Mol Gen Genet (1999) 261: 21–30

ORIGINAL PAPER

A. H. Auchincloss · A. I. Loroch · J.-D. Rochaix

The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: cloning of the cDNA and its characterization as a selectable shuttle marker

Received: 2 June 1998 / Accepted: 25 September 1998

Abstract We describe the cDNA sequence for *ARG7*, the gene that encodes argininosuccinate lyase – a selectable nuclear marker – in *Chlamydomonas reinhardtii*. The 5′ end of the cDNA contains one more exon and the organisation of the mRNA is different from that predicted from the genomic sequence. When expressed under the control of the endogenous *RbcS2* promoter, the 2.22-kb cDNA complements the *arg7* mutation as well as the genomic DNA. A linear cDNA fragment lacking promoter sequences is also able to complement, suggesting that it could be used in promoter-trapping experiments. Despite the presence of a sequence encoding a potential chloroplast transit peptide in the cDNA the protein is not targeted to the chloroplast, nor can it complement the *arg7* mutation when expressed there. By inserting a T7 bacteriophage promoter into the plasmid, a version of the cDNA which is able to complement both the *C. reinhardtii arg7* mutant and the *Escherichia coli argH* mutant has been created. This modified *Arg7* cDNA provides two advantages over the genomic DNA currently in use for gene tagging: it is shorter (6.2 kb versus 11.9 kb for pARG7.8ϕ3), and the selectable marker used in *C. reinhardtii* is the same as that used in *E. coli*, making plasmid rescue of the tag much more likely to succeed.

Key words *Chlamydomonas* · Argininosuccinate lyase cDNA · Transformation · Shuttle vector · Plasmid rescue

Communicated by R. G. Herrmann

A. H. Auchincloss · A. I. Loroch · J.-D. Rochaix (✉)
Department of Molecular Biology
University of Geneva, 30 Quai Ernest-Ansermet
CH-1211 Geneva 4, Switzerland
e-mail: jean-david.rochaix@molbio.unige.ch
Tel.: +41-22-7026187, Fax: +41-22-7026868

Introduction

The ability to carry out efficient nuclear transformation of *Chlamydomonas reinhardtii* (Kindle 1990) has dramatically altered our ability to study nuclear mutations in this organism. The transforming DNA usually integrates non-homologously into the nuclear genome (Debuchy et al. 1989; Kindle et al. 1989; Tam and Lefebvre 1993), allowing one to create "tagged" mutations; integration into a functional gene leads to a stable mutant phenotype. The disrupted DNA surrounding the integration site can then be cloned by "plasmid rescue" – by restricting the DNA with an enzyme that does not cut within the plasmid sequences, ligating and transforming the circular plasmid into *E. coli* (Tam and Lefebvre 1993). One recurrent problem is that the bacterial origin of replication and/or the antibiotic marker can become uncoupled from the *C. reinhardtii* selectable marker upon integration of the transforming DNA into the nuclear genome (Tam and Lefebvre 1993; Pazour et al. 1995; Smith and Lefebvre 1996). When this occurs, cloning of the integration site requires screening of partial genomic libraries for clones that hybridize to the selectable marker, which is much more time-consuming than plasmid rescue.

ARG7, which encodes argininosuccinate lyase (ASL, EC 4.3.2.1), is one of several selectable nuclear markers available for *C. reinhardtii*. The genomic DNA has been cloned and sequenced (Debuchy et al. 1989; Purton and Rochaix 1995), and gives a high transformation rate in *arg7* strains (Day et al. 1990; Purton and Rochaix 1995). It has been used in tagging mutagenesis schemes to generate new mutants (Adam et al. 1993; Davies et al. 1994), to introduce non-selectable genes by cotransformation (Davies et al. 1992; Tam and Lefebvre 1993), and to study homologous recombination (Gumpel et al. 1994). The enzyme catalyzes the last step in arginine biosynthesis, the cleavage of argininosuccinate to arginine and fumarate. Hence *arg7* strains cannot grow in the absence of exogenous arginine.

ASL has been recruited for use as a lens crystal protein (δ-crystallin) in many avian, reptile and amphibian species (Wistow and Piatigorsky 1987); some of these retain ASL function (Barbosa et al. 1991b). ASL and/or δ-crystallin genes have been sequenced from *C. reinhardtii* (Purton and Rochaix 1995), chicken (Ohno et al. 1985; Nickerson et al. 1986), the fungi *Saccharomyces cerevisiae* (Beacham et al. 1984) and *Candida albicans* (Hoyer et al. 1994), and bacteria (*E. coli*; Genbank AE000470), while cDNAs have been sequenced from man (O'Brien et al. 1986), rat (Amaya et al. 1988), duck (Wistow and Piatigorsky 1990) and amphibians (Iwase et al. 1995). The locus is approximately 35 kb long in mammals, with 16 exons (Matsubasa et al. 1989; Abramson et al. 1991), while the chicken gene has 17 exons, the extra exon being 5′ and non-coding (Ohno et al. 1985; Nickerson et al. 1986). In *C. reinhardtii* the gene is 7.8 kb long, and is thought to contain 13 exons (Purton and Rochaix 1995). Four of the introns are in similar positions in *C. reinhardtii* and animals. The 50-kDa homotetrameric enzyme is cytoplasmic (Dhanakoti et al. 1992), and may be localized to the mitochondrial outer membrane (Cohen and Kuda 1996). The structure of turkey δ-crystallin has been solved to 2.5 Å resolution (Simpson et al. 1994, 1995), while recombinant human ASL has been solved to 4.0 Å (Turner et al. 1997).

Genetic deficiencies in the ASL locus cause urea cycle disorders in man, which vary in age of onset and severity (McInnes et al. 1984). At least 12 alleles of the human ASL locus are known (McInnes et al. 1984). Ten mutations have been identified in cDNAs from affected patients (Walker et al. 1990, 1997; Barbosa et al. 1991a); the severity of the disease may depend in part on whether interallelic complementation between the inherited alleles is possible (McInnes et al. 1984; Turner et al. 1997). Similar genetic complexity is seen at the *C. reinhardtii ARG7* locus, for which 14 mutants are known (Matagne 1978).

We have characterised an *Arg7* cDNA from *C. reinhardtii*. This cDNA has allowed us to generate vectors which, owing both to their smaller size and decreased content of repetitive DNA, are less likely to rearrange upon integration into the *C. reinhardtii* genome (Tam and Lefebvre 1993; Cerutti et al. 1997), and should thus facilitate plasmid rescue from mutagenized strains. The more compact cDNA is easier to manipulate in bacteria, and a second non-selectable marker could be cloned in the vector, increasing the probability of marker expression (Haring and Beck 1997; Periz and Keller 1997). The cDNA encodes one more exon and the organisation of the first two exons is different from that predicted based on the genomic sequence. We have found that the cDNA complements the *arg7* mutation well in the absence of introns, unlike the case for some other *C. reinhardtii* genes.

Materials and methods

C. reinhardtii strains and growth conditions

C. reinhardtii strains were grown on TAP medium (Gorman and Levine 1966), an enriched medium which allows mixotrophic growth, under illumination with 50 µmol photons/m^2/s, unless otherwise indicated. Cells were grown on minimal medium (HSM; Harris 1989), to select for photosynthetic growth. Media were supplemented with 110 µg/ml arginine for growth of *arg7* strains. The *FuD50:arg7* strain was obtained by crossing the appropriate strains (*FuD50:mt+*, CC1185 and *arg7:mt-*, CC1861, obtained from the Chlamydomonas Stock Center, Durham, N.C.) as described (Harris 1989).

E. coli strain construction

In order to create an *argH*, *recA*, T7 lysogenic strain of *E. coli*, a C600 *argH* strain (*thr-1, leuB6*, *LAM-*, *thi-1*, *argH*) was conjugated with strain JC10240 (*thr-300*, *recA56*, *srl-300*::Tn*10*, *ilv-318*, *thi-1*; (Csonka and Clark 1980), obtained from the *E. coli* Stock Center, New Haven, Conn.) according to standard protocols (Miller 1992). The cells were plated on M9 minimal medium containing tetracycline, threonine, leucine, arginine and thiamine. Colonies were tested for sensitivity to UV light – indicating loss of *recA* function – as described (Csonka and Clark 1980). The T7 RNA polymerase lysogen was created using a kit from Novagen.

General methods

Cloning was performed according to standard techniques (Sambrook et al. 1989). Polymerase chain reactions (PCR) were performed on 5 ng/µl template with 0.5 µM primers in 50 mM TRIS-HCl pH 8.3, 1 mM $MgCl_2$, 250 µg/ml BSA, 200 µM dNTP, 5% DMSO, using *Pfu* polymerase (Stratagene). Total RNA was isolated by the acid guanidinium thiocyanate/phenol-chloroform method (Chomczynski and Sacchi 1987; Puissant and Houdebine 1990), poly(A)$^+$ RNA was isolated on oligo-dT columns as described (Sambrook et al. 1989) and formaldehyde RNA gels were blotted to Hybond N+ (Amersham). Probes for screening the cDNA library, RNA and DNA gel blots were made by random priming (Sambrook et al. 1989) using [α-^{32}P]dATP or dCTP (for RNA blots) and hybridized as described (Church and Gilbert 1984). Primer extension analysis was performed on 80 µg of total RNA with 1.25 U of AMV reverse transcriptase as described (Johanningmeier et al. 1987), using the oligonucleotide 5′-GCGGGGACGCGAGCC-3′, labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Sambrook et al. 1989).

The cDNA library was constructed by Hans Sommer (Max-Planck-Institut für Züchtungsforschung, Cologne). Reverse-transcribed cDNA fragments were blunt ended and *Eco*RI/*Not*I adapters (Pharmacia) were ligated to the ends. The cDNAs were cloned into the *Eco*RI site of λNM1149 and amplified in strain BHB2600.

Construction of cDNA-containing plasmids

The cDNA-containing insert was removed from the λ phage vector by digestion with *Eco*RI, and cloned in both orientations into the *Eco*RI site of pBluescriptKS(-) (Stratagene), yielding pKS18 and pKS19. To clone the cDNA in both orientations in the *C. reinhardtii* nuclear expression vector pSP105 (Stevens et al. 1996), the pKS constructs were digested with *Xba*I and *Kpn*I and the cDNA was cloned into the corresponding sites in pSP105. The *RbcS2* stop codon, 3′ UTR and poly(A) signal of pSP105 were thus replaced by the *Arg7* cDNA sequences. The expression vector constructs are not protein fusion constructs. Plasmid pRb-ARGs contains the *Arg7* cDNA in the sense orientation, and pRb-ARGαs in the antisense orientation (see Fig. 1B).

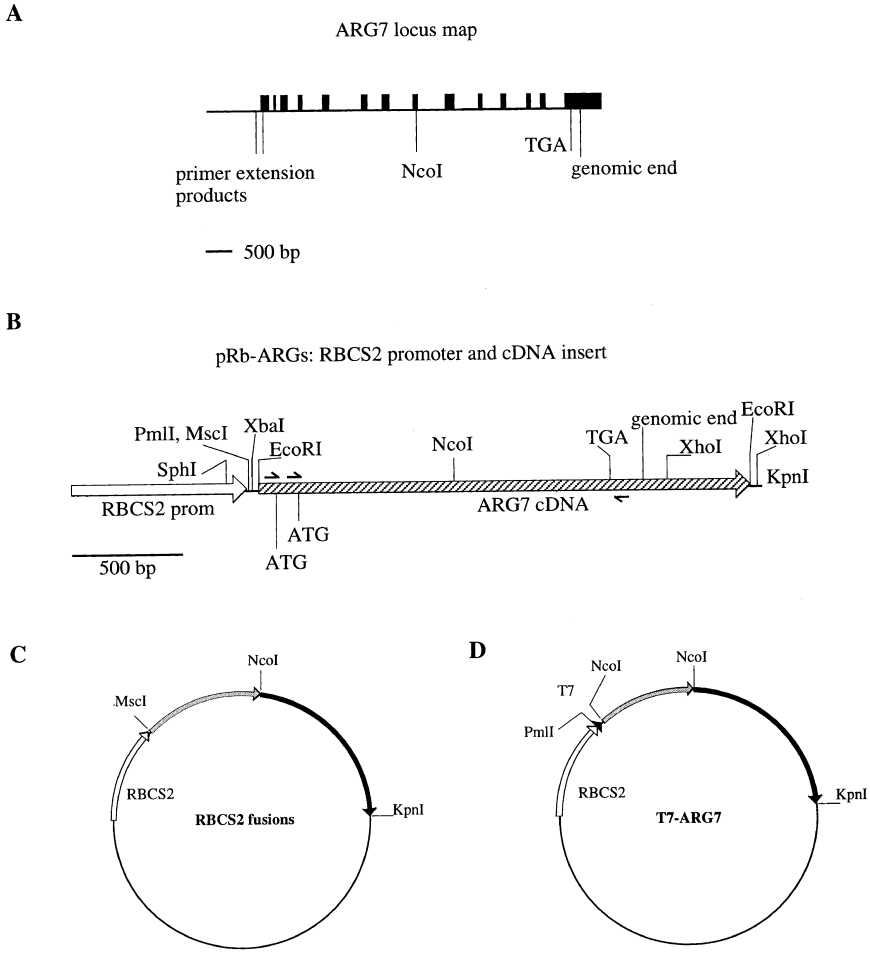


Fig. 1A–D Maps and constructions. **A** The structure of the *ARG7* locus. Sequences found in the cDNA (including 5′ and 3′ UTRs) are indicated by *filled boxes*, intron sequences by *thin lines*. The origins of the primer extension products, the endogenous *Nco*I site used in cloning, the stop codon (TGA) and the previously determined end of the genomic sequence are indicated. **B** The *RbcS2-Arg7* cDNA insert in pRb-ARGs. Sites used in cloning are indicated, as well as the two potential start codons (ATG), the stop codon (TGA) and the end of the cloned genomic sequence. Short stretches of pKS(-) derived polylinker are found between the *Xba*I and *Eco*RI sites 5′ of the cDNA and between the *Eco*RI and *Kpn*I sites 3′ of the cDNA. *Small arrows* indicate oligonucleotides used as primers to generate the different PCR products. Two different versions of each of the 5′ oligonucleotides were used, giving different ends for subsequent cloning steps. Not all *Sph*I sites in *RbcS2* are shown. **C** *RbcS2* fusions. Blunt-end/*Nco*I-digested PCR products with or without the putative transit peptide (*stippled segment*) were cloned into *Msc*I + *Kpn*I-digested pSP105, in a three-way ligation with the *Nco*I-*Kpn*I fragment containing the 3′ end of the cDNA (*black segment*). **D** T7-ARG7 Annealed T7 promoter-encoding oligonucleotides with a blunt and an *Nco*I end were ligated with the large *Pml*I-*Nco*I fragment of pRb-ARGs (containing the 3′ half of the coding sequence and the 3′ UTR). A 711-bp *Nco*I fragment containing the 5′ coding sequence (without the transit peptide; *stippled bar*) was subsequently cloned into the *Nco*I site and selected for complementation of an *E. coli argH* strain. The orientation of the 711-bp fragment was confirmed by sequencing

To make precise fusions of the putative precursor or mature cDNA with the *RbcS2* start codon in pSP105, the cDNA was amplified by PCR with oligonucleotides which inserted an *Msc*I site at the 5′ end of the gene (5′-CCCCGCTCGCTCTCCAGCAT-3′ for the precursor form, or 5′-CCCAGGCAGCTGCTGCG-3′ for the mature form) and an oligonucleotide which inserted a *Sma*I site after the stop codon (5′-CCCCCCTCCCGGGACCCGCTCAGT-3′). The PCR product was digested with *Nco*I (which cuts within the cDNA at nucleotide 897, position 5341 of the genomic sequence), the 5′ end-containing fragment was purified and ligated to a gel-purified *Nco*I-*Kpn*I fragment (containing the 3′ part of the gene) and pSP105 digested with *Msc*I and *Kpn*I. Thus only the 5′ half of the cDNA (700 bp for the mature construct, 800 bp for the precursor) was derived by PCR and subsequently sequenced (Fig. 1B, C).

In order to create chloroplast expression vectors, the cDNA with or without the potential transit peptide sequence was amplified and cloned under the control of the *psbD* promoter found in p*atpB*-INT-cg12 (cg12 is identical to cg11 (Nickelsen et al. 1994), except that the *Sph*I site far from the *aadA* gene of cg11 has been deleted). The oligonucleotides used to amplify from the 5′ end (5′-TCGGATAGCCCCATGGCCCCGCTCGC-3′ for the precursor or 5′-CGTCTCAGCGCCATGGCCCAGGCA-3′ for the mature cDNA) added an *Nco*I site, and the oligonucleotide used to amplify from the 3′ end added a *Sma*I site just after the stop codon (described above). The complete cDNA-containing fragment (obtained by complete digestion with *Sma*I and partial digestion with *Nco*I) was cloned into the *Nco*I and blunt-ended *Sph*I sites of cg12, which encodes the 3′ end of the *atpB* gene and complements the 3′ deletion of the *atpB* gene found in *FuD50* (Woessner et al. 1984). The constructs were transformed into *FuD50*:*arg7* strains using the particle gun method (Boynton et al. 1988). Due to the high rate of homologous recombination in the chloroplast, the DNA is integrated at the *atpB* locus, rescuing the photosynthetic defect and inserting the cDNA nearby. The presence of the *Arg7* cDNA in the transformants was confirmed by Southern analysis (not shown; see Boynton et al. 1988).

To insert a T7 promoter-containing fragment into pRb-ARGs, the oligonucleotides 5′-TAACACGACTCACTACTGGGAGAA-CGTGAAGGAGGAGATCTC-3′ and 5′-CATGGAGATCTCC-TCCTTCACGTTCTCCCAGTAGTGAGTCGTGTTA-3′ were annealed at 10 μM in 150 mM NaCl, yielding a blunt end and an *Nco*I site. They were cloned into the large *Pml*I-*Nco*I fragment of pRb-ARGs, which contains the 3′ half of the cDNA. A 711-bp *Nco*I fragment containing the 5′ half of the cDNA (without the putative transit peptide; see previous paragraph and Fig. 1C) was cloned into the *Nco*I site and transformed into an *E. coli argH* strain to select for those clones that were able to rescue the *argH* mutation. The orientation of the 711-bp fragment was confirmed by sequencing. To bring the plasmid origin of replication closer to the selectable marker, the DNA was digested with *Afl*III, which cleaves 420 bp upstream of the *RbcS2* promoter, and *Pst*I, which cuts immediately upstream of the promoter. The DNA was blunted and ligated. The resulting plasmid is called T7-ARG del to ori. The absence of this 420-bp *Afl*III-*Pst*I fragment does not affect the ability of the plasmid to rescue either the *C. reinhardtii* or *E. coli* ASL mutation (data not shown).

Sequencing of both strands of pKS19 and all PCR-amplified fragments was performed using the di-deoxy method followed by terminal deoxy-nucleotide transferase extension of the non-terminated ends (Kho and Zarbl 1992), or by automated sequencing. Oligonucleotides designed to hybridize to coding sequences were used as sequence primers. The sequences were aligned using AssemblyLIGN software (Kodak, IBI).

C. reinhardtii transformation

Some 3×10^7 cells of strain *cw15:arg7:mt-* (Pierre Bennoun, IBPC, Paris) was transformed with 2 μg of uncut DNA as described (except for the gel-purified linear cDNA fragment; Kindle 1990) using baked glass beads (0.4 mm; Thomas Scientific). The chloroplast genome of *FuD50:arg7* was transformed using a particle gun as described (Boynton et al. 1988) and selected for restoration of *atpB* gene function on minimal medium supplemented with arginine, or simultaneously selected for *FuD50* and *arg7* rescue on minimal medium alone.

Argininosuccinate lyase enzyme assays

Assays were performed on 10-ml liquid cultures as described (Matagne 1978) with the following modifications. Centrifuged cells were resuspended in 80 μl of 20 mM sodium phosphate pH 7.5. Glass beads (0.4 mm) were added to make a thick slurry and the cells were broken by vortexing four times for 15 s each. The beads were rinsed with 50 mM sodium phosphate pH 7.5 and the lysate was spun at $12{,}000 \times g$ for 15 min. The supernatant was used directly in assays. Protein concentrations were determined using bicinchoninic acid (Smith et al. 1985; Brown et al. 1989).

Results

Gene structure

Some 200,000 phage from a *C. reinhardtii* cDNA library were screened with a 243-bp fragment of the *Arg7* gene encoding the eighth conserved exon with surrounding intron sequences (nucleotides 4429 to 4672 of the genomic sequence; Purton and Rochaix 1995). Five positive phages were identified and further screened with a 1.6-kb fragment encoding the first two conserved exons and intron sequences (nucleotides 817 to 2413 of the genomic sequence; Purton and Rochaix 1995). Two phages that hybridized to both probes were selected for further study. The cDNA inserts in these phages were estimated to be 3 and 2.3 kb in length. The inserts were excised from the phage DNA by digestion with *Eco*RI and cloned into pBluescriptKS(-).

Upon sequencing, the 3-kb insert was found to contain two cDNAs, one for ASL, the other derived from an unknown mRNA. Only the 2.3-kb cDNA was sequenced in its entirety; the sequence has been deposited in EMBL under the Accession No. AJ010110. This cDNA is 2217 nucleotides long, extending from nucleotide 1146 of the genomic sequence to a position 468 bases beyond the end of the previously reported genomic sequence (see Fig. 1A, Purton and Rochaix 1995). The 5′ UTR is 169 bp long (measured from the second, presumptive, start codon, see Discussion), while the 3′ UTR is 626 bp in length. No poly(A) tail was found in either cDNA. The ARG7 cDNA found in the 3-kb cDNA extends 20 nucleotides further 5′ than the 2.3-kb cDNA (see Fig. 2).

The cDNA is composed of 14 exons, one more than previously proposed (Purton and Rochaix 1995); the location of the second exon is not the same as formerly suggested. The newly identified exons are 142 and 36 nucleotides long (see Fig. 2). Both contain an in-frame start codon, and would give rise to full-length translation products of 55.7 kDa (508 amino acids) and 52.0 kDa (473 amino acids), respectively. Although the region containing the first ATG codon matches the consensus sequence for an initiation codon slightly better than the second (see Fig. 2 and Silflow 1998), for reasons discussed below, we believe the downstream ATG to be the functional start codon. The protein begins with the sequence MAQAAAAPAD, with splicing occurring between the codons for the third and fourth amino acids. Exon 3 (formerly exon 2) is one codon longer than previously suggested. No differences in nucleotide sequence were found between the cDNA and the genomic sequence.

The exon/intron junctions were found to match those predicted from the genomic sequence, with the exception of the junctions between exon 1/2 and exon 2/3, which had not been previously identified. The junctions are shown in Table 1. As noted previously (Purton and Rochaix 1995), only the tenth intron does not match the *C. reinhardtii* consensus, having a C instead of a T as the second nucleotide of the intron (Silflow 1998).

Primer extension analysis was performed on total RNA isolated from *arg7* and wild-type strains to determine the 5′ end of the RNA. A primer which spans the splicing junction between exons 1 and 2 was used in this analysis (see Fig. 2). Note that the *arg7* lane in Fig. 2 was intentionally overloaded in order to identify the start site; the difference in intensity between the lanes does not reflect an increase in the relative amount of mRNA in the *arg7* mutant. Two possible transcription start sites were identified in both wild-type and mutant strains (Fig. 3A). The smaller product mapped to nucleotide 52 of the cDNA (nucleotide 1197 of the genomic sequence) while the other, slightly more abundant product mapped about 175 bases further upstream

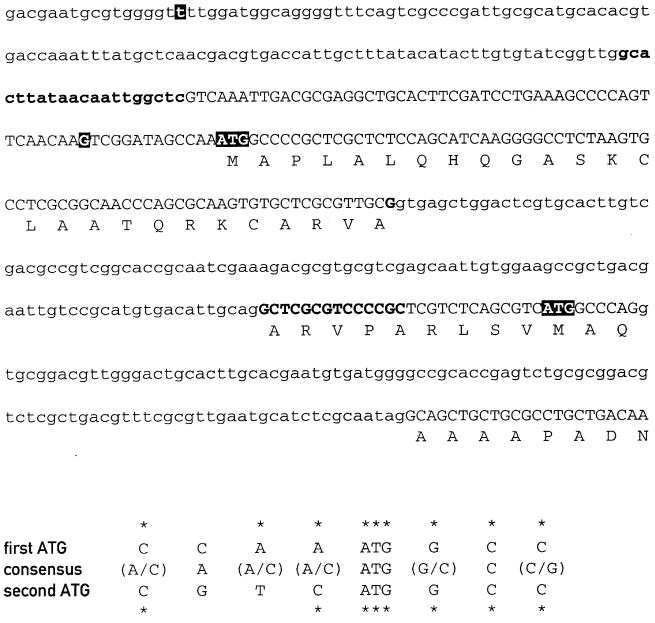


Fig. 2 5′ end of the cDNA. The genomic sequence of *Arg7* from nucleotides 1140 to 1559 is shown in the *upper* part of the Figure. cDNA-derived sequences are in *upper case*, with exons 1, 2 and part of exon 3 indicated. The 20 extra nucleotides found in the longer cDNA are indicated in *bold lower case* letters. The two in-frame initiation codons are indicated as *white letters* (ATG) on a *black background*. The sequence of the oligonucleotide used for primer extension analysis is in bold *upper case letters*, while the identified sites of transcription initiation are indicated as *single white letters* on a *black background* (the G 69 nucleotides upstream of the second start codon and the "t" at about nucleotide 1023 of the genomic sequence). The bottom part of the Figure shows the match between the two ATGs and the consensus sequence for translation initiation in *C. reinhardtii* (Silflow 1998)

(approximately nucleotide 1023 of the genomic sequence). Northern analysis performed on poly(A)$^+$ RNA identified a mRNA of approximately 2.3 kb, present in similar abundance in both wild-type and *arg7* strains (Fig. 3B); no signal can be seen in total RNA. This suggests that although the cDNA is probably not complete at its 5′ end, it is very close to full length (the length of poly(A) tails in *C. reinhardtii* is on the order of 100 to 150 bp, A. Auchincloss, unpublished results). The finding of normal amounts of approximately full-length mRNA was not unexpected, as the *arg7* allele used in this study does not show any gross rearrangements at the DNA level (Purton and Rochaix 1995). Only one poly(A) signal is found in the cDNA, at nucleotide 1640 of the cDNA as previously noted (Purton and Rochaix 1995); this is 578 bases from the 3′ end.

Transformation of *arg7* with the cDNA

To determine whether the 2.3-kb cDNA encodes functional ASL, the ability of the cDNA to rescue the *arg7* mutation present in the strain *cw15:arg7* was tested. Several constructs were made for this purpose, as shown

Table 1 Exon / intron junctions

Position	Sequence
Consensus[a]	G↓GTGAG.....(G/A)CAG↓G
Exons 1/2	G↓GTGAG.....GCAG↓G
Exons 2/3	G↓GTGCG.....ATAG↓G
Exons 3/4	G↓GTGCG.....ACAG↓G
Exons 4/5	G↓GTGCG.....GCAG↓G
Exons 5/6	G↓GTGAG.....AAAG↓G
Exons 6/7	G↓GTGAG.....ACAG↓G
Exons 7/8	G↓GTGGG.....CCAG↓G
Exons 8/9	G↓GTGAG.....GCAG↓G
Exons 9/10	G↓GTGCG.....GCAG↓G
Exons 10/11	G↓GCGAG.....GCAG↓G
Exons 11/12	G↓GTGAG.....CCAG↓G
Exons 12/13	G↓GTGCG.....ACAG↓C
Exons 13/14	G↓GTGAG.....GCAG↓G

[a] *Chlamydomonas* consensus splicing sequence, with invariant nucleotides *underlined*. The consensus is taken from Silflow (1998)

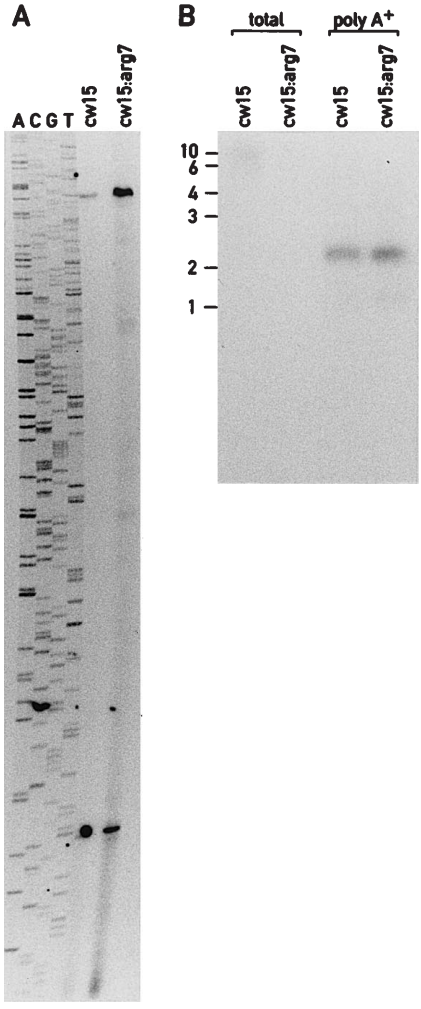


Fig. 3A, B RNA analysis. **A** Primer extension analysis was performed on total RNA using the oligonucleotide indicated in Fig. 2. The four lanes on the left show a sequencing run to estimate the size of the products, while the right-most lanes show the results for either *cw15* or *cw15:arg7*. We estimate the sizes of the products to be 52 and about 190 bases. **B** Northern analysis. Total RNA or poly(A)$^+$ RNA from the indicated strains was probed with radiolabelled cDNA and autoradiographed. The numbers on the left indicate size markers in kb

in Table 2. The 2.3-kb gel-purified linear cDNA fragment was found to transform about 1% as well as the genomic DNA present in the plasmid pARG7.8ϕ3 (Gumpel and Purton 1994; Gumpel et al. 1994). This presumably results from integration of the cDNA downstream of a functional promoter and thus would represent an instance of "promoter trapping" (O'Kane and Gehring 1987). The cDNA cloned in pBluescriptKS(-) transformed the *arg7* mutant with differing efficiencies depending on its orientation – 16% as well as the genomic sequences in one orientation and 34% as well in the other orientation. The increased transformation efficiency with respect to the linear cDNA is probably due to protection of the coding sequences from nuclease digestion by the vector DNA; that the two orientations in the cloning vector consistently give different efficiencies may be due to sequences in pKS(-) that are able to serve as a promoter and/or enhancer in *C. reinhardtii*.

The cDNA was also cloned in both orientations under the control of a strong nuclear promoter (pSP105, Stevens et al. 1996) to increase its expression in *C. reinhardtii*. These constructs contain a 794-bp fragment of the *RbcS2* promoter and 5′ UTR, including the transcription initiation site and start codon (Goldschmidt-Clermont and Rahire 1986). These clones were constructed before the orientation or sequence of the cDNA was known, and are not protein fusion constructs.

The cDNA cloned into pSP105 in the sense orientation (pRb-ARGs, Fig. 1B) transformed almost as well as the genomic clone, whereas the cDNA cloned in the anti-sense orientation transformed about 65% as well as the genomic clone (Table 2). There may be sequences present in pSP105 that are able to function as either promoters or enhancers in both orientations. Linearization of either the sense or anti-sense construct near the 5′ end of the cDNA, prior to transformation, decreased the transformation efficiency, while cleavage beyond the end of the cDNA 3′ UTR, or far from the cDNA sequence either did not alter or increased the efficiency of transformation by up to 20%. This probably indicates that the 170-bp 5′ UTR does not protect the cDNA from exonucleolytic degradation as well as the 627-bp 3′ UTR, prior to integration of the linear cDNA into the genome via its ends (Blankenship and Kindle 1992; Cerutti et al. 1997; data not shown).

We made deletions in the promoter and 3′ UTR in order to create shorter plasmids. The first 681 bp of the *RbcS2* promoter was deleted as a *Sph*I fragment, leaving a promoter of 54 bp, while the 3′ end of the cDNA was truncated at the *Xho*I site (nucleotide 1853 of the cDNA, found 104 bp beyond the end of the cloned genomic DNA, Fig. 1). The deletions were made separately and together in pRb-ARGs. Deletion of *RbcS2* promoter sequences decreased the transformation efficiency to 55% of that of genomic DNA, while deletion of the last 365 bp of the 3′ UTR decreased transformation efficiency to 23% that of the genomic DNA (see Table 2). Combining the deletions decreased transformation efficiency to 13% that the genomic DNA. These deletions were not further pursued. The nature of the 3′ UTR sequences which augment the transformation efficiency fourfold has not been further elucidated; however, it is interesting to note that they are not found in the genomic clone.

Table 2 Transformation efficiencies

Transforming DNA	Orientation	Percentage efficiency (*n*)
pARG7.8ϕ3	NA	100 ± 10 (12)
Linear cDNA	NA	1 ± 0.5 (2)
pKS18[a]	NA	16 ± 6 (3)
pKS19[a]	NA	34 ± 20 (3)
pRb-ARGs	Sense	83 ± 19 (12)
pRb-ARGøs	Antisense	65 ± 9 (4)
pRb-ARGs Δ5′	Sense	55 ± 11 (2)
pRb-ARGs Δ3′	Sense	23 ± 6 (3)
pRb-ARGs Δ5′,Δ3′	Sense	13 ± 2 (2)
Nuclear "precursor" fusion[b]	Sense	30 ± 8 (3)
Nuclear "mature" fusion[b]	Sense	19 ± 10 (3)
Chloroplast "precursor" fusion[c]	Sense	0 (2)
Chloroplast "mature" fusion[c]	Sense	0 (2)
T7-ARG del to ori	Sense	30 ± 6 (4)

[a] pKS18 and pKS19 contain the cDNA fragment cloned in opposite orientations in pKS(-). NA, not applicable
[b] The "precursor" or "mature" protein translationally fused to the *RbcS2* start codon
[c] The "precursor" or "mature" protein translationally fused to the *psbD* start codon

Possible chloroplast localization of ASL

Upon examination of the cDNA sequence, two possible in-frame translation start sites can be identified (Fig. 2). Initiation from the first of these would yield a protein with a 35-amino acid N-terminal extension that resembles a *C. reinhardtii* chloroplast transit peptide (Franzen et al. 1990). However, ASL is thought to be a cytoplasmic protein, possibly closely associated with the outer mitochondrial surface (Cohen and Kuda 1996). To determine whether the protein is targeted to the chloroplast several experiments were performed. Attempts to import the protein into isolated *C. reinhardtii* chloroplasts were unsuccessful (data not shown). We made in-frame fusions to either nuclear or chloroplast expression vectors in order to test for expression in these compartments. The protein-coding region, with or without the putative transit peptide, was amplified by PCR using primers designed to make translational fusions with the start codons found in either pSP105 or the chloroplast expression vector p*atpB*-INT-cg12. The constructs were transformed into either *cw15:arg7* using glass beads (for nuclear expression, Kindle 1990) or into *FuD50:arg7* using a particle gun (for chloroplast expression, Boynton et al. 1988). The chloroplast expression vector includes the 3′ end of the *atpB* gene and downstream sequences which are deleted in *FuD50*. Integration of the plasmid

by homologous recombination rescues the *FuD50* photosynthetic defect and transformants can be selected on minimal medium (Boynton et al. 1988).

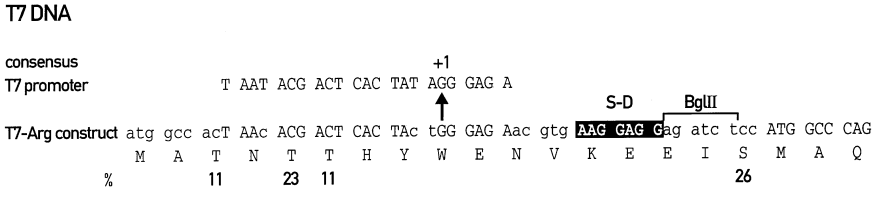
Both constructs expressed in the nucleus (i.e. with or without the putative transit peptide) were functional, based on their ability to rescue the *arg7* mutation, with the putative transit peptide-containing construct transforming slightly more efficiently than the mature construct (30% vs 19% of the efficiency of the genomic DNA, see Table 2). Thus, ASL is probably not targeted to the chloroplast. To test this further, we examined the chloroplast transformants. Although selection for restoration of the *atpB* locus alone gave numerous colonies on minimal medium supplemented with arginine, double selection of the chloroplast transformants for restoration of photosynthetic activity and arginine prototrophy gave no colonies. The gene was transcriptionally active, as an abundant 1.8-kb RNA species which hybridized to the *Arg7* cDNA was detected in the phototrophic transformants (data not shown). When the transformants were passaged on minimal or enriched plates in the absence of arginine, they were in fact able to grow slowly, and grew slightly better with each successive passage. On both minimal and enriched media, cells containing the mature construct grew better than those bearing the putative transit peptide-containing construct in the absence of arginine. The ASL specific activity also increased with each successive passage, albeit only up to about 10% of wild-type activity (data not shown). Whether this slow growth is due to leakage of the enzyme from the chloroplast or to synthesis of arginine within the chloroplast is not clear. The low specific activity observed may in part be due to the differences in codon bias between chloroplast and nuclear genes in *C. reinhardtii* (Nakamura et al. 1996). It is also possible that the ASL substrate (argininosuccinate) crosses the chloroplast envelope inefficiently. Due to this low specific activity in the chloroplast, *Arg7* cannot be used as a chloroplast marker. These combined data indicate that ASL is not a chloroplast-targeted protein in *C. reinhardtii* and that the putative transit peptide is not utilized, although the protein can be expressed and even complement the *arg7* mutation, albeit weakly, in the chloroplast. The ability of the cDNA without the putative transit peptide to complement from the nucleus is clear evidence that the protein functions in the cytosol. However, from these data it is still not clear which start codon is used in vivo (see Discussion).

Use of *ARG7* as a *C. reinhardtii-E. coli* shuttle marker

One of the frustrations of tagging mutagenesis is that the tagging gene can become uncoupled from either the *E. coli* selectable marker (usually *bla*), and/or the *E. coli* origin of replication, upon integration into the nucleus of *C. reinhardtii*. Subsequent plasmid rescue of the tagged gene in *E. coli* is therefore hampered (Tam and Lefebvre 1993; Pazour et al. 1995; Smith and Lefebvre, 1996). We noticed that the *Arg7* cDNA cloned under the control of the chloroplast *atpA* promoter is able to complement an *argH* deletion in *E. coli* (data not shown; *argH* encodes ASL in *E. coli*; Maas 1961). This observation led us to construct a *C. reinhardtii-E. coli* shuttle vector, in which promoters for *C. reinhardtii* and *E. coli* are present upstream of the cDNA. This vector should be easier to rescue from *C. reinhardtii* after tagging transformation, since the selectable marker is the same in both organisms, and thus must be intact. This obviates the need for close physical linkage between the *Arg7* DNA and a bacterial selectable marker in the tagged mutant.

To construct this vector we inserted a phage T7 promoter and a Shine-Dalgarno sequence between the *RbcS2* start codon and the second initiation codon, so that the T7 promoter should be retained after *C. reinhardtii* transformation (see Fig. 4). We chose the T7 promoter as it is short and has been well characterised (Ikeda et al. 1992); individual nucleotide changes at each of the three positions altered in the T7 promoter moderately reduce the ability of the promoter to function in vivo (Ikeda et al. 1992). We inserted the extra sequences such that they encode an in-frame 17-amino acid N-terminal extension. ASL proteins with an N-terminal extension are able to complement the *arg7* mutation (Table 2 and data not shown). Some of the codons inserted into the cDNA by this process are poorly represented in *C. reinhardtii* nuclear DNA (Nakamura et al.

Fig. 4 Construction of T7-ARG. The T7 consensus promoter (−17 to +6) is shown in *upper case letters* on the *upper* line. Changes from the consensus are shown as *lower case letters* on the *second* line, the expected translation product is shown on the *third* line. The initial ATG is the *RbcS2* start codon, the T7 transcription start site is indicated by +1. The Shine-Dalgarno sequence (S-D) is highlighted as *white letters* on a *black background*. A *Bgl*II site used to diagnose the presence of the T7 promoter is found immediately after the S-D. Other *lower case letters* indicate nucleotides chosen to maximize the representation of frequently-used nuclear codons and GC content. The numbers on the *bottom line* indicate the frequency of codon usage in nuclear genes when they are used less than 30% of the time (Nakamura et al. 1996)



1996); however, they are used in the *Arg7* cDNA at least twice each (Purton and Rochaix 1995 and present work). A further 420-bp of DNA between the bacterial origin of replication and the *RbcS2* promoter was removed in order to increase the probability that the origin of replication would remain coupled to the selectable marker (see Materials and methods).

Appropriate recipient strains of *E. coli* and *C. reinhardtii* were transformed with the T7-ARG-containing DNA. The T7 promoter-containing construct rescues the *argH* mutation in a non-T7 RNA polymerase dependent manner. However, rescue is absolutely dependent upon the presence of the T7 promoter sequences in the construct, suggesting that a functional *E. coli* promoter has been inserted into the rescuing plasmid. The T7 promoter-containing construct rescues the *arg7* mutation in *cw15:arg7* 24% as well as the genomic DNA, giving approximately 100 colonies per plate.

Discussion

We describe here the characterization of a cDNA for the selectable *C. reinhardtii* nuclear marker *ARG7*. When cloned under the control of the *RbcS2* promoter the 2217-bp cDNA transforms the nuclear mutant *arg7* almost as well as the genomic clone. We have examined some of the transformants by Southern and PCR analysis; the vector sequences insert at random into the genomic DNA, and the endogenous copy of *Arg7* is not disrupted by the cDNA sequences, making it a useful marker for transformation mutagenesis (data not shown). An integrated copy of the cDNA can be distinguished from the endogenous gene by its size, lack of introns, and, in the case of the T7 construct, by the T7 sequence which can be used as a tag. The availability of the cDNA may facilitate gene knock-out by homologous recombination, since the absence of repetitive sequences (which may increase the probability of insertion of partial copies of the DNA; Gumpel et al. 1995) will reduce the incidence of integration into the many sites that have the same sequences. Interestingly the cDNA will also complement the *arg7* mutation when it is introduced as a linear fragment of DNA, suggesting that it could be used in experiments designed to identify promoters – for example, under certain growth conditions (“promoter trapping”; O’Kane and Gehring 1987; Haring and Beck 1997).

An additional advantage of the cDNA is that its comparatively small size would allow a second, non-selectable gene to be cloned into the same vector. Such vectors containing two cDNAs could be used in cotransformation studies, greatly increasing the probability that the second cDNA would be cotransformed into the same region of the genome, and making it more likely that the non-selectable marker will be expressed at a high level (Haring and Beck 1997; Periz and Keller 1997).

The sequences found in the vector pSP105 (−788 to +6 of the *RbcS2* promoter, where +1 is the start codon; Goldschmidt-Clermont and Rahire 1986; Stevens et al. 1996), drive expression when the cDNA is cloned in either orientation in the plasmid, indicating that the *RbcS2* promoter may also contain enhancer sequences. This was previously suggested in studies with the *CRY1-1* gene, where an *RbcS2* promoter fragment from −905 to −309 was cloned in a reverse orientation upstream of *CRY1-1* (Nelson et al. 1994). In a study in which different lengths of *RbcS2* promoter were fused to the *aadA* reporter gene, a 300-bp promoter fragment gave a higher transformation frequency than 1030, 650 or 180 bp of promoter (Cerutti et al. 1997). In the present study, when the promoter was truncated upstream of −54 bp, the construct transformed poorly, suggesting that some sequences required for transcription had been deleted. These combined data suggest that there are important promoter functions between −300 and −54 bp, and possible enhancer functions between −788 and −54 in the *RbcS2* promoter.

It has been suggested that the presence of introns is required for high-level expression of nuclear genes in *C. reinhardtii* (Stevens et al. 1996). Thus we were initially surprised to find that the cDNA under the control of the *RbcS2* promoter transforms well in the absence of introns. Expression of the cDNA for radial spoke protein 3 restores motility in vivo, showing that introns are not required for the expression of this gene (Diener et al. 1993), but its ability to restore motility has not been directly compared to that of the genomic DNA. Several selectable *C. reinhardtii* cDNAs recently cloned in our laboratory complement their respective mutations (J. Nickelsen, E. Boudreau, F. Vaistij, K. Perron, and C. Rivier, unpublished results), although it is not yet clear if they complement as well as their genomic DNAs. The non-selectable *psaF* cDNA could not be expressed in cotransformation studies with *CRY1-1*, although the genomic DNA is expressed under identical circumstances. In this case the presence of introns appears to increase the expression of the cDNA significantly (M. Hippler, D. Stevens, J.-D. Rochaix, unpublished results). In *Volvox carteri* (a closely related alga) insertion of either the first, or ninth and tenth introns into the *nitA* cDNA increases the transformation efficiency of *nitA* mutants by two orders of magnitude (Gruber et al. 1996). Thus it is not yet clear whether this high transformation capacity will be a general characteristic of *C. reinhardtii* cDNAs.

There are two in-frame start codons present in the *Arg7* cDNA. If translation were to initiate from the first ATG, the protein would include an N-terminal extension which has the hallmarks of a chloroplast transit peptide. This would be unusual, as ASL is a cytoplasmic enzyme involved in the urea cycle in animals (Takiguchi et al. 1989) and may be localized on the outer surface of the mitochondrion (Cohen and Kuda 1996). Deletion of this possible transit peptide did not inhibit the ability of the enzyme to complement the *arg7* mutation, suggesting that it is not a transit peptide. Transformation of the chloroplast genome with the cDNA coding sequence

under the control of a chloroplast promoter did not complement the *arg7* mutation in direct selection experiments, although these strains do transcribe the cDNA, contain some ASL activity and will grow slowly in the absence of exogenous arginine. These data strongly suggest that the protein is not targeted to the chloroplast, but they do not demonstrate which ATG is used in vivo. Comparisons of the *Volvox* and *C. reinhardtii* cDNA sequences show that both the length and sequence of the second exon are conserved between the two organisms, but these similarities do not extend further upstream into the first exon. Furthermore, the *Volvox* cDNA contains neither a possible transit peptide nor the first in-frame ATG (W. Mages, R. Schmitt and A. Schoberth, personal communication), strengthening the suggestion that the second ATG is the true start codon.

Inspection of Table 2 shows that the constructs in which the cDNA is translationally fused to the *RbcS2* start codon (nuclear and chloroplast fusions, T7-ARG del to ori) give a lower transformation efficiency than the non-fused constructs (pRb-ARGs, and Δ5′, pRb-ARGαs). The reason for this difference is not clear, although it is possible that expression of the ASL gene is higher when the ARG start site is preceded by a short stretch of the *Arg7* 5′ UTR rather than by the entire *RbcS2* 5′ UTR.

When expressed under the control of an appropriate promoter, the cDNA will complement the *E. coli argH* mutation. This observation has led us to create a plasmid in which the cDNA is under the control of both *C. reinhardtii* and *E. coli* promoters. The *E. coli* promoter is found just downstream of the *C. reinhardtii* promoter, within the region coding for an N-terminal extension. Upon transformation of an *arg7* strain and selection for arginine prototrophy, the *E. coli* promoter should remain intact, and therefore, during plasmid rescue, the cDNA should be able to complement the *E. coli argH* mutation. By using partial digests, pieces of the *C. reinhardtii* integration site should also be cloned. This use of the same cDNA as the selectable marker in both organisms increases the probability of cloning interrupted genes, which may be hampered by uncoupling of the selectable marker (usually ampicillin resistance) from the *Arg7* DNA (Tam and Lefebvre 1993; Pazour et al. 1995; Smith and Lefebvre 1996).

Acknowledgements We thank H. Sommer for constructing the cDNA library, W. Mages, R. Schmitt and A. Schoberth for communicating unpublished *V. carteri* sequences, J. Geiselmann for discussions about *E. coli* RNA polymerase promoters, S. Purton for oligonucleotides and N. Roggli and P. Damay for figures. We also thank the members of the Rochaix lab for their many suggestions, especially K. Redding and W. Zerges for critical reading of the manuscript. This work was supported by grant 31–50895.97 from the Swiss National Science Foundation.

References

Abramson RD, Barbosa P, Kalumuck K, O'Brien WE (1991) Characterization of the human argininosuccinate lyase gene and analysis of exon skipping. Genomics 10:126–132

Adam M, Lentz KE, Loppes R (1993) Insertional mutagenesis to isolate acetate-requiring mutants in *Chlamydomonas reinhardtii*. FEMS Microbiol Lett 110:265–268

Amaya Y, Matsubasa T, Takiguchi M, Kobayashi K, Saheki T, Kawamoto S, Mori M (1988) Amino acid sequence of rat argininosuccinate lyase deduced from cDNA. J Biochem (Tokyo) 103:177–181

Barbosa P, Cialkowski M, O'Brien WE (1991a) Analysis of naturally occurring and site-directed mutations in the argininosuccinate lyase gene. J Biol Chem 266:5286–5290

Barbosa P, Wistow GJ, Cialkowski M, Piatigorsky J, O'Brien WE (1991b) Expression of duck lens delta-crystallin cDNAs in yeast and bacterial hosts. Delta 2-crystallin is an active argininosuccinate lyase. J Biol Chem 266:22319–22322

Beacham IR, Schweitzer BW, Warrick HM, Carbon J (1984) The nucleotide sequence of the yeast *ARG4* gene. Gene 29:271–279

Blankenship JE, Kindle KL (1992) Expression of chimeric genes by the light-regulated *cabII*-1 promoter in *Chlamydomonas reinhardtii*: a *cabII*-1/*nit1* gene functions as a dominant selectable marker in a *nit1*$^-$*nit2*$^-$ strain. Mol Cell Biol 12:5268–5279

Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark KB, Sanford JC (1988) Chloroplast transformation in Chlamydomonas with high velocity microprojectiles. Science 240:1534–1538

Brown RE, Jones KL, Hyland KJ (1989) Protein measurement using bicinchoninic acid: elimination of interfering substances. Anal Biochem 180:136–139

Cerutti H, Johnson AM, Gillham NW, Boynton JE (1997) A eubacterial gene conferring spectinomycin resistance on *Chlamydomonas reinhardtii*: integration into the nuclear genome and gene expression. Genetics 145:97–110

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Cohen NS, Kuda A (1996) Argininosuccinate synthetase and argininosuccinate lyase are localized around mitochondria: an immunocytochemical study. J Cell Biochem 60:334–340

Csonka LN, Clark AJ (1980) Construction of an Hfr strain useful for transferring *recA* mutations between *Escherichia coli* strains. J Bacteriol 143:529–530

Davies JP, Weeks DP, Grossman AR (1992) Expression of the arylsulfatase gene from the β_2-tubulin promoter in *Chlamydomonas reinhardtii*. Nucleic Acids Res 20:2959–2965

Davies JP, Yildiz F, Grossman AR (1994) Mutants of *Chlamydomonas* with aberrant responses to sulfur deprivation. Plant Cell 6:53–63

Day A, Debuchy R, van Dillewijn J, Purton S, Rochaix J-D (1990) Studies on the maintenance of cloned DNA fragments in the nuclear genome of the green alga *Chlamydomonas reinhardtii*. Physiol Plant 78:254–260

Debuchy R, Purton S, Rochaix J-D (1989) The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the *ARG7* locus. EMBO J 8:2803–2809

Dhanakoti SN, Brosnan ME, Herzberg GR, Brosnan JT (1992) Cellular and subcellular localization of enzymes of arginine metabolism in rat kidney. Biochem J 282:369–375

Diener DR, Ang LH, Rosenbaum JL (1993) Assembly of flagellar radial spoke proteins in *Chlamydomonas*: identification of the axoneme binding domain of radial spoke protein 3. J Cell Biol 123:183–190

Franzen LG, Rochaix J-D, von Heijne G (1990) Chloroplast transit peptides from the green alga *Chlamydomonas reinhardtii* share features with both mitochondrial and higher plant chloroplast presequences. FEBS Lett 260:165–168

Goldschmidt-Clermont M, Rahire M (1986) Sequence, evolution and differential expression of the two genes encoding variant small subunits of ribulose bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii*. J Mol Biol 191:421–432

Gorman DS, Levine RP (1966) Cytochrome *f* and plastocyanin: their sequence in the photoelectric transport chain. Proc Natl Acad Sci USA 54:1665–1669

Gruber H, Kirzinger SH, Schmitt R (1996) Expression of the *Volvox* gene encoding nitrate reductase: mutation-dependent activation of cryptic splice sites and intron-enhanced gene expression from a cDNA. Plant Mol Biol 31:1–12

Gumpel NJ, Purton S (1994) Playing tag with *Chlamydomonas*. Trends Cell Biol 4:299–301

Gumpel NJ, Rochaix J-D, Purton S (1994) Studies on homologous recombination in the green alga *Chlamydomonas reinhardtii*. Curr Genet 26:438–442

Gumpel NJ, Ralley L, Girard-Bascou J, Wollman F-A, Nugent J, Purton S (1995) Nuclear mutants of *Chlamydomonas reinhardtii* defective in the biogenesis of the cytochrome b_6f complex. Plant Mol Biol 29:921–932

Haring MA, Beck CF (1997) A promoter trap for *Chlamydomonas reinhardtii*: development of a gene cloning method using 5′ RACE-based probes. Plant J 11:1341–1348

Harris EH (1989) The *Chlamydomonas* source book: a comprehensive guide to biology and laboratory use. Academic Press, San Diego

Hoyer LL, Magee BB, Rikkerink EH, Scherer S (1994) The *ARG4* gene of *Candida albicans*. Gene 142:213–218

Ikeda RA, Ligman CM, Warshamana S (1992) T7 promoter contacts essential for promoter activity *in vivo*. Nucleic Acids Res 20:2517–2524

Iwase K, Yamauchi K, Ishikawa K (1995) Cloning of cDNAs encoding argininosuccinate lyase and arginase from *Rana catesbeiana* liver and regulation of their mRNAs during spontaneous and thyroid hormone-induced metamorphosis. Biochim Biophys Acta 1260:139–146

Johanningmeier U, Bodner U, Wildner GF (1987) A new mutation in the gene coding for the herbicide-binding protein in *Chlamydomonas*. FEBS Lett 211:221–224

Kho C-J, Zarbl H (1992) A rapid and efficient protocol for sequencing plasmid DNA. BioTechniques 12:228–229

Kindle KL (1990) High-frequency transformation of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 87:1228–1232

Kindle KL, Schnell RA, Fernandez E, Lefebvre PA (1989) Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. J Cell Biol 109:2589–2601

Maas W (1961) Studies on repression of arginine biosynthesis in *Escherichia coli*. CSH Symp Quant Biol 26:183–191

Matagne RF (1978) Fine structure of the *arg-7* cistron in *Chlamydomonas reinhardi*. Mol Gen Genet 160:95–99

Matsubasa T, Takiguchi M, Amaya Y, Matsuda I, Mori M (1989) Structure of the rat argininosuccinate lyase gene: close similarity to chicken delta-crystallin genes. Proc Natl Acad Sci USA 86:592–596

McInnes RR, Shih V, Chilton S (1984) Interallelic complementation in an inborn error of metabolism: genetic heterogeneity in argininosuccinate lyase deficiency. Proc Natl Acad Sci USA 81:4480–4484

Miller JH (1992) A Short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Nakamura Y, Wada K-n, Wada Y, Doi H, Kanaya S, Gojobori T, Ikemura T (1996) Codon usage tabulated from the international DNA sequence databases. Nucleic Acids Res 24:214–215

Nelson JA, Savereide PB, Lefebvre PA (1994) The *CRY1* gene in *Chlamydomonas reinhardtii*: structure and use as a dominant selectable marker for nuclear transformation. Mol Cell Biol 14:4011–4019

Nickelsen J, van Dillewijn J, Rahire M, Rochaix J-D (1994) Determinants for stability of the chloroplast *psbD* RNA are located within its short leader region in *Chlamydomonas reinhardtii*. EMBO J 13:3182–3191

Nickerson JM, Wawrousek EF, Borras T, Hawkins JW, Norman BL, Filpula DR, Nagle JW, Ally AH, Piatigorsky J (1986) Sequence of the chicken $\delta 2$ crystallin gene and its intergenic spacer. J Biol Chem 261:552–557

O'Brien WE, McInnes R, Kalumuck K, Adcock M (1986) Cloning and sequence analysis of cDNA for human argininosuccinate lyase. Proc Natl Acad Sci USA 83:7211–7215

O'Kane CJ, Gehring WJ (1987) Detection in situ of genomic regulatory markers in *Drosophila*. Proc Natl Acad Sci USA 84:9123–9127

Ohno M, Sakamoto H, Yasuda K, Okada TS, Shimura Y (1985) Nucleotide sequence of a chicken δ-crystallin gene. Nucleic Acids Res 13:1593–1606

Pazour GJ, Sineshchekov OA, Witman GB (1995) Mutational analysis of the phototransduction pathway of *Chlamydomonas reinhardtii*. J Cell Biol 131:427–440

Periz G, Keller LR (1997) DNA elements regulating α_1-tubulin gene induction during regeneration of eukaryotic flagella. Mol Cell Biol 17:3858–3866

Puissant C, Houdebine L-M (1990) An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. BioTechniques 8:148–149

Purton S, Rochaix J-D (1995) Characterisation of the *ARG7* gene of *Chlamydomonas reinhardtii* and its application to nuclear transformations. Eur J Phycol 30:141–148

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Silflow CD (1998) Organization of the nuclear genome. In: Rochaix J-D, Goldschmidt-Clermont M, Merchant S (eds) Molecular biology of Chlamydomonas: chloroplasts and mitochondria. Kluwer, Dordrecht

Simpson A, Bateman O, Driessen H, Lindley P, Moss D, Mylvaganam S, Narebor E, Slingsby C (1994) The structure of avian eye lens delta-crystallin reveals a new fold for a superfamily of oligomeric enzymes. Nature Struct Biol 1:724–734

Simpson A, Moss D, Slingsby C (1995) The avian eye lens protein delta-crystallin shows a novel packing arrangement of tetramers in a supramolecular helix. Structure 3:403–412

Smith EF, Lefebvre PA (1996) *PF16* encodes a protein with armadillo repeats and localizes to a single microtubule of the central apparatus of *Chlamydomonas* flagella. J Cell Biol 132:359–370

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85

Stevens D, Rochaix J-D, Purton S (1996) The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. Mol Gen Genet 251:23–30

Takiguchi M, Matsubasa T, Amaya Y, Mori M (1989) Evolutionary aspects of urea cycle enzyme genes. BioEssays 10:163–166

Tam L-W, Lefebvre PA (1993) Cloning of flagellar genes in *Chlamydomonas reinhardtii* by DNA insertional mutagenesis. Genetics 135:375–384

Turner MA, Simpson A, McInnes RR, Howell PL (1997) Human argininosuccinate lyase: a structural basis for intragenic complementation. Proc Natl Acad Sci USA 94:9063–9068

Walker DC, Christodoulou J, Craig HJ, Simard LR, Ploder L, Howell PL, McInnes RR (1997) Intragenic complementation at the human argininosuccinate lyase locus. J Biol Chem 272:6777–6783

Walker DC, McCloskey DA, Simard LR, McInnes RR (1990) Molecular analysis of human argininosuccinate lyase: mutant characterization and alternative splicing of the coding region. Proc Natl Acad Sci USA 87:9625–9629

Wistow G, Piatigorsky J (1987) Recruitment of enzymes as lens structural proteins. Science 236:1554–1556

Wistow GJ, Piatigorsky J (1990) Gene conversion and splice-site slippage in the argininosuccinate lyases/delta-crystallins of the duck lens: members of an enzyme superfamily. Gene 96:263–270

Woessner JP, Masson A, Harris EH, Bennoun P, Gillham NW, Boynton JE (1984) Molecular and genetic analysis of the chloroplast ATPase of Chlamydomonas. Plant Mol Biol 3:177–190