates is even less well understood than that of diatoms. Only a handful of genes have been isolated from these taxa, most encoding plastid proteins, rRNAs, and other genes useful for phylogenetic comparison (Palmer and Delwiche 1996, see Gray 1999 for reviews on dinoflagellate phylogeny). Most dinoflagellates are characterized by a lack of histones and an unusually large number of chromosomes that remain permanently condensed (for reviews see Herzog and Soyer 1981, Soyer-Gobillard 1996). Many dinoflagellates also contain the rare nucleic acid hydroxymethyl uracil as a major component of their total nucleic acid content (Steele and Rae 1980).

In this study, we isolate and characterize two new algal cDNAs from the diatom *Phaeodactylum tricorutum* and from the dinoflagellate *Cryptocodinium cohnii* by heterologous gene-functional complementation. This analysis enhances our understanding of these organisms at the molecular biological level and establishes the validity of this gene discovery approach in diatoms and dinoflagellates.

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MATERIALS AND METHODS

Strains and media. Plate and liquid cultures of *P. tricornutum* (UTEX 640) were grown as previously described (Apt et al. 1996). Plate and liquid cultures of *C. cohnii* (MK8840) were grown in Instant Ocean™ artificial seawater (Aquarium Systems, Mentor, OH, USA) supplemented with 50 g·L⁻¹ glucose and 6 g·L⁻¹ yeast extract (Difco/Becton Dickson, Sparks, MD, USA); for solid media, agar was added to 1.3% (w/v). All cultures were harvested during mid-log phase growth. The haploid *S. cerevisiae* strains MKP-o (*MATα*, *can1-100*, *ade2-1*, *lys2-1*, *ura3-52*, *leu2-3*, *leu2-112*, *his3-Δ200*, *trp1-Δ901*, *SUP4-o*) (Pierce et al. 1987) and W303 (*MATα*, *can1-100*, *ade2-1*, *ura3-1*, *leu2-3*, *leu2-112*, *trp1-1*) (Thomas and Rothstein 1989) were tested for complementation with both the *P. tricornutum* and *C. cohnii* cDNA libraries using methods described below. Yeast cultures were grown in YB glucose media with the appropriate amino and/or nucleic acids omitted (Mount et al. 1996). The *SUP4-o* gene of yeast strain MKP-o is an ochre suppressor allele of a yeast tyrosine tRNA gene. The ochre suppressible markers in this yeast are *can1*, *ade2*, and *lys2*. By design, these markers were subject to frequent spontaneous reversion to the wild-type phenotype. For this reason, only the *leu2*, *his3*, and *trp1* phenotypes were tested for complementation using MKP-o, whereas yeast strain W303 (which was not subject to problematic reversions of any of its markers) was used for generation of potential adenine or leucine prototrophs. In both strains, the *ura3* marker was used only for primary selection and quantification of transformants. Bacterial cultures were grown in standard LB media containing the appropriate antibiotics.

cDNA library construction. For the preparation of total RNA, *P. tricornutum* and *C. cohnii* cells in mid-log phase of growth were chilled rapidly to 4° C in a liquid nitrogen bath and pelleted at 1000g. Supernatants were discarded; the pellets were flash frozen and then pulverized in a mortar under liquid nitrogen. The resulting powder was resuspended in an extraction buffer consisting of 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 50 mM dithiothreitol at a ratio of 1:10 (w/v). A total of 0.1 volumes of 2 M sodium acetate was added before extraction with 1 volume of phenol and 0.2 volumes of chloroform/isoamyl alcohol (24:1). RNA was then precipitated from the aqueous phase with an equal volume of isopropanol, washed with 75% ethanol, dried, and resuspended in water (Maniatis et al. 1989). Further purification of mRNA was performed using the Oligotex™ mRNA purification kit (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. A plasmid cDNA library was constructed by the manufacturer's protocol with the Superscript™ Choice System for cDNA Synthesis (Life Technologies, Gaithersburg, MD, USA) using 5' phosphorylated *Bst*XI restriction site adaptors B1 (CTAAATTACTCACAA) and B2 (AGTAATTTAG) (d'Enfert et al. 1995). These adaptors were ligated to cDNA, which was then size fractionated to purify full-length transcripts from truncated products. These purified cDNAs were separated into two size-range pools, 200–1500 base pair (bp) and >1500 bp of which only the larger pool was used for further experiments. The adapted cDNA was then ligated to the *S. cerevisiae* transformation vector pFL-61 (Minet et al. 1992) that had previously been digested with *Bst*XI and gel purified. Ligated cDNA-containing plasmids were then propagated in *Escherichia coli* on solid media (to ensure that large insert-containing vectors were not under-represented in the plasmid preparation). Colonies were harvested and plasmid DNA was purified by standard methods (Maniatis et al. 1989).

Transformations. All bacterial transformations were done with UltraMax™ DH5-αFT™ Competent cells (Life Technologies) as described by the manufacturer. Yeast cells were grown to an A₆₆₀ between 0.4 and 0.6 from a single colony inoculum in a 100-mL culture. Cells were pelleted at 2000g and washed in 10 mL of 100 mM LiOAc, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4). To 100-μL aliquots of cells, 5 μg of transforming DNA was added with 100 μg of salmon sperm carrier DNA (>1 kilobase pair [kbp] average molecular weight). To this, 700 μL of a solu-

tion containing 400 μg·mL⁻¹ PEG 3350, 100 mM LiOAc, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4) was also added. The cells were incubated at 30° C for 60 min after which 100 μL of dimethyl sulfoxide was added and the mixture incubated at 42° C for an additional 20 min. Cells were pelleted and resuspended in 200 μL of a solution containing 1 M sorbitol, 6.5 mM CaCl₂, and 1.75 mg·mL⁻¹ yeast extract (Braun et al. 1992). After plating on omission medium lacking uracil and either adenine or tryptophan, the cells were incubated at 30° C for 2–12 days until prototrophic colonies appeared. Plasmids were recovered by standard protocols (Piper 1996) and amplified from individual yeast transformants by subcloning and transformation of the *E. coli* strain described above. True complementation was confirmed by retransforming yeast strains with recovered plasmid to regain the prototrophic phenotype. The vector pFL-61 has two *Not* I restriction sites flanking the cDNA insertion site, so cDNAs can be readily removed for size screening. All restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Plasmids were sequenced and compared to the GenBank sequence database using BLAST algorithms (Altschul et al. 1997).

RESULTS AND DISCUSSION

Isolation of *Phaeodactylum tricornutum* cDNAs capable of curing tryptophan auxotrophy. The *P. tricornutum* cDNA library was used to transform *S. cerevisiae* strain MKP-o. Of 25,000 primary transformants generated for the *trp1* marker of MKP-o, only two were found to be prototrophic when grown in the absence of exogenous tryptophan. The two clones were designated 1A and 2A. No complementation was observed for the other auxotrophic phenotypes of MKP-o tested (*leu2* and *his3*). For each marker, no fewer than 50,000 primary transformants were generated before abandoning the effort. Plasmids were isolated and sequenced from transformed cultures where the *trp1* phenotype was complemented. The plasmid from clone 1A yielded two fragments when digested with *Not* I, 1.4 kbp and 0.8 kbp in size. *Not* I restriction enzyme digestion of the plasmid extracted from clone 2A liberated a 1.4-kbp fragment. After retransformation, both plasmids were capable of curing the tryptophan auxotrophy of both MKP-o and W303 yeast strains, although prototrophic colonies derived from clone 2A were always smaller and grew more slowly than those from 1A. Sequence data from clone 1A (GenBank accession AY030294) revealed an insert of exactly 2097 bp with a *Not* I restriction enzyme site at 756 bp, but it did not include a poly-adenosine tail. The sequence of the insert from clone 2A (GenBank accession AF472597) was 1348 bp long, including a poly-adenosine tail. The inserts shared exact identity between bases 1139–2097 of 1A and 1–957 of 2A (Fig. 1). Both inserts are probably derived from the same gene. It seems most likely that during construction of the library, both cDNAs were truncated on at least one end before being ligated to the *Bst*XI restriction site adaptors. As a result, the putative 3' untranslated region and poly-adenosine tail of the transcript is not included in the insert of clone 1A, and nearly half of the 5' end of the ORF is not found in the insert of clone 2A.

The nucleic acid sequence of the entire clone 1A cDNA was compared with the GenBank database us-

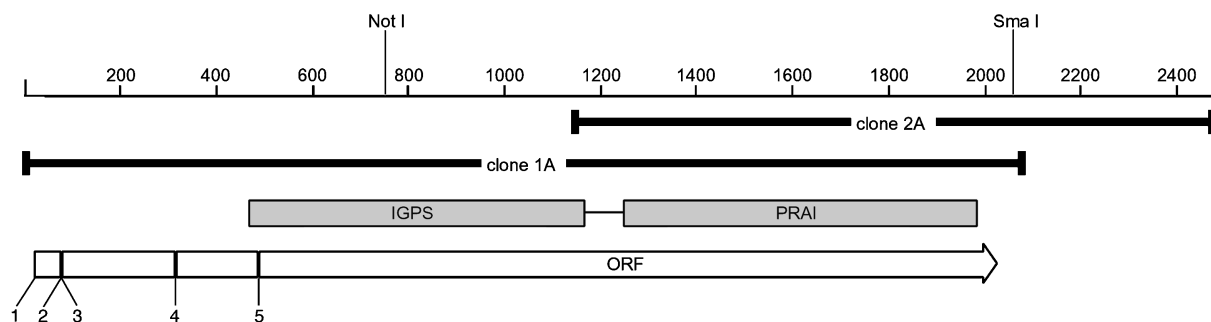


FIG. 1. The positions of various features from the cDNA sequences of clones 1A and 2A are shown. Restriction sites are indicated above the base pair ruler, which represents the entire length of the theoretical combined 2487-bp transcript. Black bars: sequences of cDNA inserts derived from clones 1A (AY030294) and 2A (AF472597) match precisely in overlapping areas. Shaded boxes: conserved domains elucidated by comparing the longest hypothetical protein (corresponding to the frame between bases 22 and 2029) to the GenBank protein database using the BLASTP algorithm. Open box (bottom): locations of the first five potential, in-frame, ATG start codons are indicated by numbered vertical black slashes (at bp 22, 79, 82, 319, and 490). Each open reading frame will terminate at a TAA stop codon in position 2029.

ing the BLASTX algorithm. The most similar sequence returned from the search (42% identical, 57% positive, and 1% gaps) was of the *Phytophthora parasitica* indole-3-glycerol phosphate synthase-*N*-(5'-phosphoribosyl) anthranilate isomerase protein (IGPS-PRAI), a bifunctional enzyme involved in tryptophan biosynthesis. There are five in-frame ATG start codons located in the 5' end of clone 1A that could be used for translation initiation of the IGPS-PRAI protein. The first of these will generate a deduced protein sequence of 669 amino acids. However, no significant similarities to the first 140 amino acids of this deduced protein were found when compared with GenBank using the BLASTP algorithm. It is unclear then which of these ATGs represents the true translation start site, although the fourth at position 319 may be the most likely as it is the first start codon preceding the conserved IGPS domain.

Gene domains responsible for enzyme activities involved in tryptophan biosynthesis are organized differently in other organisms. In enteric bacteria, each activity is confined to a single enzyme, whereas in many filamentous fungi the IGPS and PRAI enzymes are fused to the C-terminus of a third domain possessing glutamine amidotransferase activity. In yeast and some thermophilic bacteria, the glutamine amidotransferase domain is often fused to the IGPS domain

alone, with a separate polypeptide retaining PRAI activity (Karlovsky and Prell 1991, Sterner et al. 1995). In *S. cerevisiae*, the gene that encodes the PRAI enzyme is more commonly referred to as *trp1*, which has been disrupted to obtain tryptophan auxotrophy in MKP-o. It is interesting to note that the truncated insert of clone 2A lacks the IGPS domain; however, it was still able to functionally complement the PRAI deficiency in the tryptophan-requiring yeast used in this study. The insert of clone 2A has an in-frame ATG codon at position 66 (corresponding to position 1205 of clone 1A) that could have been used as a translation initiation site for the expression of this PRAI domain as an autonomously functional protein.

Isolation of a Cryptocodium cohnii cDNA capable of curing adenine auxotrophy. The *C. cohnii* cDNA library was used to transform the yeast strain W303. Four prototrophic colonies grew on adenine deficient media after the initial transformation (of approximately 90,000 primary transformants). Plasmids were isolated from each prototrophic clone and used for retransformation of the yeast. Only one was found capable of curing adenine auxotrophy. Again, no complementation was observed for the other auxotrophic phenotypes of W303 tested (*leu2* and *trp1*) among more than 100,000 primary transformants. Sequence analysis of

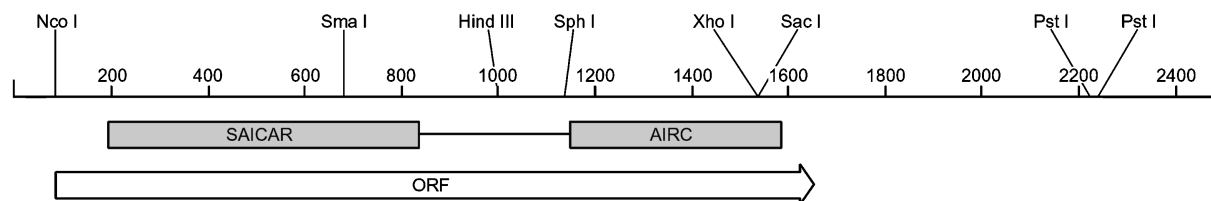


FIG. 2. The positions of features from the ccAde-A cDNA (AY032657) are shown. Restriction sites are indicated above the base pair ruler, which represents the entire length of the 2468-bp ccAde-A cDNA. Shaded boxes: conserved domains elucidated by comparing the ccAde-A deduced protein with the GenBank protein database with the BLASTP algorithm. Black box (bottom): the only feasible open reading frame found in the transcript, from bp 77 to 1645.

the *ade2* complementing cDNA (designated ccAde-A) revealed an insert of 2468 bp (accession number AY032657). The insert encoded a protein with high sequence similarity (BLASTX) in its N-terminal half to phosphoribosylamidoimidazole-succinocarboxamide (SAICAR) synthetase of *Agrobacterium tumefaciens* (37% identical, 55% positive, and 2% gaps) and in its C-terminal half to the phosphoribosylaminoimidazole carboxylase (AIRC) domain of the *G. gallus* SAICAR-AIRC protein (32% identical, 48% positive, and 3% gaps). In *S. cerevisiae*, the enzyme activities of these two domains correspond with the *ade1* and *ade2* loci, respectively (Stotz and Linder 1990, Davies and Hutchison 1991). Even though we only tested the ability of this cDNA to complement the *ade2* phenotype, we believe that this cDNA probably encodes a fusion of both the SAICAR and AIRC domains due to the strong sequence similarities found in the N terminal half of the deduced protein to many other SAICAR domains (Fig. 2). One last interesting trait of this cDNA is its unusually long 3' untranslated region of almost 800 bp.

Fusion of these two domains is uncommon among bacteria, fungi, or plants. However, it is not unusual to see these domains fused in metazoans; several other examples of this have been demonstrated (Chen et al. 1990, Minet and Lacroute 1990, Chapman et al. 1994). This type of domain fusion has not been observed in a nonmetazoan organism until now. It is not known if this fusion is typical for organisms related to the dinoflagellates because no identities were found among the known gene sequences of other alveolates, including those in the large apicomplexan databases. However, it has been suggested that apicomplexan parasites use host-supplied purines and thus may have lost genes involved in purine biosynthesis (Krug et al. 1989).

Dinoflagellates can have genome sizes of 10^9 bp and higher (Haapala and Soyer 1974). Log-phase cultures of *Cryptocodinium* double in cell number about once every 8 h (data not shown). One could hypothesize that the enzymes responsible for nucleic acid biosynthesis in this organism should be plentiful and possess high activity. If so, perhaps this correlates with our success in the isolation of this SAICAR-AIRC cDNA.

We established that heterologous gene functional complementation is a valid technique for the isolation of genes derived from two different phyla of algae. It is hoped that this technique can be used for the eventual identification and characterization of many more algal genes involved in other important cellular processes.

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