Nuclear Transformation and Gene Expression in

Chlamydomonas reinhardtii.

A thesis submitted for the degree of

Doctor of Philosophy

by

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ABSTRACT

Although nuclear transformation of *C. reinhardtii* using homologous markers for mutant rescue is now a routine procedure, attempts to express foreign genes in this alga have been conspicuously unsuccessful. Several reasons have been proposed including a biased codon usage in *C. reinhardtii* nuclear genes, the methylation of foreign DNA or the incompatibility/absence of untranslated elements in the introduced DNA such as promoters, polyadenylation signals and introns.

In this thesis evidence is presented for the expression of a bacterial gene in *C. reinhardtii*. A construct in which the phleomycin resistance gene (*ble*) of *Streptoalloteichus hindustanus* is flanked by the 5' and 3' untranslated regions of a *C. reinhardtii* nuclear gene, was introduced into an *arg* mutant by cotransformation with the *ARG* marker. Screening of *arg* transformants revealed that ~3% are resistant to Pm. However, Southern analysis showed that ~90% of the transformant genomes contain the *ble* gene. Western analysis of co-transformants using antibodies against the Sh.ble protein revealed that it is present in the PmR cells. Crosses between Pm65 (a PmR transformant containing a single copy of the *ble* gene) and wild type cells demonstrated that the PmR phenotype segregates in predicted Mendelian ratios in progeny. Southern analysis of these progeny has revealed that the *ble* gene co-segregates with the PmR phenotype.

By modification of the transformation method a protocol has been developed that allows the direct selection of nuclear transformants at a low level using the *ble* construct. Studies into parameters affecting the rate of
transformation were conducted and the results presented.

The sequence of a cDNA clone for the gene *RPL41* encoding the ribosomal protein L41 subunit gene is presented. The potential of using a mutated version of *RPL41* as a selectable marker in nuclear transformation to confer resistance to the translation inhibitor cycloheximide is considered.
For Yvonne, Caitlin and Joseph.
ACKNOWLEDGEMENTS

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Finally, by way of an apology, I would like to thank Yvonne for her tolerance over the past few months and her stoic acceptance of my abandonment of any domestic responsibility.
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<td>Ap</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ars</td>
<td>autonomously replicating sequence</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bp (kbp)</td>
<td>base pairs (kilobase pairs)</td>
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<tr>
<td>CaMv</td>
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<td>cDNA</td>
<td>copy deoxyribonucleic acid</td>
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<td>Da (kDa)</td>
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<td>dATP (A)</td>
<td>2' deoxyadenosine 5'-triphosphate</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA.Na₂</td>
<td>diaminoethanetetra-acetic acid. disodium salt</td>
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</tr>
<tr>
<td>EtBr</td>
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<tr>
<td>IPTG</td>
<td>isopropylthio-β-galactoside</td>
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<tr>
<td>Kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth medium</td>
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MBN
MIC
mRNA
mt
Neo
OLB
PCR
PEG
Pm
REMI
RNA
rpm
SAR
SDS
SSC
Tn
TAP
TBE
Tc
TE
Tricine
Tris
U
UTR
v/v
wt
w/v
X-gal
XSO₄
YAC
YCp
YPEp
YEp
YRp
YT

mung bean nuclease
minimum inhibitory concentration
messenger ribonucleic acid
mating type
neomycin
oligo labelling buffer
polymerase chain reaction
polyethylene glycol
phleomycin
restriction enzyme-mediated integration
ribonucleic acid
revolutions per minute
scaffold attachment region
sodium dodecyl sulphate
salt sodium citrate
transposon
tris acetate phosphate medium
tris borate EDTA
tetracycline
Tris-EDTA
tris(hydroxymethyl)methylglycine
tris(hydroxymethyl)aminoethane
unit (of restriction enzyme)
untranslated region
volume for volume
wild type
weight for volume
5-bromo-4-chloro-3-indolyl-β-D-galactoside
5-bromo-4-chloro-3-indolyl-sulphate
yeast artificial chromosome
yeast centromere plasmid
yeast episomal plasmid
yeast replicating plasmid
yeast tryptone medium
The force that through the green fuse drives the flower
Drives my green age; that blasts the roots of trees
Is my destroyer.

Dylan Thomas
CHAPTER 1

INTRODUCTION
1.1 *Chlamydomonas reinhardtii* as a model system

1.1.1 Historical perspective

The unicellular green biflagelate alga *Chlamydomonas reinhardtii* (figure 1.1) has been used (for many years) as a model system for studying diverse aspects of biochemistry, cell biology and genetics and has itself been the subject of investigation for over a century (Trainor & Cain, 1986).

Of the order Volvocales and family Chlamydomonadaceae (Bold and Wynne, 1985), the genus *Chlamydomonas* contains several hundred described species (Ettl, 1976). The genus has a wide distribution (Bishoff, 1959), and species are not limited to marine and freshwater environments but are also found in soil (Hilton & Trainor, 1963) and in the atmosphere (Brown *et al.* 1964). The species *C. reinhardtii* was isolated from nature by Reinhardt in 1876 who believed it to be an example of *Chlamydomonas pulvisculus* (Reinhardt, 1876). However, studies by Dangeard in 1888 revealed the isolate to be a separate species and he renamed it *C. reinhardtii* in honour of Reinhardt (Dangeard, 1888).

Although the presence of a mating cycle and even the necessity for starvation to induce a mating reaction was discovered quite early (Klebs, 1869), the majority of early work with *Chlamydomonas* was descriptive (*e.g.* Kater, 1929; Strehlow, 1929), although Pascher (1918) had reported the segregation of various characteristics in sexual crosses. The use of *C. reinhardtii* as a genetics model progressed when Smith and others began isolating zygospores from nature and germinating these (*e.g.* Smith, 1946; Lewin, 1949) thus obtaining mating pairs from a single isolate. The majority of the diverse collection of laboratory strains available in the different culture collections worldwide (*e.g.* Harris, 1984) belong
Figure 1.1 Schematic diagram of a *Chlamydomonas reinhardtii* cell. C, chloroplast; E, eyespot; ER, endoplasmic reticulum; F, flagella; G, golgi; M, mitochondria; N, nucleus; P, pyrenoid; V, vacuole. From a drawing by Keith Roberts.
to a traceable lineage descended from an original mating pair, 137c (mt+) and 137c (mt-) isolated by Smith in 1945. Genetic analysis using *C. reinhardtii* blossomed in the 1950s. The discovery of the non-Mendelian inheritance of certain antibiotic resistances (Sager, 1954) and the construction of the first genetic map for the species (Ebersold and Levine, 1959; Ebersold et al., 1962) raised the status of *C. reinhardtii* as a genetics model, and on this foundation the experimental work of subsequent decades has been built.

1.1.2 *Advantages of C. reinhardtii as a model system*

*C. reinhardtii* has a number of advantages that make it an ideal choice as a model system for studying a wide variety of biological phenomena. Firstly the ease of culture: *C. reinhardtii* can be maintained on inexpensive and easily prepared media (Gorman and Levine, 1965; Surzycki, 1971). Stocks can be maintained on media solidified with agar for up to eight weeks without restreaking, however these vegetative cells can be rapidly cultured in liquid medium (mean generation time of approximately eight hours) to produce large quantities of material for analysis. There are large culture collections available worldwide (e.g. Harris, 1984), containing a broad spectrum of both wild type and well characterised mutant strains.

*C. reinhardtii* has a small (1x10^5 kbp) haploid genome (Weeks, 1992), and both dominant and recessive mutants can therefore be produced without being masked by a wild type allele. Under appropriate environmental conditions, *C. reinhardtii* undergoes a simple sexual cycle (Sager and Granick, 1954) (figure 1.2). In conditions of nitrogen starvation, vegetative cells undergo gametogenesis. These haploid gametes can fuse with a gamete of the opposite mating type to form a diploid zygote. Following meiosis, a tetrad containing four
Figure 1.2 Life cycle of *Chlamydomonas reinhardtii*. From a drawing by Karen VanWinkle Swift.
haploid progeny is produced in which nuclear genes are inherited in Mendelian ratios (2:2) and organellar genes are inherited uniparentally (chloroplast genes from mt+ parent and mitochondrial genes from mt- parent) thus allowing classical genetic analysis from crosses.

Several features of C. reinhardtii make it well suited to studying specific biological phenomena. For example, the photosynthetic apparatus of C. reinhardtii closely resembles that of higher plants. The fact that cells are able to dispense with photosynthesis when grown on a reduced carbon source such as acetate (Levine, 1968) means that otherwise lethal photosynthetic mutations can be produced. This allows a genetic study of photosynthetic function. Furthermore, C. reinhardtii possesses a single, large (ca. 40% of the total cell volume), chloroplast with a relatively low copy number (80 - 100) of the chloroplast genome. This has led to the development of an efficient transformation system for this organelle (Boynton et al., 1988) and the study of chloroplast-encoded photosynthetic components using reverse genetic techniques.

Another fruitful area of Chlamydomonas research is motility. C. reinhardtii possess flagella with a classic 9+2 axoneme structure (Ringo, 1967), that exhibit both flagellar and ciliary motility. The dissection of flagellar motility using Chlamydomonas has been studied since the generation of motility mutants in the 1950s (Lewin, 1954) and Chlamydomonas is now one of the main organisms used for genetic and biochemical analysis of eukaryotic flagellar assembly and motility (Johnson, 1995).

The possession of an eyespot (Sager and Palade, 1957) means that Chlamydomonas is also used as a model for many photoresponses (Witman, 1993) and the attractiveness of a single cell system that allows for combined genetic and biochemical studies has led to Chlamydomonas being used for the
investigation of a wide range of metabolic pathways including, respiration, nitrogen assimilation, lipid metabolism and sulphur metabolism (Harris, 1989).

1.2 The need for a transformation system

It became apparent from the early 1980s that a major limiting factor in using C. reinhardtii as a model system was the absence of a procedure for introducing modified or novel DNA sequences into the genome of this alga (Harris, 1989). The ability to introduce and express exogenous DNA is a powerful tool and is a necessity for many molecular biological techniques. The availability of transformation systems in other organisms has increased the scope of investigations that can be undertaken and increased our knowledge of the molecular basis underlying many biological phenomena.

1.2.1 Transformation of other organisms

Protocols for transforming the bacterium Escherichia coli have been available since the early 1970s when it was demonstrated that treating cells with CaCl₂ promoted uptake of bacteriophage λ DNA (Mandel & Higa, 1970) and plasmid DNA (Cohen et al., 1972). The whole science of genetic manipulation is based on the concurrent development of techniques to cut and join DNA molecules in vitro and this ability to introduce exogenous DNA into bacterial cells where it is stably maintained. The first cloning experiments using these techniques were reported over twenty years ago (Jackson et al., 1972; Lobban & Kaiser, 1973), and E. coli remains the major host organism in which genetic manipulation is performed. The amplification of specific 'clonal' fragments of DNA in E. coli is desirable for a number of reasons, for example: the amplification
of a fragment that can subsequently be used as a template for radiolabeling as a probe or as a template in a sequencing reaction; the amplification of a variety of different clones in separate hosts representing a ‘library’ of DNA from another organism, and the expression of exogenous genes and foreign protein production by linking the gene to *E. coli* controlling elements. Whilst the construction of hybrid molecules *in vitro* and their amplification in *E. coli* is routine, for certain investigations it is desirable to introduce the DNA into a different organism. This may be for the following reasons;

i) The modification of gene activity by the introduction of additional copies into the genome, or the introduction of antisense constructs.

ii) The manipulation of endogenous genes via gene disruption (gene knockout) or site directed mutagenesis.

iii) The generation of ‘tagged’ mutants as a result of the integration of transforming DNA into the genome.

iv) The complementation of mutated genes with the cloned wild-type version or homologous foreign genes - either by transformation with individual clones or by ‘shot gun’ transformation using gene libraries.

v) The expression of foreign genes, either to confer a novel phenotype in the organism or to allow production of recombinant protein.

For the synthesis of foreign proteins, choice of host is particularly important. Prokaryotic and eukaryotic genes have different structures, both in the structure and arrangement of non-translated controlling elements and notably the presence of non-translated elements (introns) which interrupt many eukaryotic coding sequences and are removed from RNA transcripts prior to translation. Eukaryotic proteins in addition often undergo post translational modification for example glycosylation. For these reasons active eukaryotic proteins will often
need to be expressed in a eukaryotic host that can splice introns and effect post translational modifications.

As a consequence of these reasons, transformation systems have been developed in organisms other than *E. coli*. Other bacteria can be transformed, notably the gram positive, spore forming, aerobe *Bacillus subtilis* in which transformation is understood in some detail (Venema, 1979). Transformation systems are also available for yeast and other lower eukaryotes. The budding yeast *Saccharomyces cerevisiae* was first transformed by fusing yeast spheroplasts with polyethylene glycol (PEG) in the presence of DNA and CaCl₂. Leucine auxotrophs were transformed to prototrophy by the integration of a wild type copy of the Leu2 gene within a chromosome (Hinnen et al., 1978). Yeast nuclei have also been transformed by agitating cells in the presence of glass beads and the transforming DNA (Costanzo & Fox, 1988). A variety of ‘shuttle vectors’ have been developed for use in *S. cerevisiae* that have the ability to replicate in *E. coli* and which can exist extrachromosomally as circular plasmid molecules in *S. cerevisiae*: The yeast episomal plasmid (YEp) which is based on the naturally occurring 2μm plasmid (Beggs, 1978), the yeast replicating plasmid (YRp) which contains a chromosomally derived autonomously replicating sequence (*ars*) (Struhl et al., 1979) and the yeast centromere plasmid (YCp) which was developed from the YRp vectors but contains a centromere and thus stabilises the plasmid in mitosis and meiosis (Clarke and Carbon, 1980). The culmination of this work has led to the production of yeast artificial chromosomes (YACs) which are linear molecules bounded by cloned telomeres (Szostak & Blackburn, 1982). As these molecules behave as mini-chromosomes very large fragments of DNA (greater than 40 kb) can be cloned into them.
The fission yeast *Schizosaccharomyces pombe* can be transformed by a number of methods including; the spheroplast method (Beach & Nurse, 1981) the use of lithium acetate (Ito *et al*., 1983), lithium chloride (Broker, 1987), electroinjection (Hashimoto *et al*., 1985) and electroporation (Hood & Stachow, 1990). *S. pombe* have been used in many molecular genetic studies. Genes have been cloned by functional complementation whereby genomic or cDNA libraries are introduced into mutant cells which are then either selected by or screened for the complementation of the mutant phenotype. Because homologous recombination occurs readily in *S. pombe* gene replacement is possible. A gene fragment containing a selectable marker can be used to transform *S. pombe* to create a null mutant (Rothstein, 1991). Alternatively genes that are essential for viability can be mutated *in vitro* and then transformed into a host and the wild type gene replaced by homologous recombination (Shortle *et al*., 1984). *S. pombe* has also been used to clone genes from other eukaryotic organisms, for example the human homologue of the fission yeast cell control cycle gene *cdc2* which was cloned by complementing an *S. pombe* *cdc2*-mutant with a human cDNA library (Lee & Nurse, 1987).

The slime mould *Dictyostelium discoideum*, which is used as a model organism for studying cell differentiation (Loomis, 1982), is another example of a lower eukaryote for which a transformation system has been developed. The first readily reproducible transformation of *D. discoideum* involved introducing a hybrid construct containing 5’ untranslated region (UTR) sequence from an endogenous gene linked to the bacterial neomycin resistance (NeoR) gene from the bacterial transposon Tn5, using a CaPO₄ precipitation method (Nellen *et al*., 1984). Transformation rates were subsequently improved by including on the
construct 3’ UTR sequences from an endogenous D. discoideum gene (Nellen & Firtel, 1985). Disruption of an endogenous gene by homologous recombination with transforming DNA has been achieved in D. discoideum (De Lozanne & Spudich, 1987) and it has also been demonstrated by pulse field gel electrophoresis that in this system transforming DNA can integrate into chromosomes at a number of separate sites within a single transformant (Cole & Williams, 1988). Interestingly, when transformation of D. discoideum is performed in the presence of a restriction enzyme together with transforming plasmid DNA that has been linearised by the same enzyme, transformation rates increase 20-fold. The DNA is found to integrate at sites determined by the added recognition site of the added enzyme in >70% of transformants. This approach has led to the cloning of genes by insertional mutagenesis (Kuspa & Loomis, 1992).

Transformation systems are not limited to prokaryotes and lower eukaryotes. Both higher plants and animals have also been transformed, thus enabling the manipulation of their genomes and an extension of the scope of molecular genetic investigations available in these organisms.

The transformation of plants is primarily achieved by the introduction of foreign DNA carried on the modified Ti plasmids of Agrobacterium tumefaciens. In nature A. tumefaciens is able to transfer the T-DNA region of the Ti plasmid into the nuclear genome of cells adjacent to a wound site. These cells are then transformed to oncogenic growth (Zaenen et al., 1974). The use of Ti plasmids of A. tumefaciens has been exploited to introduce foreign DNA to plant cells (Herrera-Estrella et al., 1983a) and the inclusion of selectable markers on the construct (Herrera-Estrella et al., 1983b) has led to the direct selection of transformed plant cells which, under appropriate hormonal regimes, can be regenerated into whole plants. Transformation with Ti plasmid vectors is limited to
dicotyledons and a few monocotyledonous species but other methods of transformation have been developed including; direct gene transfer to protoplasts (Paszkowski et al., 1984), the fusion of protoplasts to liposomes containing DNA (Deshayes et al., 1985), microinjection of protoplasts (Crossway et al., 1986), macroinjection into meristematic inflorescences (De la Pena et al., 1987) and particle bombardment (Klein et al., 1987), thus allowing the transformation of monocotyledonous plants (e.g. Gordon-Kamm et al., 1990). This technology has been used for crop improvement and has led to the production of transgenic plants that are resistant to plant pathogens (e.g. Gehrlach et al., 1987) or are resistant to herbicides (e.g. De Block et al., 1987). In addition the introduction of ‘antisense’ constructs (genes in the reverse orientation linked to a promoter such that an RNA molecule complementary to the wild type gene transcript is produced) has led to the silencing of specific genes in plants, for example the antisense inhibition of the polygalacturonase gene in tomatoes to slow the ripening process (Smith et al., 1988).

Although homologous recombination occurs efficiently in lower eukaryotes like yeast, other fungi and D. discoideum (Hinnen et al., 1978; De Lozanne & Spudich, 1987; Miller et al., 1987), the integration of transforming DNA into the genomes of higher eukaryotes predominantly by random (non-homologous) recombination. This makes the selection of rare homologous recombination events dependant upon an efficient selection system (Paszkowski et al., 1988). This allows gene replacement experiments in higher plants although a low ratio of homologous to random integration (less than 10-4) means that it is far from routine. The availability of transformation systems for plants has also made possible the study of developmental (Forde et al., 1989) and tissue specific (Edwards et al. 1990) gene expression in plants by the introduction of reporter
genes such as the bacterial $\beta$-glucuronidase ($uidA$) gene (Jefferson et al., 1986) linked to the promoter of the gene under investigation.

The transformation of animal cells was first achieved by the introduction of exogenous DNA to mammalian cells in tissue culture (Szybalski & Szybalski, 1962). DNA is co-precipitated with calcium phosphate and the insoluble granules are taken up by the cells by phagocytosis. Some of the DNA is stably integrated into the nuclear genome and expressed (Graham & Van der Eb, 1973). Modification of the basic protocol has led to increased transformation efficiency with this method (Chu & Sharp, 1981). Other methods for the transformation of animal cells in culture include: the fusion of cells with bacterial protoplasts containing the transforming DNA (Schaffner, 1980); microinjection (Kondoh et al., 1983); electroporation (Neumann et al., 1982) and lipofection (Felgner et al., 1987). One of the first selectable markers used was the thymidine kinase ($tk$) gene which was introduced to TK- cell lines. Transformants were selected by growth in HAT medium (containing hypoxanthine, aminopterine and thymidine) as aminopterine & hypoxanthine are toxic to $tk$-cells*. The introduction of a second, unlinked, non-selected DNA species was performed by co-transformation (Wigler et al., 1979). However this limited transformation to cell lines with a TK- phenotype. Subsequently many dominant selectable markers have been developed, for example the bacterial Neo$^R$ gene (Berg, 1981). Vectors for the transformation of mammalian cell culture lines have also been developed including ones based on the promoters and 3' UTR sequences of simian virus (pSV2) and Rous sarcoma virus (pRSV) genes. These vectors have been engineered to contain selectable markers such as the Neo$^R$ gene e.g. pSV2-neo (Southern & Berg 1982) and pRSV-neo (Gorman et al., 1983). In co-

* Thymidine is included in the media as the substrate for thymidine kinase for the synthesis of dTTP by the salvage pathway.
transformations (e.g. Wigler et al., 1979) the selected DNA, the unselected DNA and the carrier DNA form large concatameric structures that are integrated at (usually) a single random site in the genome but are susceptible to deletion at a high frequency (Perucho et al., 1980). Using the pSV2 or pRSV vectors results in the integration of mainly single copies, again at random chromosomal locations (e.g. Southern & Berg, 1982).

In animal cells (like plants) the predominant integration pattern is random or non-homologous recombination. To select for rare homologous recombination events one needs a system in which transformants produced in this way can be distinguished from the more common random integration. The introduction of genes with selectable phenotypes allows for such a system; either by the selective inactivation of an introduced gene or the activation of a mutated introduced gene that will occur only if integration is by homologous recombination between the endogenous and introduced copies. Thomas et al. (1986), demonstrated homologous recombination in animal cells via the recombination and activation of of a mutated test gene (neo) that would only be activated upon recombination with another mutated copy within the cell, thus enabling the selective detection of rare homologous recombination events. This system relies however upon the gene to be replaced having a selectable phenotype. An alternative system developed by Mansour et al. (1988) relies on introducing two selectable markers: one a positive marker flanked by homologous sequences, the other a negative marker located outside the region of homology. In this system transformants are selected by the positive marker. The inclusion of a negative marker on the construct allows for the ‘suicide’ selection of all illegitimate recombination events. This is achieved by including a non-toxic substrate in the medium which can be metabolised by the negative selection marker to produce a product that is
toxic to the host cell. Transformants generated by homologous recombination will result in the introduction of the positive marker but not the negative marker. Such transformants are therefore resistant to suicide selection. In the system pioneered by Mansour et al., (1988) the NeoR gene is cloned within an intron of the gene to be modified and the tk gene from herpes simplex virus (HSV) is also included at a separate location on the construct. Transformants are selected on the basis of a NeoR phenotype. Rare homologous recombinants are then selected from this population by gancyclovirR since HSV-tk+ cells are sensitive to gancyclovir.

The techniques developed for the introduction of exogenous DNA to cultured mammalian cells has led to the production of whole organisms that carry foreign DNA, for example transgenic mice. Transgenic mice can be generated in two ways. DNA can be microinjected directly into the male pronucleus of a fertilised egg which is subsequently reimplanted into pseudopregnant mice (Brinster et al., 1981). Pregnancy is allowed to go to full term and transgenic progeny are identified by isolation of DNA from a short segment of tail and hybridisation to the transgene labelled as a probe. This method leads to the production of transgenic mice in which the transgene has generally integrated at a random chromosomal location. To generate transgenic mice with homologously integrated transgenes, pluripotent embryonic stem (ES) cells must first be transformed in tissue culture and homologous recombinants selected as described above (Mansour et al., 1988). These cells can then be introduced into a developing embryo at the blastocyst stage and the embryo reintroduced to the mother as before. Chimeric mice thus generated are then crossed until a mouse derived entirely from the transgenic ES cells is produced.

The development of transgenic mice (and other animals) have opened up
many avenues of research in the field of mammalian molecular genetics: the
generation of tagged mutants by insertional mutagenesis (Palmiter & Brinster,
1986); the study of gene regulation (e.g. Grosveld et al., 1991); the production of
models for oncogenesis (Sinn et al., 1987) and as models for inherited human
diseases (Stacey et al., 1988).

1.2.2 The need for a transformation system in C. reinhardtii.

The range of investigations and applications that the transformation of
other organisms has enabled, demonstrates both the importance and the
desirability of a transformation system for C. reinhardtii. The utility of an
organism for research is greatly enhanced by a transformation system and without
it the type of experiments that are described above in section 1.2.1 would be
impossible.

1.3 Early transformation attempts in C. reinhardtii.

The first demonstration of the uptake of exogenous DNA by C.
reinhardtii was reported in 1975 (Lurquin & Behki, 1975). 3H labelled bacterial
DNA was introduced into C. reinhardtii cells by incubating them with the DNA
in the presence of the polycations diethylaminoethyl (DEAE)-dextran, poly-L-
lysine and poly-L-orthinine. No evidence for the integration of detectable
amounts of [3H]DNA was obtained, however radioactivity corresponding to the
integration of [3H]thymine recycled from the degradation of the incoming
[3H]DNA was detected. Using the poly-L-orthinine method (Lurquin & Behki,
1975) and the PEG method developed for the transformation of yeast (Hinnen et
Rochaix and colleagues presented evidence for the transformation of the *C. reinhardtii* strain *arg7-cw15* to arginine prototrophy using the corresponding yeast gene *arg4* (Rochaix & van Dillewijn, 1982; Rochaix *et al.*, 1984). However, the transformation rate was close to the reversion rate of *arg7* and although it was shown that some transformants contained the foreign DNA sequences, it was not demonstrated that this DNA was responsible for the Arg+ phenotype. Several laboratories have reported the use of bacterial genes to transform *C. reinhardtii* to kanamycin resistance (KanR). For example, Hasnain *et al.* (1985) used the pSV2neo construct that contains the aminoglycoside 3' phosphotransferase gene under the control of the simian virus early promoter (Southern & Berg, 1982) and Bingham *et al.* (1989) used the neomycin phosphotransferase II gene under the control of the octopine synthetase promoter from a Ti plasmid (*ocs-npt II*). Transformation was again at a very low level and of all the putative transformants, only one appeared to be stable. In addition, whilst bacterial DNA sequences were shown to be present, in neither case was it demonstrated that it was responsible for the KanR phenotype. The use of KanR as a selectable marker for the nuclear transformation of *C. reinhardtii* has been further questioned since it has been discovered that spontaneous kanamycin resistance appears at a high frequency due to mutations in chloroplastic ribosomal DNA (Harris *et al.*, 1989). Neither the use of *arg4* or NeoR as a selectable marker was found to be reproducible by other laboratories.
1.4 Nuclear transformation of *C. reinhardtii*.

1.4.1 The use of endogenous genes to transform mutants of *C. reinhardtii*

A breakthrough in the nuclear transformation of *C. reinhardtii* occurred when cloned endogenous genes and new transformation techniques were used to rescue mutant strains. A 'biolistic' transformation system in which recipient cells are bombarded with tungsten microprojectiles coated with DNA (Klein *et al.*, 1987) had previously been used previously to transform the chloroplast genome of *C. reinhardtii* (Boynton *et al.*, 1988). The use of this 'biolistic' technique led to the rescue of mutants with wild type copies of the argininosuccinate lyase gene *ARG1* (Debuchy *et al.*, 1989), the nitrate reductase gene *NIT1* (Kindle *et al.*, 1989) and the oxygen evolving enhancer protein 1 gene *OEE1* (Mayfield & Kindle, 1990). Using *ARG7* plates containing ~10^7 *arg7* cells were bombarded with the transforming DNA. After 24 hours recovery, cells were resuspended in one millilitre of liquid medium and replated on a selective medium (lacking exogenously added arginine). After a week, transformants appeared at 20-50 colonies/plate. Experiments using supercoiled and linear DNA were found not to affect the transformation rate. Genetic analysis of DNA from transformants revealed that integration was not at the *ARG7* locus however the possibility of homologous recombination via the extensive repetitive DNA sequences found in the *ARG7* introns and throughout the genome was not ruled out (Debuchy *et al.*, 1989). Using the *NIT1* gene, which is required for growth when nitrate is the sole nitrogen source, ~2x10^8 *nit1* cells were bombarded and subsequently selected on medium containing nitrate as the sole nitrogen source. Transformants were observed at 2-10 colonies/plate and, as with *ARG7*, the use of linear or
supercoiled DNA did not effect transformation efficiency. In Southern and northern analysis the transforming DNA and a transcript were detected and again, as with ARG7 transformants, the transforming DNA was not linked to the nit1 locus indicating non-homologous recombination. Furthermore when a second nonselected DNA species was introduced in a co-transformation with NIT1 this DNA was found to be present in some transformants (Kindle et al., 1989). The OEE1 gene was used to transform Fud44 cells (mutants lacking the OEE1 protein due to the insertion of a transposon into the OEE1 gene - Day et al., 1988). 4x10^7 or 1x10^8 cells were bombarded with OEE1 and subsequently plated on minimal medium to select for photoautotrophic growth. Transformant colonies were recovered at ~two/plate but Southern analysis revealed that ~75% were revertants due to the excision of the transposon. Again the use of linear or supercoiled DNA appeared to have no effect on efficiency of transformation and the mode of integration was non-homologous. Northern analysis of the true transformants revealed that an OEE1 transcript was present at similar levels to the endogenous OEE1 gene in WT cells (Mayfield and Kindle, 1990).

The experiments described above represent the first reproducible transformation protocols for *C. reinhardtii* and, whilst the transformation rates were low, represented a significant advance for *Chlamydomonas* research. Improvement in the transformation efficiency came with the development of the 'glass bead method' (Kindle, 1990). This is based on a method originally used to transform yeast (Costanzo & Fox, 1988). Cell-wall deficient *C. reinhardtii* cells are agitated in the presence of glass beads and the transforming DNA, and are then plated to a selective medium. A double mutant *nit1-305 cw15* lacking both a cell wall and nitrate reductase activity was transformed using this method and
plated to medium containing nitrate as the sole nitrogen source. A number of parameters affecting the efficiency of transformation were investigated and the optimum conditions were obtained by using mid log phase cells (1-2 \times 10^6 \text{ cells/ml}) resuspended at a concentration of 3 \times 10^8 \text{ cells/ml}, and agitating for 15 seconds. Linearised DNA was found to improve transformation rates and plating the cells in soft agar rather than spreading directly also showed an improvement. The most significant increase in transformation efficiency (~100-fold) was obtained by including 5% polyethylene glycol (PEG) in the transformation. However, subsequent experiments using the double mutant \textit{arg7-8 cw^d} demonstrated no change in transformation rates +/- PEG (Purton & Rochaix, 1995). Kindle also demonstrated that this technique is suitable for co-transformation by introducing a separate non-selected gene for radial spoke protein 3 along with \textit{NIT1} DNA into a \textit{nit1-}, \textit{rsp3-} double mutant (this was first treated with autolysin to remove the cell wall). Cells selected for their ability to grow on nitrate as a sole nitrogen source were subsequently screened for transformation to a motile phenotype and up to 60% were found to be co-transformants (Kindle, 1990).

\textit{C. reinhardtii} has subsequently been transformed by other methods. Electroporation has been used to introduce plasmids up to 14 \text{ kb} in size into cells with and without cell walls. Using this method, cells lacking nitrate reductase activity were transformed with the \textit{NIT1} gene although transformation rates were low (Brown \textit{et al.}, 1991). Silicon carbide whiskers have also been used to transform walled cells (Dunahay, 1993). \textit{nit1-} cells were agitated in the presence of \textit{NIT1} DNA and silicon carbide whiskers and transformants were obtained at rates comparable to the glass bead method.

Recently a chimeric gene containing \textit{RBCS2} promoter sequence fused to a
mutant form of the endogenous \textit{CRY1} gene (encodes ribosomal protein S14) has been used to transform wild type cells (Nelson \textit{et al.}, 1994). The mutated form of the gene (\textit{cry}1\textsubscript{1}) carries a mutation conferring resistance to the translation inhibitors cryptopleurine and emetine. However, as the turnover of ribosomes in vegetative cells is low, selection requiring incorporation of the mutant S14 subunit can only be achieved by inducing cells to undergo gametogenesis, where 90\% of ribosomal proteins are degraded (Siersma & Chang, 1971), and then allowing them to dedifferentiate back to the vegetative state where ribosomes are reassembled containing the mutant S14 protein (Nelson \textit{et al.}, 1994).

\textbf{1.4.2 Applications of nuclear transformation in C. reinhardtii}

Following the development of the reproducible transformation systems described above, experiments previously impossible in \textit{C. reinhardtii} have now been performed. The observation that a second, unselected DNA species could be introduced along with the selectable marker (Kindle \textit{et al.}, 1989) has led to the rescue of paralysed-flagella mutants both by biolistic transformation (Diener \textit{et al.}, 1990) and by the glass bead method (Kindle, 1990). Similarly, cells that were deficient in plastocyanin were rescued to photosynthetic growth by a co-transformation with a wild type copy of the plastocyanin gene (Quinn \textit{et al.}, 1993). This work also determined the molecular basis of the plastocyanin deficiency in the mutant to be a frame shift mutation within the gene. Transformation in \textit{C. reinhardtii} has also been used to identify the gene for which a known mutant phenotype exists. A motility mutant \textit{oda6} was identified as a dyenin mutant by transformation with a genomic clone that had previously been mapped by classical genetic techniques (Mitchell & Kang, 1991).

The observation that the integration of transforming DNA is essentially
random in *C. reinhardtii* (Debuchy *et al*., 1989; Kindle *et al*., 1989; Mayfield & Kindle, 1990) has led to the generation of mutants and the cloning of nuclear genes by insertional mutagenesis. For example, cadmium sensitive mutants and acetate requiring mutants have been isolated (Pfeifer McHugh & Spanier, 1994; Adam *et al*., 1993) and the cloning of genes has been achieved by insertional mutagenesis following transformation with the *NIT1* and *ARG7* markers (*e.g.* Tam & Lefebvre, 1993; Gumpel *et al*., 1996). An overview of the generation of mutants and cloning of genes by this method has recently been presented (Gumpel and Purton, 1994).

Chimeric genes have been constructed for transformation into *C. reinhardtii* for a variety of reasons. A fusion of the chlorophyll *a/b* binding protein gene (*CABII-1*) promoter and the *NIT1* gene coding region was used to transform *nit1* cells and it was demonstrated that accumulation of *NIT1* mRNA was dramatically stimulated by light (Blankenship & Kindle, 1992). This construct was also shown to function as a dominant selectable marker in a *nit1*·*nit2*· strain as the *CABII-1/NIT1* hybrid is expressed in the presence of ammonium whilst the endogenous *NIT1* gene is repressed in such an environment. Chimeras of this sort have also been used to study mRNA stability. A chimeric, deflagellation-induced mRNA consisting of the 5' UTR and coding region of the rubisco small sub-unit 2 gene (*RBCS2*) and the 3' UTR of the α-1 tubulin (α-1 *TUB*) gene was found to be subject to post induction degradation in the same way as the WT α-1 *TUB* gene, thus locating the stability determinant to the 3' UTR region (Baker, 1993). To investigate the role of the acetylation of α-tubulin, an *RBCS2/α-1 TUB* chimera in which the acetylatable lysine codon had been mutated was transformed into *C. reinhardtii* (Kozminski *et al*., 1993). The *RBCS2* promoter was chosen due to its
strong constitutive expression so that the gene product of the chimera would compete with WT α-tubulin.

Reporter genes for use in expression studies are widely used in other systems, particularly transgenic plants (e.g. Forde et al., 1989). Their use in *Chlamydomonas* has been investigated by a number of researchers. The bacterial β-glucuronidase gene (*uidA*) under the control of the *CABII-1* promoter was used to transform *C. reinhardtii* but transformants did not express β-glucuronidase at the level of enzyme activity or mRNA detection (Blankenship & Kindle, 1992). Methylation of the transgene in the promoter region was found to be extensive and it was suggested that this may be a mechanism by which genes recognised by *Chlamydomonas* as foreign are silenced. A more suitable reporter gene was identified in the endogenous arylsulphatase (*ARS*) gene (Davies et al., 1992). The *ARS* gene under the control of its own promoter is only expressed in sulphur-limiting conditions (De Hostos et al., 1988) and can cleave the chromogenic substrate 5-bromo-4-chloro-3-indolyl sulphate (*XSO₄*) to produce an insoluble blue product. The *ARS* gene was placed under the control of the β-2 tubulin gene (*TUB2*) promoter and introduced into *Chlamydomonas* in a co-transformation procedure. Colonies expressing the *TUB2/ARS* chimeric construct were selected by treating with *XSO₄* and selecting those in which a blue colour was generated. It was suggested that chimeric genes incorporating *ARS* could be used to investigate regulated gene expression in *Chlamydomonas* (Davies et al., 1992). This system has indeed been used to investigate the transcriptional control of *TUB2* by deleting or mutating sequences within the promoter in the chimeric *TUB2/ARS* construct and assaying the level of *ARS* activity in transformants (Davies & Grossman, 1994).
In *Chlamydomonas*, as in higher eukaryotes, homologous recombination in nuclear transformation is rare. In higher eukaryotes this type of integration was first detected by employing a system whereby the selective inactivation of an introduced gene or the activation of a mutated introduced gene will occur if integration is by homologous but not by random recombination (*e.g.* Thomas *et al.*, 1986). This approach has also been fruitfully employed to select rare homologous recombination events in *C. reinhardtii* (Sodeinde & Kindle, 1993; Gumpel *et al.*, 1994). Recently the targeted disruption of a negatively selectable gene with a copy disrupted by a selectable marker, has also been demonstrated (Nelson & Lefebvre, 1995). The *NIT8* gene which encodes a protein necessary for the assimilation of nitrate and nitrite was disrupted by cloning the *RBCS2-CRY1-1* marker within it. This construct was introduced to *NIT8*+ cells and transformants to emetine resistance were selected. By including chlorate in the selection medium transformants generated by homologous recombination between the endogenous *NIT8* gene and the disrupted copy were able to be detected because *NIT*+ cells are sensitive to chlorate. (Illegitimate recombination events would maintain the endogenous copy of *NIT8* and thus be chlorate sensitive).

Another technique that is now possible in *C. reinhardtii* is the cloning of genes by complementation. This involves the transforming of a mutant with clones from a WT genomic library. Genomic cosmid libraries of *C. reinhardtii* have been constructed and these have been used to isolate genes following the complementation of mutants (Purton & Rochaix, 1994; Zhang *et al.*, 1994).

It can be seen from the type of experiments discussed above that the development of an efficient transformation system for *C. reinhardtii* has greatly increased the scope of investigations that can be undertaken in this useful organism.
1.5 Foreign gene expression in *C. reinhardtii*.

Despite the success at transforming *C. reinhardtii* with cloned endogenous genes to complement mutants, the ability to express a foreign gene as a dominant selectable marker is still a desirable goal for a number of reasons. Firstly the markers currently used are large: *NIT1* is 9 kb (Nelson et al., 1994); *ARG7* is 7.6 kb (Purton & Rochaix, 1995) and *RBCS2-CRY1-1* is 4.5 kb (Nelson et al., 1994) In the case of *ARG7*, the marker contains much repetitive DNA (Debuchy et al., 1989). This often leads to recombination within the vector and deletion of genomic sequences flanking the site of integration (Gumpel & Purton, 1994). With the exception of the *RBCS2-CRY1-1* chimera (Nelson et al., 1994), the use of endogenous genes to transform *C. reinhardtii* requires that all transformations be performed in a mutant background and when one wishes to transform a particular strain, this must first be crossed to the transformation host strain to create the relevant double mutant (Kindle et al., 1989). Furthermore, although homologous recombination in nuclear transformants has now been demonstrated to occur at a very low frequency (Sodeinde & Kindle, 1993; Gumpel et al., 1994), gene targeting experiments for the replacement or disruption of genes with non-selectable phenotypes would require the development of positive-negative selection techniques of the type that are used in mammalian systems (e.g. Mansour et al. 1988). One prerequisite for the development of such a system for *Chlamydomonas* would be the availability of a small dominant selectable marker such as a bacterial antibiotic resistance gene.

The first reports of nuclear transformation in *C. reinhardtii* relied on the introduction and expression of foreign genes. The transformation of the *C. reinhardtii* strain *arg7-cw15* to arginine biosynthesis with the corresponding
yeast gene \textit{arg4} was reported (Rochaix & van Dillewijn, 1982; Rochaix \textit{et al.} 1984), and several laboratories have reported the use of bacterial genes to transform \textit{C. reinhardtii} to kanamycin resistance (Hasnain \textit{et al.}, 1985; Bingham \textit{et al.}, 1989). However transformation rates in all of these experiments were low (at a level similar to reversion or spontaneous resistance) and putative transformants were usually unstable. Neither of these methods making use of \textit{arg4} or Neo$^R$ as a selectable marker were found to be reproducible by other laboratories and the use of Kan$^R$ as a selectable marker for the nuclear transformation of \textit{C. reinhardtii} has been further questioned since it has been discovered that spontaneous kanamycin resistance appears at a high frequency due to mutations in chloroplastic ribosomal DNA (Harris \textit{et al.}, 1989). Attempts have also been made to introduce and express foreign genes in a co-transformation with endogenous markers. For example, the hygromycin resistance ($hph$) gene of \textit{E. coli} flanked by the 35S promoter and 35S polyadenylation signal from cauliflower mosaic virus (CaMv) (Pietrzak \textit{et al.}, 1986) was introduced in a co-transformation but despite the presence of the DNA no co-transformants were resistant to hygromycin (Day \textit{et al.}, 1990). Similarly, attempts to express the bacterial \(\beta\)-glucuronidase gene (\textit{uidA}) under the control of the \textit{Chlamydomonas} \textit{CABII-1} promoter following co-transformation were also unsuccessful (Blankenship & Kindle, 1992). More recently the bacterial neomycin phosphotransferase II (\textit{NPTII}) gene flanked by the Ti plasmid nopaline synthase (\textit{nos}) promoter and polyadenylation signal were used in a co-transformation to transform \textit{C. reinhardtii} to Kan$^R$ (Hall \textit{et al.}, 1993). Kan$^R$ clones contained the transgene and NPTII activity was demonstrated in these transformants but the linkage of the genotype and phenotype in sexual crosses
was not shown.

The inability to reproducibly express foreign genes in the nucleus of *C. reinhardtii* has been a persistent problem and many reasons have been proposed to explain this. Firstly, the silencing of introduced genes by methylation (Blankenship & Kindle, 1992; Ferris, 1995). Certainly this is a widely observed phenomena in higher plants (e.g. Weber et al., 1990; Inglebrecht et al., 1994). Secondly, the nuclear genome of *C. reinhardtii* exhibits a high GC content (61.6%) (Jarvis et al., 1992) and this is reflected in a biased codon usage (Campbell & Gowri, 1990). It has been suggested that this codon bias may result in an inability to efficiently express heterologous genes (Mayfield & Kindle, 1990). Thirdly, integration at ectopic locations has been cited as a potential problem (Kindle & Sodeinde, 1994). Certainly the level of expression of intact and chimeric *Chlamydomonas* genes reintroduced in transformation is often significantly below that of the WT gene (Kindle et al., 1989; Blankenship & Kindle 1992; Quinn et al., 1993). Finally, the lack of foreign gene expression may be due to the incompatibility of controlling elements used or the absence of introns (Stevens & Purton, 1994). Studies of foreign gene expression have demonstrated the importance of introns for the efficient expression of transgenes in plants and animals (Kyozuka & Shimamoto, 1995; Webb & Nisbet, 1995). All *C. reinhardtii* nuclear genes sequenced to date contain introns, for example *ARG7* which has eleven (Purton & Rochaix, 1995). It is therefore reasonable to assume that introns may be required for the efficient expression of transgenes in *Chlamydomonas*. Furthermore Gruber & Schmitt (University of Regensburg) reported at a recent meeting findings that in the related alga *Volvox carteri* an endogenous cDNA for the nitrate reductase gene would only be expressed upon the reintroduction of an intron within the coding sequence.
1.6 Aims of the study

The aim of this investigation is to develop a dominant selectable marker for use in the nuclear transformation of *C. reinhardtii*. Specifically:

i) To stably express a foreign gene in *C. reinhardtii* such that the gene has the potential to act as a dominant selectable marker.

ii) To develop a transformation and selection system for the marker.

iii) To investigate parameters that affect the efficiency of transformation.

iv) To sequence the endogenous L41 ribosomal protein gene (*RPL41*) and investigate its potential as an additional dominant selectable marker in *C. reinhardtii*. 
CHAPTER 2

MATERIALS AND METHODS
2.1 Algal material

2.1.1 C. reinhardtii strains

The wild-type (wt) \textit{C. reinhardtii} strains CC-1021 mating type+ (mt+) and CC-125(mt+), the cell wall deficient strain cw15 (mt-) (Davies and Plaskitt, 1971), the oxygen evolving enhancer subunit 1 (OEE1) deficient, cell wall deficient strain FuD44 \textit{cwd} and the cycloheximide resistant strains \textit{act-1} (Sager and Tsubo, 1961) and \textit{act-2} (Smyth \textit{et al.}, 1975) were obtained from Dr. E. Harris at the Chlamydomonas Genetics Center at Duke University, North Carolina. The cell wall deficient strain \textit{cwd}(mt-) (Loppes and Deltour, 1975) and the arginine requiring, cell wall deficient strain \textit{arg7-8cwd} (mt-) were obtained from Dr. R. Loppes at the University of Liege.

2.1.2 Growth and maintenance of C. reinhardtii strains

Media used to culture \textit{C. reinhardtii} are detailed in table 2.1. \textit{C. reinhardtii} strains were maintained on Tris-acetate phosphate (TAP) medium (Gorman and Levine, 1965) solidified with 2% (w/v) bacto agar under a photon flux of 20\(\mu\)E/m\(^2\)/s at 18\(^\circ\)C and were streaked to fresh plates every 6-8 weeks. Working stocks were maintained at 45\(\mu\)E/m\(^2\)/s at 25\(^\circ\)C and these were restreaked weekly. Liquid cultures were grown in the appropriate medium in Erlenmeyer flasks in a Gallenkamp illuminated orbital incubator in the light (80\(\mu\)E/m\(^2\)/s) at 25\(^\circ\)C and aerated by shaking at 150 rpm. Starter cultures of 25 ml volume were inoculated from stock cultures and grown to stationary phase (~2\(\times\)10\(^7\) cells/ml) and an appropriate amount of this culture was subsequently used to inoculate a larger volume of medium. Appropriate aseptic techniques were employed.
Table 2.1 C. reinhardtii growth media.

<table>
<thead>
<tr>
<th>for 1 litre</th>
<th>TAP Medium</th>
<th>Tris minimal medium</th>
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<tbody>
<tr>
<td>H₂O</td>
<td>975 ml</td>
<td>975 ml</td>
</tr>
<tr>
<td>Tris</td>
<td>2.42g</td>
<td>2.42g</td>
</tr>
<tr>
<td>4x Beijerin Salts*</td>
<td>25 ml</td>
<td>25 ml</td>
</tr>
<tr>
<td>1M (K)PO₄ ph7.0#</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Trace Elements†</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>~1 ml to pH 7.0</td>
<td>~1 ml to pH 7.0</td>
</tr>
<tr>
<td>Concentrated HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>References</td>
<td>Gorman and Levine (1965)</td>
<td>Surzycki (1971)</td>
</tr>
</tbody>
</table>

---

* 4x Beijerin Salts
  16g NH₄Cl (omitted in TAP ·N media)
  2g CaCl₂
  4g MgSO₄
  Dissolve in 1 litre distilled H₂O

# 1M (K)PO₄
  250 ml 1M K₂HPO₄
  170 ml 1M KH₂PO₄ (titrate to pH 7.0)

† Trace Elements
i. Dissolve in 550 ml distilled water in the order indicated below, then heat to 100°C
  11.4g H₂BO₄
  22g ZnSO₄·7H₂O
  5.06g MnCl₂·4H₂O
  4.99g FeSO₄·7H₂O
  1.61g CoCl₂·6H₂O
  1.57g CuSO₄·4H₂O
  1.1g (NH₄)₆Mo₇O₂₄·4H₂O
ii. Dissolve 50g EDTA·Na₂ in 250 ml H₂O by heating and add to the above solution. Reheat to 100°C. Cool to 80 - 90°C and adjust to pH 6.5 - 6.8 with 20% KOH.
iii. Adjust to 1 litre. Incubate at room temperature for two weeks and allow a rust coloured precipitate to form. The solution will change from green to purple.
iv. Filter through three layers of Whatman No. 1 paper under suction until the solution is clear. Store at 4°C.
throughout.

2.1.3 Measurement of cell density.

The cell density of *C. reinhardtii* in liquid culture was determined by removing a 1ml sample from a well-mixed culture and adding to it 10μl of tincture of iodine (0.25g iodine in 95% ethanol). Duplicate 10μl aliquots of cells killed in this way were then counted using a haemocytometer (Webber Scientific International Ltd) and multiplied by 10^4 to give cell density/ml.

2.2 Bacterial strains

2.2.1 *Escherichia coli* strains

The *E. coli* strains used and their genotypes are listed in table 2.2

2.2.2 Growth and maintenance of bacterial strains

Media used to culture *E. coli* are detailed in table 2.3. Long term storage of *E. coli* cultures was in the form of frozen glycerol stocks. 1.2 ml of an overnight liquid culture was mixed with 0.8 ml of sterile glycerol in a 2 ml screw cap Eppendorf tube, and stored at -70°C. *E. coli* strains were maintained in the short term by growing overnight at 37°C on LB media solidified with 1.6% agar (supplemented with appropriate antibiotics were applicable) and then storing at 4°C. Appropriate aseptic techniques were employed throughout.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>F' traD36 proA+ proB+ lacIq lac\Delta M15/recA1 end A1 gyrA96 (Nalr) thi hsdR17 supE44 relA1 Δ(lac-proAB) mcrA</td>
</tr>
<tr>
<td>XL1 Blue</td>
<td>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac-F'[pro AB+ lacIq lac\Delta M15 Tn10(tet)]</td>
</tr>
<tr>
<td>C600</td>
<td>thi-1 thr-1 leuB6 lacY1 tonA21 supE44 mcrA</td>
</tr>
</tbody>
</table>
Table 2.3 *E. coli* growth media.

<table>
<thead>
<tr>
<th>For 1 litre</th>
<th>LB</th>
<th>2YT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactotryptone</td>
<td>10g</td>
<td>16g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5g</td>
<td>10g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10g</td>
<td>5g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 litre</td>
<td>to 1 litre</td>
</tr>
</tbody>
</table>

Media supplemented with the following antibiotics where necessary:
- Ampicillin (Ap) 50mg/ml in ddH₂O
- Tetracycline (Tc) 15mg/ml in 50% ethanol
- Phleomycin (Pm) 5mg/ml in ddH₂O

Stock solutions were filter-sterilised using a leur lock syringe and a 0.45μm filter and stored in aliquots at -20°C. Stock solutions diluted 1000x in media to achieve working concentrations.
2.3 Reagents and enzymes

The radiolabelled 2’deoxynucleoside 5’triphosphates (dNTPs); [α-32P] 2’deoxycytidine 5’triphosphate (dCTP) and [α35S] 2’deoxyadenosine 5’ triphosphate (dATP) were purchased from Amersham International. Restriction endonucleases and other DNA modifying enzymes and polymerases were obtained from; New England Biolabs (Hitchin), Stratagene (Cambridge), Promega (Southampton) and Boehringer Mannheim (Lewis). All chemicals used were of the highest grade available wherever possible. Solutions, media and reagents were sterilised by autoclaving at 15 psi above atmospheric pressure at 1210C for 20 minutes.

2.4 Recombinant DNA techniques

2.4.1 Restriction enzyme digestion

Analytical digestion of plasmid DNA (0.5-2μg) using 5-10 units of restriction endonuclease was performed in buffer supplied by the manufacturer diluted to 1x in double distilled water (ddH2O). Digestions were incubated for 1-2 hours at 370C. C. reinhardtii DNA was digested in 20 units of restriction endonuclease for 2 hours. An additional 5 units of enzyme were added and the digestion allowed to proceed for a further 30 minutes to ensure complete digestion.

2.4.2 Agarose gel electrophoresis of DNA

Restriction enzyme digestion generated DNA fragments of greater than 0.5 kbp were separated according to size on 1% agarose gels in 0.5x TBE buffer
(45mM Tris-HCl, 45mM boric acid, 1.25mM EDTA.Na₂). Samples were mixed at the appropriate ratio with 6x loading buffer: 40% (w/v) glycerol, 0.1%(w/v) SDS, 0.1mM EDTA.Na₂ 0.01%(w/v) bromophenol blue, 0.01%(w/v) xylene cyanol and subjected to electrophoresis in a Hoefer (Newcastle-under-Lyme) 10 cm cooled minigel apparatus at 75 or 100V constant voltage for 1-2 hours or in a Hybaid 30 cm maxigel apparatus at 40V constant voltage for 14-18 hours. Fragments smaller than 500 bp were separated as above but on 1.5 - 2% agarose gels at a maximum constant voltage of 50V. 0.5|g of bacteriophage λ DNA digested with either HindIII or AvaI and BglII or 0.5|g of pUC19 plasmid DNA digested with Hinfl were used as size markers. Sizes of markers in bp are given in table 2.4. Following electrophoresis, DNA bands were visualised by staining the agarose gels for 15 minutes in 1μg/ml ethidium bromide (EtBr), washing in distilled water (dH₂O) and illuminating with UV light at 302nm on a UVP inc. (California) TM-20 transilluminator. Photographic records of gels were taken using Polaroid 665 film in a Polaroid DS-34 camera fitted with a TIFFEN 40.5mm 15 orange filter.

2.4.3 Recovery of DNA fragments from agarose gels.

DNA fragments were recovered from agarose gels using a 'geneclean' kit (Stratech) according to the manufacturers protocol and resuspended in TE buffer or ddH₂O depending on use. DNA recovered in this way was suitable for cloning and as the template for the production of random oligo labelled probes.

2.4.4 Construction of recombinant plasmids

Recombinant plasmids were prepared by ligating DNA fragments into compatible restriction sites within parental vectors. 10μg of vector DNA was
Table 2.4 Sizes of markers for agarose gel electrophoresis. (Sizes are in bp).

<table>
<thead>
<tr>
<th>λ: Hind III</th>
<th>λ: Ava I, Bgl II</th>
<th>pUC19: Hinf I</th>
</tr>
</thead>
<tbody>
<tr>
<td>23,130</td>
<td>14,866</td>
<td>1,419</td>
</tr>
<tr>
<td>9,416</td>
<td>8,778</td>
<td>517</td>
</tr>
<tr>
<td>6,557</td>
<td>5,432</td>
<td>396</td>
</tr>
<tr>
<td>4,361</td>
<td>4,333</td>
<td>214</td>
</tr>
<tr>
<td>2,322</td>
<td>3,780</td>
<td>75</td>
</tr>
<tr>
<td>2,027</td>
<td>2,425</td>
<td>65</td>
</tr>
<tr>
<td>564</td>
<td>2,215</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>1,917</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,629</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,463</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,076</td>
<td></td>
</tr>
<tr>
<td></td>
<td>540</td>
<td></td>
</tr>
</tbody>
</table>
digested with an appropriate enzyme or enzymes, electrophoresed as described in section 2.4.2 to separate any contaminating uncut molecules, and recovered by the 'geneclean' method (section 2.4.3). Vectors digested with a single enzyme were treated with calf intestinal alkaline phosphatase (Boehringer-Manheim) to prevent religation of parental plasmids: 'genecleaned' vectors were resuspended in 90μl of ddH2O. 10μl of 10x reaction buffer and 1μl of alkaline phosphatase were added and the reaction incubated at 55°C for 1 hour and then at 75°C for 5 minutes to inactivate the phosphatase. The total sample was then subjected again to agarose gel electrophoresis and 'genecleaned' as detailed above and resuspended in TE buffer at 1μg/μl. Vectors digested with two incompatible enzymes or with a blunt cutting enzyme were resuspended in TE buffer at 1μg/μl without treating with alkaline phosphatase.

DNA inserts with 5' or 3' overhangs (‘sticky ends’) needing to be ligated into a blunt site were first treated with Mung Bean Nuclease (MBN). The DNA to be blunted was resuspended in 15.5μl of ddH2O. To this was added 2μl of 10x MBN buffer and 2.5μl MBN (diluted to 1.5U/μl in MBN dilution buffer) and incubated at 37°C for 30 minutes. The blunt ended insert DNA was subsequently recovered by subjecting the entire sample to agarose gel electrophoresis and 'genecleaning' as previously detailed and finally resuspending in TE buffer.

Ligation reactions were performed using 2x Molar excess of DNA insert molecules as detailed below;

i - For sticky ended ligations:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restricted vector</td>
<td>1μl</td>
</tr>
<tr>
<td>DNA insert</td>
<td>2μl</td>
</tr>
<tr>
<td>10 x buffer</td>
<td>1μl</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>0.5μl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>5.5μl</td>
</tr>
</tbody>
</table>
Reaction incubated at 170°C overnight.

ii - For blunt ended ligations:  
- Restricted vector: 1μl  
- DNA insert: 2μl  
- 10 x buffer: 1μl  
- T4 DNA ligase: 1μl  
- ddH2O: 5μl

Reaction incubated at 37°C for 30 minutes.

2.4.5 Transformation of E. coli.

Recombinant plasmids were introduced into competent *E. coli* strains (XL1 Blue or JM109 - see table 2.2) by a transformation method derived from that of Cohen *et al.* (1972). 100μl of a stationary phase overnight culture of the appropriate strain was used to inoculate 10 ml of LB medium (supplemented with antibiotics where appropriate) and this was grown at 37°C in an orbital shaker at 200 rpm for 3 hours. Cells were harvested by centrifugation in a Mistral 3000 centrifuge at 3,000 rpm for 5 minutes, resuspended in 10ml of cold (4°C) 100mM MgCl₂ and incubated on ice for 5 minutes. Cells were repelleted as above and then resuspended in 0.1 volume of cold (4°C) 50mM CaCl₂. Following incubation on ice for a further 30 minutes, 100μl aliquots of cells were transferred to 1.5ml Eppendorf tubes and to these were added the transforming plasmid (typically 5μl of a ligation reaction or 0.1μg of a parental plasmid). The competent cell / plasmid mixture was incubated on ice for 30 minutes and then heat shocked at 42°C for 90 seconds. After cooling briefly on ice, 900μl of LB medium was added to the cells which were then incubated at 37°C for 30 - 45 minutes to allow expression of plasmid encoded antibiotic resistance markers. 10% of the
transformed cell suspension was then plated to LB-1.6% agar plates supplemented with appropriate antibiotic(s). For pUC based vectors (Vieira & Messing, 1982) agar plates were also pre-treated with 4μl of a 200mg/ml stock solution of the non-substrate lac inducer IPTG and 40μl of a 20mg/ml in dimethylformamide stock solution of the chromagenic substrate X-gal as described in Sambrook et al. (1989) to allow blue/white selection. The remaining 90% of the transformed cell suspension was then concentrated by centrifuging and discarding most of the medium prior to gently resuspending the cells in the remaining medium and plating as above. The plates were incubated inverted at 37°C overnight and resultant recombinants were picked to 10 ml of LB medium supplemented with the appropriate antibiotic(s) and grown up for 'miniprep' analysis as described in section 2.5.2. A sample of the culture was used to prepare a glycerol stock as detailed in section 2.2.2.

2.5 Preparation of DNA

2.5.1 Preparation of DNA from C. reinhardtii.

Preparation of total DNA from C. reinhardtii was by a rapid 'miniprep' method adapted from Rochaix et al. (1988). Cells from 25 ml of a culture at a density of 1x10^7 cells/ml were harvested by centrifugation in a 25ml Sterilin sample tube at 3000 rpm for 5 minutes in a Mistral 3000 centrifuge. The supernatant was discarded and the cell pellet resuspended in 1ml of TAP medium and transferred to a 1.5ml Eppendorf tube and the were cells repelleted by centrifugation in an MSE microcentrifuge. Again the supernatant was discarded and the cell pellet was resuspended in 0.35 ml of TEN buffer (50mM EDTA.Na₂
pH 8.0, 20mM Tris-HCl pH 8.0, 0.1M NaCl). 50μl of pronase at 10mg/ml and 25μl of 20% sodium dodecyl sulphate (SDS) were added and the cells were incubated at 550°C for 2 hours. 2μl of diethylpyrocarbonate (DEPC) were added and incubation continued for a further 15 minutes at 70°C in a fume hood. The tube was briefly cooled on ice and 50μl of 5M potassium acetate added. The contents of the tube were mixed by shaking thoroughly and the tubes were incubated on ice for a further 30 minutes. The precipitate which formed was removed by centrifuging the tubes for 15 minutes in an MSE microcentrifuge and transferring the supernatant to a fresh Eppendorf tube. Contaminating proteins were removed by phenol extraction: one volume of TE (10mM Tris-HCl pH 8.0, 1mM EDTA, Na₂ pH 8.0) saturated phenol and one volume of chloroform:isoamyl alcohol (24:1; v/v) was added and the phases were mixed by vortexing. Following centrifugation for 5 minutes in a microcentrifuge the upper, aqueous phase was transferred to a fresh Eppendorf and the extraction repeated. The DNA in the recovered aqueous phase was subsequently precipitated by adding 2.5 volumes of cold (-20°C) absolute ethanol and incubating at -20°C for 30 minutes. The DNA was recovered by centrifuging the tubes for 10 minutes in a microcentrifuge to pellet the DNA. The supernatant was discarded and the pellets were washed with 70%(v/v) ethanol and then dried in a Rotovac vacuum drier. DNA pellets were finally resuspended in 50μl TE pH 8.0, 1μg/ml RNAaseA. 20μl of this was used for Southern blot analysis.

2.5.2 Isolation of plasmid DNA

Small scale preparations of plasmid DNA were performed using a modified version of the alkaline SDS method of Birnboim and Doly (1979). E. coli cells
harbouring plasmid DNA were grown overnight at 37°C in 10ml of LB medium containing the appropriate antibiotic(s). Cells were pelleted in a Mistral 3000 centrifuge at 3000 rpm for 5 minutes, resuspended in 100μl of solution I (50mM glucose, 25mM Tris-HCl pH8.0, 50mM EDTA.Na₂) and transferred to a 1.5ml Eppendorf tube. 200μl of freshly made solution II (1% SDS, 0.2M NaOH) was added and the tube inverted gently to mix. After a 5 minute incubation at room temperature, 150μl of solution III (2M acetic acid, 3M potassium acetate) was added and left on ice for 5 minutes. Precipitated bacterial chromosomal DNA was pelleted by centrifugation and the supernatant transferred to a fresh tube. Phenol / chloroform extraction and plasmid DNA recovery from the aqueous phase was as detailed in section 2.5.1. The DNA pellet was finally resuspended in 40μl TE/RNAase A buffer.

Larger scale isolation of plasmid DNA was performed using a Qiagen (Dorking) maxiprep kit according to manufacturer’s instructions; finally resuspending the DNA in 200μl of TE pH8.0. The concentration of the DNA was determined by diluting 5μl of the concentrated stock into 1ml of ddH₂O and measuring the absorbance at 260 nm. DNA at a concentration of 1mg/ml gives a reading of 20 absorbance units at 260 nm therefore the concentration could be calculated and the sample diluted to a working concentration of 1mg/ml.

2.5.3 Preparation of λ DNA by a plate lysate method

Preparation of bacteriophage λ DNA was achieved by a plate lysate method adapted from Sambrook et al. (1989) and a method described in the Amersham International cDNA cloning system booklet. Cells from an overnight culture of E.coli C600 were diluted 100x in LB medium supplemented with filter
sterilised maltose to 0.2% and MgSO₄ to 10mM and grown for 3 hours at 37°C in an orbital shaker at 200 rpm. 200μl of this cell suspension was then added to 200μl of phage particles at 10^6/ml in a sterile universal bottle and left for 20 minutes at 37°C to allow adsorption of the phage. 6.5 ml of top agarose (LB, 0.2% maltose, 10mM MgSO₄, 0.7% agarose) at 42°C was added to the infected bacteria and these were plated to LB agarose plates (LB + 1.6% agarose in 13cm petri dishes). When the top agarose had set these were incubated inverted overnight at 37°C. The following day, 5ml of λ diluent (10mM Tris-Hcl pH7.5, 10mM MgSO₄) was added to the surface of the agarose and the plates were agitated gently on a shaking platform for 1-2 hours to allow diffusion of phage particles. The diluent was recovered with a pipette, transferred to a sterile universal bottle and centrifuged at 4,000 rpm to pellet any bacterial cells and debris. The supernatant containing phage was transferred to a corex tube and an equal volume of 20% polyethylene glycol (PEG) 8,000, 2M NaCl in λ diluent was added and the tubes were incubated in an iced water bath (0°C) for 1 hour to precipitate the phage. The tubes were centrifuged for 10 minutes at 10,000xg in a Sorvall SS-34 rotor to pellet the phage. The tubes were drained then briefly respun and all remaining PEG solution was carefully removed using a pipette. The phage pellet was resuspended in 750μl of LB and transferred to an Eppendorf tube. To this 750μl of a suspension of DEAE cellulose (Whatman) DE52 in LB

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Diethyaminoethyl (DEAE) cellulose (Whatman) DE52 in LB was prepared in the following manner: 100g of DE52 was weighed into a beaker and several volumes of 0.05N HCl were added slowly whilst checking to ensure that the pH dropped below pH4.5. Concentrated NaOH was added with constant gentle stirring until pH=7.0. The resin was allowed to settle, the supernatant decanted and the resin was washed several times in LB by gently resuspending in 2 volumes of LB allowing the resin to settle and decanting the excess medium. This was repeated until the pH=LB(pH7.0). The DE52 was finally resuspended to make a slurry of ~75% resin and 25% LB. Sodium azide was added to 0.1% to prevent the growth of microorganisms and the suspension stored at 4°C.
was added and gently mixed by inversion 20-30 times. The tube was then centrifuged for 5 minutes in a microcentrifuge and the supernatant transferred to a fresh tube (twice). To 1ml of supernatant 17.5\(\mu\)l of a 0.1mg/ml solution of proteinase K and 42.5\(\mu\)l of 10% SDS were added. The contents were mixed and incubated at room temperature for 5 minutes. 173\(\mu\)l of 3M potassium acetate was added (precipitate formed) and the tubes were incubated for 20 minutes at 88\(^\circ\)C (precipitate redissolved), then cooled on ice for 10 minutes (precipitate reformed). Tubes were centrifuged for 10 minutes in a microcentrifuge and the supernatant was split between two fresh tubes. An equal volume of isopropanol was added and the tubes incubated at -70\(^\circ\)C for 10 minutes to precipitate the phage DNA. The tubes were warmed to room temperature and then centrifuged to pellet the DNA. The pellets were washed with 70% ethanol and then dried under a vacuum as described above in section 2.5.1 and the combined phage DNA was resuspended in 40\(\mu\)l TE pH8.0.

2.6 Transformation of \textit{C. reinhardtii}.

\textit{C. reinhardtii} cells were transformed by a method based on that reported by Kindle (1990). Cells to be transformed were grown to mid-log phase (2-4x10\(^6\) cells/ml) in 200ml of TAP medium in 500ml Erlenmeyer flasks under the conditions detailed in section 2.1.2. Accurate determination of the cell density was achieved by aseptically removing 1ml of culture and counting cells using a haemocytometer as described in section 2.1.3. Cultures were then transferred to sterile 200ml nalgene centrifuge bottles and centrifuged in a Sorval GSA rotor at 3,000x\(\text{g}\), 4\(^\circ\)C for 5 minutes to pellet the cells. The medium was discarded and the
cells resuspended in fresh medium to a concentration of 2x10^8 cells/ml. 300μl of this cell suspension was transferred to a sterile 5ml test tube containing 0.3g of sterile 0.4mm diameter glass beads (BDH) and 2μg of the transforming plasmid. The cell/glass bead/DNA suspension was then vortexed for 15 seconds at top speed using a Vortex genie-2 (Scientific Industries) vortex. 4ml of soft TAP agar (0.6%) at 42°C was added to the tubes and the cells were then plated to selective medium and grown at 22°C at a light intensity of 45μE/m²/s. Transformants were scored after 6-7 days.

For direct selection of transformants using the ble (phleomycin (Pm) resistance) marker the above protocol was slightly modified. Glass beads for transformation were sterilised by autoclaving and then introduced to 25ml Sterilin universal bottles and the transformations were performed in these. Following vortexing the cells were diluted in 20ml of TAP medium and allowed to grow for 18 hours in a Gallenkamp illuminated orbital incubator in the light (80μE/m²/s) at 25°C and aerated by shaking at 150 rpm. This allowed the recovery of the cells and expression of the ble gene. Cells were pelleted by centrifugation for 5 minutes at 3,000 rpm in a Mistral 3000 centrifuge. The cell pellet was gently resuspended in 0.5 ml TAP medium mixed with 4ml of TAP 0.6% agar supplemented with Pm at 2μg/ml, and spread on the surface of TAP 2% agar also supplemented with Pm at 2μg/ml. Transformants were scored after 7-9 days.

2.7 Filter hybridisation of nucleic acids

2.7.1 Transfer of DNA to nylon membranes.

DNA fragments that had been separated by agarose gel electrophoresis
were transferred to Hybond-N nylon membranes (Amersham International) in a method derived from that of Southern (1975). Following the production of a photographic image of the DNA as described in section 2.4.2, the gel was soaked in 1.5M NaCl, 0.5M NaOH for 30 minutes to denature the DNA duplex to single strands. Gels were then soaked 2x15 minutes in a neutralising buffer (1M Tris-HCl pH8.0, 1.5M NaCl). The gels were then assembled on a blotting stack using 20xSSC (3M NaCl, 0.3M sodium citrate) as the transfer buffer. Two sheets of Whatman 3MM paper (pre-soaked in 20xSSC) were used as wicks to draw the SSC from a reservoir to a supported glass plate. The gel was placed inverted onto the 3MM and any remaining wick exposed at the edge of the gel was masked with Nescofilm (Nipon Shoji Kaisha Ltd - Japan) to prevent SSC transfer buffer bypassing the gel. A sheet of Hybond-N cut to the exact size of the gel was placed on top of it and on this were placed three sheets of 3MM (again cut to the exact size). Finally a stack of paper towels 5cm in height was placed on the stack and the whole assembly was weighed down with a glass plate and a 0.5 kg weight. Transfer was allowed to take place for 15 hours following which the filter was baked in an envelope of 3MM at 80°C for 1 hour to immobilize the DNA on the filter. Membranes were subsequently stored in the 3MM envelope at room temperature.

2.7.2 Radiolabelling of DNA probes

DNA fragments to be used as probes were labelled in a method derived from that described by Feinberg and Vogelstein (1984). 10 - 100ng of template DNA was made up to a total volume of 16μl in an Eppendorf tube with ddH₂O.
To this was added 5µl of oligo labelling buffer (OLB). The tube was heated to 100°C for 5 minutes and then cooled rapidly on ice. 1µl of bovine serum albumin, 2.5µl of 32P-α-dCTP at 10Ci/µl and 0.5µl (1U) of the Klenow subunit of DNA polymerase I were added and the reaction incubated at room temperature for 4 hours. Unincorporated 32P-α-dCTP was removed by passing the reaction down a NucTrap® push column (Stratagene) according to manufacturer’s instructions. The probe was then incubated at 100°C for 5 minutes to denature the DNA to single strands, cooled rapidly on ice and used immediately for hybridisation to DNA molecules immobilized on nylon membranes.

2.7.3 Hybridisation of labelled probes to Southern blots.

Filters harbouring immobilized DNA as prepared in section 2.7.1 were incubated in pre-hybridisation buffer (50% formamide, 5x Denhardt’s reagent, 5xSSC, 0.1% (w/v) SDS, 100g/ml sheared salmon sperm DNA) in Hybaid hybridisation bottles in a Hybaid dual hybridisation oven at 42°C for 4 hours. The radiolabelled DNA probe prepared as above in section 2.7.2 was added to the pre-hybridisation buffer and hybridised as above for 15 hours. Following hybridisation, filters were washed sequentially in 1xSSC, 0.1% (w/v) SDS for 20 minutes and 0.1xSSC, 0.1% (w/v) SDS for 20 minutes. Filters were sealed in Saran

OLB was made as detailed below: Solution A; (1ml solution O [1.25M Tris-HCl pH8.0, 0.125M MgCl₂ - stored at 40C], 18µl 2-mercaptoethanol, 5µl dATP, 5µl dTTP, 5µl dGTP each previously dissolved in 3mM Tris-Hcl pH7.0, 0.2mM EDTA.Na₂ - stored at -20°C, Solution B; 2M Hepes titrated to pH6.6 with 4M NaOH - stored at 40C, Solution C; Hexadeoxyribonucleotides evenly suspended in T.E. at 90 OD units/ml - stored at -20°C.

Solutions A,B and C were mixed in a ratio of 100:250:150 to make OLB.

50x Denhardt’s reagent (1%w/v Ficoll - type 400, 1% w/v Polvinylpyrrolidine, 1%w/v BSA - fraction V)
wrap (Dow chemical Co.) and hybridised probe was detected by autoradiography at -80°C using RX X-ray film (Fuji - Japan) backed with a Kodak X-omatic intensifying screen.

2.8 Preparation of proteins

2.8.1 Rapid preparation of soluble protein from C. reinhardtii.

25ml cultures of C. reinhardtii were grown to a cell density of 2 - 6x10^6 cells/ml and harvested by centrifugation at 3,000 rpm for 5 minutes at 4°C in a Mistral 3000 centrifuge. The cells were rinsed in protein sample buffer (5 mM HEPES pH7.5, 5mM EDTA.Na2, 2mM benzamidine, 2mM DTT). Cells were again pelleted and resuspended in 250μl of the same buffer and transferred to an Eppendorf tube. Cells were lysed by freeze-thawing and insoluble material was removed by centrifugation. The concentration of protein samples was determined by the Bio-Rad assay method according to the manufacturer’s protocol.

2.8.2 Rapid preparation of soluble protein from E.coli

Protein was prepared from 25ml cultures of E.coli as described above in section 2.8.1 for C. reinhardtii.

2.9 Western analysis of Sh. ble protein in C. reinhardtii transformants.

2.9.1 Tricine-SDS-Polyacrylamide gel electrophoresis (PAGE) of proteins.

0.2x volumes of 5x protein loading buffer (312.5mM Tris-HCl pH6.8, 50%w/v glycerol, 10%w/v SDS 0.025%w/v bromophenol blue, 25% v/v 2-
mercaptoethanol) was added to protein samples and the mixture heated to 100°C for 5 minutes. Samples were then loaded onto a 17.5% polyacrylamide gel. Tricine-SDS-PAGE was performed according to the method of Schägger and von Jagow (1987) using a Biorad 20 cm x 20 cm slab gel apparatus. The anode buffer was 200mM Tris-HCl pH8.9 and the cathode buffer was 100mM Tris pH8.25, 100mM tricine, 0.1%SDS. The stacking gel was 4.2% polyacrylamide (acrylamide:bis-acrylamide ratio of 29:1), 744mM Tris-HCl pH8.45, 0.0744% SDS and the resolving gel was 17.5% polyacrylamide (acrylamide:bis-acrylamide ratio of 29:1), 1M Tris-HCl pH8.45, 0.1%SDS, 13.3%(w/v) glycerol. Samples were electrophoresed at a constant voltage of 50V for 15 hours. Biorad low range prestained markers were used as molecular weight standards.

2.9.2 Transfer of protein to nitrocellulose membranes.

Protein samples separated according to molecular weight by Tricine-SDS-PAGE as described above in section 2.9.1 were transferred to nitrocellulose membranes by the method of Towbin et al. (1979) in a Bio-Rad semi-dry transblotter following the manufacturer's protocol. Filters were probed using anti-Shb.ble antibodies (Cayla, France) diluted 1:500. Antibody binding was visualised using an alkaline phosphatase assay (Bio-Rad) according to the manufacturer's protocol.

2.10 Genetic analysis

Crosses between a PmR transformant (Pm 65 mt-) and WT cells (either CC-1021 mt+ or CC-125 mt+) were performed according to methods developed by
Levine and Ebersold (1960) and detailed in Harris (1989). Low nitrogen medium for the production of gametes was prepared as per TAP medium in table 2.1 but 4x Beijerink salts was prepared without ammonium chloride. A loopful of cells of each mating type was resuspended in 2ml of nitrogen free TAP and left overnight to induce gametogenesis. The cell suspensions were mixed the following day and left in the light for two hours to enable mating to take place and the resultant zygotes to form. The zygotes were then plated to nitrogen free TAP 4% agar plates (maturation plates) and incubated in the dark for 5 days. Mature zygotes were then transferred to germination plates (regular TAP 2% agar). Unmated gametes were first removed from the maturation plate by scraping the surface of the agar with a sterile razor blade. Embedded zygospores were then collected under a dissecting microscope in a plug of agar using a sterile microspatula and transferred to a maturation plate where the plug of agar was wiped across the surface to release the zygospore. The plates were then incubated overnight under bright light to allow the zygospores to mature into tetrads. A sterile glass microneedle was then used to separate the four meiotic progeny from within the tetrad. Offspring were allowed to grow into colonies then transferred to fresh plates for growth and subsequent analysis.

2.11 Polymerase Chain Reaction (PCR)

Amplification of DNA fragments by PCR (Mullis & Faloona, 1987) was performed in a Hybaid Thermal Cycler using Vent DNA polymerase (NEB). All reactions were in a total volume of 100μl. Reactions contained: 1μg genomic DNA or 10ng plasmid DNA template, 50μM each dATP, dCTP, dGTP and dTTP.
1μM each oligonucleotide primer, 1U Vent DNA polymerase, 1x Vent reaction buffer (Promega) and made up to volume with ddH2O. Reactions were overlaid with 50μl of mineral oil to prevent evaporation. Conditions of the reaction cycles were determined by the length and composition of the oligonucleotide primers and by the predicted length of the product. Individual reaction conditions for each amplification are therefore detailed at the relevant sections in the results chapters.

2.12 Nucleotide sequence analysis

DNA sequence analysis was performed by a procedure derived from the dideoxy chain termination method of Sanger et al. (1977) and the use of plasmid DNA as a template (Chen & Seeburg, 1985) using a Sequenase II sequencing kit (USB) and [α³⁵S]dATP (Amersham International). 10μg of plasmid DNA template was made up to a volume of 18μl with ddH2O, 2μl of 2M NaOH was added and the mixture heated to 68°C for 20 minutes to denature the DNA strands. The DNA was precipitated by adding 10μl of 3M Sodium acetate pH6.0, 100μl of absolute ethanol and incubating at -20°C for 30 minutes. The DNA was pelleted in a microcentrifuge, washed with 70% ethanol and dried under a vacuum. The pellet was resuspended in 7μl of ddH2O. 1μl of oligonucleotide primer at 40ng/μl and 2μl of 5x Sequenase reaction buffer were added. The mixture was heated to >65°C in a water bath for 2 minutes and allowed to cool slowly to <35°C to allow annealing of the primer. Sequencing was then performed according to the manufacturer’s protocol. Sequencing reactions were heated for 2 minutes at 80°C and half of each sample was loaded onto a 6% polyacrylamide (acrylamide:bis-
acrylamide, 19:1), 7M urea, 1xTBE gel (Scotlab-Strathclyde), using 30 cm vertical electrophoresis apparatus (Cambridge Electrophoresis). Gels were run at a constant power of 35W until the bromophenol blue dye reached the bottom of the gel. The remainder of the sample was then loaded and the run allowed to proceed until the bromophenol blue dye from this had reached the bottom. Gels were then fixed in 10% methanol, 10% acetic acid and dried onto Whatman 3MM paper on a Bio-Rad gel drier at 80°C. An autoradiogram of the gel was obtained by exposing it to Fuji RX X-ray film overnight at room temperature.
CHAPTER 3

Expression of the bacterial phleomycin resistance gene *ble* in *Chlamydomonas reinhardtii*. 
3.1 Introduction

The glycopeptide phleomycin belongs to a family of antibiotics that include bleomycins and tallysomycins which are effective against both prokaryotic and eukaryotic organisms at low concentrations (Berdy, 1980). The phleomycin resistance gene (ble) from the bacterium *Streptot括片ichus hindustanus* has been cloned and encodes a small (13.6 kDa), acidic protein (Drocourt *et al.*, 1990). The Sh.ble protein is a binding protein that exhibits a strong affinity for the phleomycin family of antibiotics and functions by binding in a 1:1 ratio with the antibiotic. When bound, phleomycin is unable to be activated by ferrous ions and oxygen to break down DNA (Gatignol *et al.*, 1988). Phleomycin resistance has been used as a selectable marker for the transformation of various eukaryotes including; fungi (Schuren & Wessels, 1994), yeast (Glumoff *et al.*, 1989), a trypanosome (Jefferies *et al.*, 1993), mammalian cells (Mulsant *et al.*, 1988) and plants (Hille *et al.*, 1986). The ble gene of *S. hindustanus* is GC rich (71%) and exhibits a similar codon usage to *C. reinhardtii* nuclear genes (Drocourt *et al.*, 1990).

The broad activity range of phleomycin, the number of different eukaryotes for which ble has proved to be an effective marker and the similar codon usage of the ble gene to that found in *C. reinhardtii* genes suggests that it may be a suitable marker for nuclear transformation in *Chlamydomonas*. 
3.2 Results

3.2.1 Sensitivity of C. reinhardtii to phleomycin

The sensitivity of a wild-type strain (CC-125) and a cell-wall-deficient strain (cw15) of C. reinhardtii to phleomycin (Pm) was tested both in liquid and on solid media. Cultures at stationary phase were used to inoculate TAP medium supplemented with Pm at a range of concentrations between 0 and 1 μg/ml. Samples of the same cultures were spotted to TAP agar plates supplemented with Pm at a range of concentrations between 0 and 5 μg/ml. Data showing the ability of the strains to grow are presented in table 3.1. The presence of a cell wall had no effect on sensitivity to the drug and minimum inhibitory concentrations (MIC) for the drug in liquid and solid media were determined to be 0.02 μg/ml and 0.6 μg/ml respectively. In order to test whether Pm would be suitable for the selection of transformants the spontaneous resistance rate of C. reinhardtii was tested. Selectable markers currently employed give transformation rates of 10^{-5} - 10^{-6}. Spontaneous resistance rates at, or below this rate would complicate the identification of transformants. 1x10^9 wild type cells were plated to media containing Pm at a concentration of 2 μg/ml. No resistant colonies were recovered indicating that the rate of spontaneous resistance is several orders of magnitude below current transformation rates.

3.2.2 Construction of pSP108

pSP105, an expression vector for C. reinhardtii had previously been constructed (Purton and Rochaix, unpublished). The vector is based on the 5' and 3' UTR of the strongly expressed RBCS2 gene (Goldschmidt-Clermont &
<table>
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<th>0.08</th>
<th>0.1</th>
<th>0.2</th>
<th>0.4</th>
<th>0.6</th>
<th>0.8</th>
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<td>+/-</td>
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<td>ND</td>
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<tr>
<td>WT (solid)</td>
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<td>ND</td>
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<tr>
<td>cw15 (solid)</td>
<td>+</td>
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<td>+/-</td>
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</table>

*Table 3.1* Growth of wild type (WT) and cell-wall-less (cw15) *Chlamydomonas reinhardtii* cells in liquid and on solid medium supplemented with phleomycin. Growth (+) and reduced or no growth (−) are indicated. ND = not determined.
Rahire, 1986). These elements are cloned into pBluescribe pBS+ (Stratagene) and separated by a multiple cloning site, thereby allowing translational fusions at the \textit{RBCS2} initiation codon (figure 3.1). The \textit{ble} gene from \textit{S. hindustanus} was cloned into pSP105 by Saul Purton in the following way: the \textit{ble} gene was amplified from pUT430 (Drocourt \textit{et al.}, 1990) using the 28mer GTATCGATGGCCAGcTGACCAGcGCCG (5' sense oligo) and the 21mer TTGGGTCTAGCAGGTATCTCC (3' antisense oligo) an initial denaturation step at 95°C for 4 minutes was followed by 15 cycles of; denaturing 95°C - 1 minute, primer annealing 55°C - 1 minute and primer extension 72°C - 2 minutes. A product of 395 bp containing a unique \textit{MscI} restriction site (underlined) at the start codon (bold) and a unique \textit{SalI} site (underlined) was cloned into the \textit{MscI} and \textit{SalI} sites of pSP105 to create pSP108 (figures 3.1 and 3.2). Lower case letters in the oligos show changes introduced to improve codon usage in \textit{C. reinhardtii}. The \textit{RBCS2/ble} junction was confirmed to be in frame by sequencing through the junction using the following 19mer 5' sense oligo CCCTCCTGATAAACCGGCC corresponding to the 5' UTR \textit{RBCS2} sequence. The full sequence of the \textit{RBCS2/ble} hybrid gene is presented (figure 3.3) and is present on pSP108 as a 1.4 kb \textit{EcoRI} cassette.

3.2.3 \textit{Co-transformations using pSP108}

The \textit{ble} gene was introduced into \textit{C. reinhardtii} cells by co-transformation as described in section 2.6. In two separate sets of experiments, pARG7.8 (Debuchy \textit{et al.}, 1989) and pSP108 were co-transformed into \textit{arg7-8 cw4} cells and transformants to arginine biosynthesis were selected by plating to TAP medium that lacked arginine. In each set of experiments 100 independent
Figure 3.1 Linear maps of the plasmids pSP105 and pSP108. pSP105 contains 5’ and 3’ untranslated regions of RBCS2 cloned into the vector pBluescribe M13+ and separated by a polylinker. pSP108 is derived from pSP105 and contains the ble gene as a translational fusion with RBCS2 sequences.
Figure 3.2 Map of plasmid pSP108 showing the RBCS2/ble marker present as a 1.4 kb EcoRI cassette and unique flanking enzyme sites.
**Figure 3.3** Nucleotide sequence of the 1.4 kb *Eco*RI cassette from pSP108 containing the hybrid *RBCS2/ble* gene. The coding sequence is shown in upper case letters.
transformants were picked and grown in 1ml of TAP medium for 48 hours. Five microlitres of each culture was spotted in duplicate to TAP agar and TAP agar supplemented with Pm at 2 μg/ml. Colonies able to grow in the presence of Pm were selected for further analysis. The first hundred co-transformants tested yielded four PmR colonies and the second hundred two PmR colonies. These clones, named Pm16, Pm19, Pm21, Pm65, Pm71 and Pm73, were subjected to further analysis as detailed below. A further experiment was performed as detailed above and from 100 potential co-transformants screened, four exhibited resistance to Pm. These clones, Pm2.2, Pm2.7, Pm2.9 and Pm2.96 have not been subjected to further analysis. All of the PmR transformants have remained stable for more than two years when grown on non-selective media.

3.2.4 Southern analysis of transformants

Total DNA from the six PmR transformants, nine randomly chosen PmS transformants and the untransformed host arg7-8 cw prepared by the miniprep method (section 2.5.1) was digested with EcoRI and separated on a 1% agarose 0.5xTBE gel by electrophoresis at 40V. The DNA was transferred to a nylon membrane (section 2.7.1) and hybridised to a probe specific for the ble gene. Figure 3.4 shows that the ble gene is present in all of the PmR transformants and, interestingly, that it is also present in eight out of the nine PmS clones analysed. The RBCS2/ble construct is present on pSP108 as a 1.4 kb EcoRI cassette and in four of the PmR clones this cassette appears to be retained intact. In two of the PmR transformants (Pm19 and Pm 73) one or both of the EcoRI sites have been lost during the integration of the marker. Three of the four PmR clones that have retained intact EcoRI cassettes (Pm 16, Pm 21 and Pm71) have also integrated
Figure 3.4 Southern analysis of DNA extracted from PmS and PmR co-transformants and the untransformed host strain arg7-8cw (arg). DNA was digested with EcoRI and hybridised with a probe specific for the coding region of ble. The 1.4 kb EcoRI fragment corresponding to the cassette in figure 3.2 is indicated. Size markers are in kilobase pairs.
further copies of the \textit{RBCS2/ble} gene in which one or both of the \textit{EcoRI} sites have been lost during integration. Further Southern analysis has shown that Pm65 (the only PmR clone that retains just the \textit{EcoRI} cassette) has integrated only a single copy. All of the PmS clones that contain the \textit{ble} gene harbour an intact \textit{EcoRI} cassette. In addition, six of these (Pms2, Pms3, Pms4, Pms5, Pms7 and Pms8) also contain further copies that have lost one or both \textit{EcoRI} sites.

As the \textit{ble} marker is flanked by 5' and 3' sequences from the \textit{C. reinhardtii RBCS2} nuclear gene, the transformants were analysed to see if a gene targeting event had taken place resulting in the disruption of the endogenous gene. The Southern blot shown in figure 3.4 was stripped and rehybridised to a probe from the coding region of \textit{RBCS2} (a 0.65 kb \textit{BamHI}, \textit{NcoI} fragment of plasmid p1.03; Goldschmidt-Clermont & Rahire 1986). Figure 3.5 shows that the probe detects intact copies of \textit{RBCS2} and the homologous \textit{RBCS1} genes in the untransformed host and all transformants. This confirms previous findings that homologous recombination events do not occur readily during the nuclear transformation of \textit{C. reinhardtii} (Sodeinde & Kindle, 1993; Gumpel \textit{et al.}, 1994). The blot also serves to indicate the relative loading of DNA in each lane.

Blankenship and Kindle (1992) attempted to express the \textit{uidA} gene from the \textit{C. reinhardtii NIT1} promoter without success. They reported methylation of the transgene to be implicated in the absence of expression so this was investigated in the PmS transformants. DNA from Pm65 (a PmR transformant containing a \textit{ble} gene present as an intact \textit{EcoRI} cassette) and PmS40 (a PmS transformant that also contained an intact \textit{EcoRI} cassette) was digested with the methyl sensitive isoschizomers \textit{HpaII} and \textit{MspI}. Blots of these digests probed with the \textit{ble} DNA revealed no difference. Furthermore growing PmS co-
Figure 3.5 Southern analysis of DNA extracted from Pm$^S$ and Pm$^R$ co-transformants and the untransformed host strain arg7-8cw$_4$ (arg). The blot shown in figure 3.4 was stripped and re-hybridised to a probe for the coding region of RBCS2. The RBCS2 and RBCS1 bands are indicated. Size markers are in kilobase pairs.
transformants in the presence of the hypomethylating base analogue 5-aza-cytidine failed to activate copies of ble putatively silenced by methylation.

3.2.5 Expression of the ble gene in transformants.

As evidenced by figure 3.4, both the PmR transformants and the majority of the PmS transformants contain ble DNA. Both populations contain transformants that have integrated intact EcoRI cassettes alone and transformants that have also integrated copies where one or more of the EcoRI sites has been lost. In addition the PmR transformants also contain a class of transformant in which no intact EcoRI cassettes are present. There is therefore no immediately apparent difference between PmR and PmS transformants, with most classes of transformant being represented in each population. In these circumstances it is essential to demonstrate that the PmR phenotype results from the expression of an integrated ble gene and is not due to spontaneous resistance resulting from mutation. To demonstrate the expression of the ble gene in transformants, northern analysis was performed. No transcript was detected. Furthermore, when a more sensitive RNAase protection assay was used, I again failed to detect a transcript.

To confirm that the PmR phenotype was caused by the expression of the ble gene, western analysis was performed. Total soluble protein from four transformants (Pm21, Pm65, Pm71 and Pm73) and untransformed host cells was extracted as described in section 2.8.1. In addition, total soluble protein was prepared from E. coli cells expressing the ble gene from pSP108. 150μg of protein from C. reinhardtii cells and 15μg of protein from the E. coli cells was separated by SDS-PAGE and electroblotted to nitrocellulose. The blot was
probed with anti-Sh.ble antibodies (Cayla, France). The blot is presented as figure 3.6. The Sh.ble protein is a small soluble protein of 13.6 kDa (Drocourt et al., 1990). A band equivalent to this is seen in the protein samples from the four transformants and from the bacterial cells, but not in the untransformed host cells. The presence of free chlorophyll at the bottom of the western blot demonstrates the approximately equal loading of all the algal samples. Western analysis of protein from Pm$^S$ transformants has failed to detect the Sh.ble protein.

3.2.6 Levels of resistance to Pm in transformants

From the Southern blot analysis presented (figure 3.4) it is evident that some Pm$^R$ transformants have several copies of the ble gene. However, although it is not possible to determine whether all copies of the gene are expressed in a transformant, it is interesting to investigate whether different levels of sensitivity to Pm occur and if so whether this may be related to copy number. To test this, cultures of the six Pm$^R$ strains and wild type cells were grown to stationary phase and 1ml of these cultures was used to inoculate 25ml of TAP medium supplemented with Pm at a range of concentrations. Growth of the cultures after five days was determined as a function of chlorophyll concentration by harvesting 1ml of the cells from each culture and extracting with 80% (v/v) acetone. The chlorophyll concentration was measured by absorbance at 652 nm. A graph of the results obtained is presented as figure 3.7. The sensitivity to Pm differed for the six transformants but fell broadly on three curves. Together with the wild type cells, these curves represent four groups that can be defined by their LD$_{50}$ (i.e. the concentration of drug required to reduce the amount of chlorophyll by 50%: A$_{562}$ = −0.6). The untransformed strain has an LD$_{50}$ of
Figure 3.6 Western analysis of four PmR C. reinhardtii transformants, the untransformed host strain arg7-8cw4 (arg7) and E. coli transformed with pSP108 and grown in the presence of IPTG. Total soluble protein was separated on a 17.5% denaturing polyacrylamide gel, transferred to nitrocellulose and probed using anti-Sh.Ble antibodies. Free chlorophyll (chl) and pre-stained markers (M) are indicated.
Figure 3.7 Resistance of WT cells and PmR transformants to increasing levels of Pm. The amount of chlorophyll in 1 ml of culture is expressed as absorbance units measured at 652 nm.
0.03μg/ml. Pm16, Pm19 and Pm65 have an LD$_{50}$ of ~0.08μg/ml. Pm21 and Pm73 have an LD$_{50}$ of ~0.19μg/ml, and Pm71 has an LD$_{50}$ of ~0.32μg/ml. Whilst further analysis of transformants would be required to determine whether the differences in the levels of sensitivity are due to copy number, it is interesting to note that Pm65, which has only one copy of the ble gene, is in the group of transformants that exhibit the lowest level of resistance and Pm71 which has at least three copies of the gene has the highest tolerance to the drug.

The variation in the levels of resistance between the clones led me to consider that Pm$^S$ transformants containing the ble gene may be resistant to Pm at greatly reduced levels. Whilst the presence of the Sh.ble protein was not detected in Pm$^S$ transformants it is conceivable that the protein is present but at a level below the sensitivity of detection and that assignment of a Pm$^S$ phenotype was as a result of the concentration of Pm used in the initial selection of co-transformants being too high. In such a scenario, transformants would appear to be Pm$^S$ when screened on media supplemented with Pm at 2μg/ml and the absence of a detectable Sh.ble protein in western analysis would serve to confirm this view. To test this, the growth of a Pm$^S$ transformant (Pm$^S40$), Pm65 and WT cells at a lower range of Pm concentrations was investigated. Stationary phase cells were used to inoculate TAP supplemented with varying concentrations of Pm and growth of the cultures after five days was determined as a function of chlorophyll concentration as detailed above. The results are presented as a graph (figure 3.8). From the graph it can observed that even at the lowest concentrations of Pm used that the growth of Pm$^S40$ is inhibited at the level observed in WT cells. This supports the belief that the Pm$^S$ transformants are not functionally expressing the ble gene at a low level.
Figure 3.8 Resistance of Pm65, PmS40 and WT cells to increasing levels of Pm. The amount of chlorophyll in 1 ml of culture is expressed as absorbance units measured at 652 nm.
3.2.7 Genetic analysis of Pm\textsuperscript{R} inheritance

Whilst a Pm\textsuperscript{R} phenotype, the presence of the \textit{ble} gene and the presence of the gene product have all been demonstrated in the transformants, the confirmation of a stable nuclear transformation event also requires that the acquired phenotype is stable in meiosis and furthermore that the phenotype and the gene co-segregate.

A demonstration of this kind is possible in \textit{C. reinhardtii} through sexual crosses and tetrad analysis. The inheritance of the \textit{ble} gene was investigated by mating Pm65 (mt-) which contains a single copy of the \textit{ble} gene with WT cells (either CC-125(mt+) or CC-1021(mt+)). It has recently been observed that the host used for transformation \textit{(arg7-8 cw4)} contains one or more uncharacterized mutations that inhibit mating and in addition prevents the recovery of complete tetrads (Gumpel \textit{et al.}, 1996). Because of this only thirteen daughter cells from seven incomplete tetrads were recovered following crosses. These daughter cells were however subjected to analysis to characterize the inheritance of the \textit{ble} gene and the Pm\textsuperscript{R} phenotype. All thirteen of the daughter cells were grown up in liquid culture and spotted in duplicate to TAP agar +/- Pm @2\textmu g/ml to test for the transmission of the phenotype. Furthermore, DNA from these progeny was isolated by the miniprep method, digested with \textit{EcoRI} and, following electrophoretic separation, blotted to a nylon membrane and hybridised to a probe made from the entire pSP108 \textit{EcoRI} cassette. This 1.4 kb fragment contains both the \textit{ble} gene and 3' and 5' UTR sequences from \textit{RBCS2} which provides therefore an internal positive control. The results of the spot tests and Southern blot analysis are presented in figure 3.9. On the Southern blot a high molecular weight band (marked with a diamond in figure 3.9) represents the endogenous

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Figure 3.9 The co-segregation of ble and the PmR phenotype in progeny from a Pm65(mt- x WT(mt+) cross. DNA from the 13 progeny was digested with EcoRI and hybridised to the 1.4 kb EcoRI RB52/ble cassette (upper panel). The endogenous RB52 gene present in all the progeny is marked with a diamond. The RB52/ble transgene derived from Pm65 is marked with a triangle. The presence of this transgene correlates with a PmR phenotype as confirmed by spot tests on to solid medium +/- Pm (lower panel). An additional fragment of RB52 (marked with a circle) which is derived from Pm65 does not co-segregate with the PmR phenotype.
*RBCS2* gene. The *ble* gene (marked with a triangle) is present in seven of the daughter cells and, by comparing to the spot tests below, can be seen to co-segregate with those cells able to grow in the presence of Pm. Interestingly, an additional band (marked with a circle) which does not co-segregate with the *ble* gene is seen in seven of the progeny. Additional Southern analysis has shown that this band is present in Pm65 but not in *arg7-8 cw^4* or in either of the WT strains used (figure 3.10). It is believed therefore that this band represents additional *RBCS2* DNA from pSP108, that has integrated at a separate, unlinked location. This is believed to be the first observation of the integration of transforming DNA at two separate sites following the transformation of *C. reinhardtii* by the glass bead method.

Although complete tetrads were not recovered from the crosses there is no evidence to suggest deviation from Mendelian ratios for the inheritance of a single copy nuclear gene in tetrads (2:2). Approximately half of the daughter cells contain the gene and in tetrad 5 from which three daughter cells were recovered there are no more than two of either phenotype/genotype. However, as so few progeny were recovered it was important to demonstrate that true daughter cells and not recovered parentals were being analysed, additional phenotypic tests were carried out on all thirteen progeny. In addition to phleomycin resistance, they were tested for the presence of a cell wall, arginine requirement and mating type. The results of these phenotype tests are presented in table 3.2. From these results, and the independent segregation of the additional *RBCS2* fragment (figure 3.9) it can be clearly demonstrated that authentic progeny were being analysed and that the Pm^R^ phenotype is not tightly linked to other markers.
Figure 3.10 Southern analysis of ectopic integration of pSP108. DNA extracted from Pm65, untransformed host arg7-8cwd and WT (CC-1021 & CC-125) cells. DNA was digested with EcoRI and hybridised to the 1.4 kb EcoRI RBCS2/ble cassette. The endogenous RBCS2 present in all cell types is marked with a diamond. The RBCS2/ble transgene of Pm65 is marked with a triangle. The presence of the additional fragment of RBCS2 (marked with a circle) is also restricted to Pm65. The positive (+ve) control is the 1.4 kb EcoRI fragment of pSP108.
Table 3.2 Phenotypes of progeny from the Pm65(mt-) x WT(mt+) cross. The following traits were scored: sensitivity (S) or resistance (R) to Pm, presence (+) or absence(-) of a cell wall (cw); requirement for arginine in the medium (this phenotypic marker derives from the arg7-8 mutation present in Pm65 but which is masked by complementation with the introduced ARG7 gene); mating type (mt).

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<tr>
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<td>S</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5A</td>
<td>S</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5B</td>
<td>R</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5C</td>
<td>S</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6A</td>
<td>R</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7A</td>
<td>R</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>
3.2.8 Direct selection of Pm\textsuperscript{R} transformants.

The evidence presented above demonstrates that the RBCS2/ble construct can be used to transform C. reinhardtii to phleomycin resistance. However, whilst this demonstrates that there is no intrinsic block on the expression of foreign genes in the nucleus, the ble construct is of little practical use to Chlamydomonas research unless it can be used as a dominant selectable marker for the direct selection of transformants. In initial attempts at direct selection transformants were plated directly to medium containing the drug in soft TAP agar that also contained phleomycin. No transformants were ever recovered, presumably because C. reinhardtii is very sensitive to Pm and cells were killed before the Sh.ble protein was expressed. In an attempt to circumvent this problem various modifications were made to the basic protocol. Cells were plated in soft TAP agar without Pm onto medium that did contain the drug in the hope that expression of the ble gene would take place before the diffusion of Pm into the soft plating agar. Again no transformants were recovered. Another variation involved plating transformants to medium that lacked Pm, leaving the cells to recover on the plates for 24 hours and then overlaying with more soft TAP agar containing Pm. This method did result in colonies being recovered but subsequent analysis (growth in liquid culture and spotting to fresh TAP plates supplemented with Pm) revealed them to be false positives. A further modification that was tested involved leaving the transformants in the tubes overnight following vortexing in the hope that this would allow the expression of the ble gene. The cells were then plated to TAP+Pm the following day. This was also unsuccessful and no transformants were recovered.

A method that did work was finally devised and this is described in section 2.6. The method is based on that used to generate transformants complemented
with an endogenous gene (Kindle, 1990) but is performed in a larger vessel such that the cells can be diluted in fresh medium following the vortexing procedure and allowed to recover overnight in an orbital incubator. They are harvested the following day and plated to selective medium. Initial transformations were performed using \( \text{arg}7-8 \ \text{cwd} \) as the host and maintaining arginine in the medium throughout. Cells were plated to medium supplemented with \( \text{Pm} \) @ 2\( \mu \text{g/ml} \) in soft agar (also supplemented with \( \text{Pm} \) @ 2\( \mu \text{g/ml} \)). \( \text{Pm}^R \) colonies appeared after 7-9 days and typically 5-10 transformants per plate were recovered. The cell wall deficient strain \( \text{cwd} \) was also transformed in this way and again 5-10 \( \text{Pm}^R \) colonies were recovered per plate. In these transformation experiments ‘no DNA’ controls were routinely performed in parallel and \( \text{Pm}^R \) colonies were never recovered. In order to confirm that these were true transformants, colonies were grown in liquid culture and spotted to fresh TAP plates supplemented with \( \text{Pm} \) @ 2\( \mu \text{g/ml} \). In addition, Southern blot analysis was performed on twelve clones picked from five separate transformations. DNA was prepared from the clones by the miniprep method and digested with \( \text{EcoRI} \). Following electrophoretic separation, the DNA was blotted to a nylon membrane and hybridised to the \( \text{ble} \) gene. This Southern blot is presented as figure 3.11. It can be seen from the blot that all the transformants tested contain the \( \text{ble} \) gene. As with the co-transformants there are clones that contain intact \( \text{EcoRI} \) cassettes, clones in which one or both of the \( \text{EcoRI} \) sites have been lost and clones that contain both type of integration pattern. One difference between co-transformants and directly selected (ds) transformants that is immediately apparent however is copy number. The majority of the ds transformants appear to have a single copy of the \( \text{ble} \) gene although D2 has at least three. This has implications for gene tagging where it is important that
Figure 3.11 Southern analysis of DNA extracted from directly selected Pm\textsuperscript{R} transformants (A1 - E3) and the untransformed host strain \textit{arg7-8cw\textsubscript{d} (arg7)}. DNA was restricted with \textit{EcoRI} and hybridised with a probe specific for the coding region of \textit{ble}. Size markers are in kilobase pairs.
only a single integrative event takes place.

3.3 Discussion

This work has demonstrated that by introducing a bacterial gene flanked by endogenous *C. reinhardtii* controlling elements into the nuclear genome by co-transformation that the expression of a foreign gene in *Chlamydomonas* is possible. Southern analysis (figure 3.4) has revealed that this transgene is present in PmR transformants but is however also present in ~90% of PmS co-transformants. ~3% of co-transformants express the ble gene. Given that such a high percentage of the co-transformants in which the ble gene had integrated are PmS it was important to demonstrate that phleomycin resistance resulted from the integrated DNA and not from spontaneous mutation caused by the transformation procedure. Attempts were made to detect evidence of transcription from the chimeric *RBCS2-ble* gene. However, neither northern blot nor RNAase protection assay analysis proved sensitive enough to detect a transcript. This is perhaps due to the transcript being unstable or having a very short half life. Certainly, it is the case that significantly reduced levels of transcription from both intact and chimeric *C. reinhardtii* genes introduced into the nuclear genome by transformation has been reported by a number of groups (Kindle *et al.*, 1989; Blankenship and Kindle, 1992; Quinn *et al.*, 1993). In order to confirm that the PmR phenotype was caused by the integration of the ble gene, evidence of expression was sought by detecting the gene product. The Sh.ble protein is a glycopeptide of 13.6 kDa and is well characterised (Drocourt *et al.*, 1990). The ble gene in pSP108 is cloned downstream of the bacterial lac
promoter. *E. coli* cells harbouring pSP108 and grown in the presence of IPTG (a gratuitous inducer of the *lac* operon) were found to express the gene and therefore exhibit resistance to Pm. Western analysis of *E. coli* cells expressing the ble gene, PmR transformants and untransformed host cells (figure 3.6) has revealed that soluble protein from these cells probed with anti-Sh.ble antibodies detects a band of the correct size in the bacterial cells and in the PmR transformants but not in untransformed host cells. It is interesting to note that the transformant that contains a single copy of the ble gene (Pm65) has the weakest signal from the detection of the anti-Sh.ble antibody. Determination of the levels of sensitivity to Pm amongst all of the co-transformants (figure 3.7) supports this observation that the ble gene is being expressed at its lowest level in Pm65. The results of sensitivity tests expressed as a graph in figure 3.7 show that there are three broad groups into which the PmR transformants fall. Given that Pm65 which is known to contain only one copy of the ble gene is in the most sensitive group and that Pm71 which has at least three copies is the least sensitive, it is tempting to speculate that differences in levels of resistance are due to differences in copy number.

Further evidence that the transforming DNA is responsible for the PmR phenotype is presented from genetic analysis. In crosses between Pm65 and WT cells a number of unlinked phenotypic markers including resistance to phleomycin are seen to segregate independently in progeny (table 3.2). Furthermore, the PmR phenotype is seen to co-segregate with the ble gene in all cases (figure 3.9). Interestingly, a band detected in Southern analysis that is present in Pm65 but not in WT or untransformed host cells (figure 3.10) and is believed to be a section of pSP108 containing RBCS2 sequences, is demonstrated
to segregate independently of the ble gene in Pm65 (figure 3.9). This is believed to be due to a separate, ectopic integration event during transformation. If this is the case then there are implications for gene tagging experiments in C. reinhardtii. Other groups have scored for a ‘tagged gene’ by co-segregation of the marker phenotype (e.g. Nit+ or Arg+) with the mutation. Hence they would miss ‘tagging’ by non-functional transforming DNA. Insertional mutagenesis experiments that lead to the cloning of a gene via the tagged disruption of a gene with a scorable phenotype (e.g. Tam & Lefebvre, 1993) require that a single integrative event has occurred to facilitate analysis. Should multiple integration events prove to be common in the transformation of C. reinhardtii, then detailed analysis of transformants would be required prior to attempting to clone out DNA flanking the marker.

The reason for the low rate of expression of the ble gene in co-transformants integrating pSP108 remains uncertain. The high incidence of integration of a second, non-selected DNA species in co-transformation is a well documented phenomenon in C. reinhardtii (Day et al., 1990; Diener et al., 1990; Blankenship & Kindle, 1992). Diener et al., (1990) introduced a radial spoke protein gene (RSP3) with the NIT1 gene in a co-transformation into a nit1-, rsp3- host. Of 21 NIT1+ transformants recovered 14 had also been restored to motility. This represents a co-transformation rate of some 67%. In addition some of the transformants that remained non-motile also contained RSP3 DNA. In the co-transformations using ARG7 and ble whilst the great majority of transformants do contain ble DNA only ~3% express the ble gene. The reason for the silencing of this transgene in so many transformants is not clear. In higher plants the silencing of transgenes by methylation is well documented (e.g. Inglebrecht et al., 1994).
Blankenship and Kindle (1992) attempted to express the uidA gene from the C. reinhardtii NIT1 promoter without success. They reported methylation of the transgene to be implicated in the absence of expression. This was also investigated in this study. By using isoschizomers no difference in the methylation pattern from PmS or PmR transformants was detected. Furthermore the use of the hypomethylating base analogue 5-aza-cytidine failed to activate copies of ble putatively silenced by methylation. I would tentatively suggest therefore that methylation does not appear to be a factor here. Another widely reported phenomenon in higher plants is the “co-suppression” of transgenes that occurs when multiple copies of a transgene are integrated during transformation (Flavell, 1994; Matzke & Matzke, 1995). Whilst much detailed analysis would be required to say for certain if this were a factor here, copy number of ble DNA in PmS and PmR transformants (figure 3.4) does not appear to be significantly different. Whilst copy number independent gene expression seems to be the norm in C. reinhardtii transformants (Kindle et al., 1989; Mitchell & Kang, 1991; Smart & Selman, 1993) it is interesting to note that increased expression following the multiple integration of OEE1 has been reported (Mayfield, 1991). Silencing of the ble gene in PmS co-transformants could also be due to small mutations such as deletions and insertions, or rearrangements within the gene that occur prior to, or during, integration and are too small to be detected by Southern blot analysis. Sequencing of the RBCS2-ble constructs from PmS co-transformants would be required to test this possibility. Alternatively the observed difference in expression between PmR and PmS clones may be due to ‘positional’ effects. The ble gene in PmS clones may have integrated into transcriptionally inactive regions of the genome or, in the clones where the gene is being expressed, the integration
event may have taken place such that the gene is placed under the influence of
an enhancer or some other cis acting element. The fact that co-transformations
with endogenous genes often results in a high level of expression of the
unselected gene (e.g. Diener et al., 1990) would argue against the former
scenario, whilst the fact that the RBCSI promoter has been used on other
constructs for efficient expression of chimeric genes (e.g. Kozminski et al., 1993)
would suggest that further elements are not required for expression. The reason
for the lack of expression in so many co-transformants remains therefore to be
established.

As reported above, a protocol has been developed that allows direct
selection of transformants using the RBCSI-ble construct. Southern analysis of
transformants generated in this way (figure 3.11) reveals that fewer copies of the
marker are integrated than when pSP108 is introduced in a co-transformation
with the ARG7 marker and that the majority appear to have received only a single
copy. This is important because, as discussed above, gene tagging procedures are
facilitated if only a single, disruptive, integrative event takes place. Whilst the
initial transformation efficiency is low (5-10 transformants per plate) the marker
represents a significant addition to the transformation arsenal available for
Chlamydomonas. The use of the ble marker means that transformation of C.
reinhardtii need no longer be restricted to the rescue of mutants with a selectable
phenotype. The availability of a dominant marker of this kind will also assist in
the development of constructs for 'positive - negative' selection of the kind that
are used to select for rare homologous recombination events in mammalian cells
(e.g. Mansour et al., 1988). An investigation of the parameters that affect
transformation efficiency and attempts to improve transgene expression are
presented in the following chapter.
CHAPTER 4

Nuclear transformation and use of the ble marker in

*Chlamydomonas reinhardtii.*
4.1 Introduction

The rate of transformation for a given system can be improved in two ways; firstly, the selectable marker used can be modified so as to make it more effective, secondly, the transformation procedure itself can be altered to make it more efficient. For example, the transformation of *Chlamydomonas* progressed when the use of endogenous genes as selectable markers delivered by the bombardment of cells was developed (Debuchy *et al*., 1989; Kindle *et al*., 1989; Mayfield & Kindle, 1990). Transformation rates were improved by the development of the glass bead system (Kindle, 1990) and, when using the *NIT1* marker, the efficiency of this system was further improved by the inclusion of 5% PEG in the transformation procedure.

Work presented in the previous chapter has demonstrated that the expression of a foreign gene in *C. reinhardtii* is possible, but the transformation rate remains relatively low. As has been demonstrated by Southern analysis (figure 3.4) although the marker has integrated in ~90% of co-transformants it is silent in the great majority. Improvements in the transformation rate could be achieved by alterations to the *ble* marker. Reasons previously proposed for the absence of foreign gene expression in *C. reinhardtii* have been only partially addressed by pSP108. For instance, whilst the construct is based on a gene with a more suitable codon usage (than other foreign genes previously tested) and is under the control of endogenous 5' and 3' UTR sequences, it remains intronless. Studies of foreign gene expression have demonstrated the importance of introns for the efficient expression of transgenes in plants and animals (Kyozuka & Shimamoto, 1995; Webb & Nisbet, 1995). All *C. reinhardtii* nuclear genes sequenced to date contain introns, for example *ARG7* which has eleven (Purton
& Rochaix, 1995). There is therefore a possibility that the presence of an intron may be required for the efficient expression of the ble gene.

It has been suggested that the use of scaffold attachment regions (SARs) on constructs may improve the expression of foreign genes in Chlamydomonas (Kindle & Sodeinde, 1994). Certainly this has been the case for other organisms where it has been demonstrated that SARs improve the expression of transgenes introduced by transformation (e.g. Allen et al., 1993; Stief et al., 1989). The presence of SARs on an construct are believed to promote a form of integration that mimics the ‘open’ or actively transcribed DNA structure within the higher order chromatin structure (Callan, 1982).

An alternative explanation for the observed differences in expression between PmR and PmS transformants harbouring the RBCS2/ble gene may be related to recombination between homologous plasmid sequences. Extrachromosomal recombination prior to the integration of transforming DNA occurs readily in higher plants (Puchta et al., 1994) and Chlamydomonas (Gumpel et al., 1994). It may be that in those co-transformants expressing the ble gene pSP108 has recombined with pARG7.8 via common plasmid sequences and that this has given some advantage. Perhaps the presence of repetitive DNA within the ARG7 introns (Debuchy et al., 1989) promotes integration.

Alternatively, improvements in the efficiency of the transformation system may prove a better way of increasing the transformation rate with a foreign gene. Kindle (1990) looked at several parameters that effected the efficiency of transformation using the glass bead method. The two main factors were demonstrated to be the culture density of recipient cells and the length of vortexing - these were optimised at 1-2x10^6 cells/ml and 10-20 seconds,
respectively. Kindle also looked at other parameters including the addition of hexamine cobalt, spermidine, carrier DNA, mannitol, dimethyl sulfoxide and PEG. Of these only the addition of PEG represented an improvement. Kindle also reported a slight increase in the rate of transformation when linearised rather than supercoiled DNA was used. However Purton & Rochaix (1995) found no difference between linear and supercoiled DNA when using the ARG7 marker.
4.2 Results

4.2.1 The effect of various treatments on transformation rate.

Transformation efficiency in *Dictyostelium* has been improved 20-fold by a process termed restriction enzyme-mediated integration (REMI), whereby the transforming DNA is introduced into cells in the presence of a restriction enzyme (Kuspa & Loomis, 1992). To test whether a similar improvement could be established in *Chlamydomonas* pARG7.8 linearised with *BamHI* was introduced into *arg7*-8 *cw* 

5 cells both with and without 100U of *BamHI*. Five separate transformations with *BamHI*, five without and one 'no DNA' control were performed. The results summarised in table 4.1 show that unlike the situation that pertains in *Dictyostelium*, the inclusion of a restriction enzyme in *Chlamydomonas* transformations results in a 10-fold decrease in the number of transformants recovered.

The addition of 5% PEG was found to increase the transformation rate 100-fold when using the *NIT1* marker (Kindle, 1990). However the use of PEG with the *ARG7* marker has no effect (Purton & Rochaix, 1995). Once a direct selection procedure was established the effect of PEG upon transformations using the *ble* marker was investigated. Mutant *cw*d and another cell wall deficient mutant *cw15* were used as the recipient cells. Five transformations were performed for each cell type both without and in the presence of 5% PEG. No DNA controls for both cell types +/- PEG were also performed. The results are summarised in table 4.2. The use of PEG with *cw*d cells has no effect and in both sets of transformations where this strain was the host approximately 10 transformants/plate were recovered. The transformation rate using *cw15* was
<table>
<thead>
<tr>
<th>marker</th>
<th>100U Bam HI</th>
<th>average number transformants/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pARG7.8: Bam HI</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>pARG7.8: Bam HI</td>
<td>-</td>
<td>104</td>
</tr>
<tr>
<td>no DNA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 4.1* Restriction enzyme mediated integration (REMI) transformation of *C. reinhardtii*. The effect of transformant recovery when transformations are performed in the presence (+) and absence (-) of 100 U of *Bam*HI.
Table 4.2 The effect of PEG upon transformation efficiency. *cw15* and *cw_d* cells were transformed in the presence (+) and absence (-) of 5% PEG.

<table>
<thead>
<tr>
<th>marker</th>
<th>host</th>
<th>5% PEG</th>
<th>average number transformants/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSP108</td>
<td>(cw_d)</td>
<td>-</td>
<td>12.6</td>
</tr>
<tr>
<td>no DNA</td>
<td>(cw_d)</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>pSP108</td>
<td>(cw_d)</td>
<td>+</td>
<td>11</td>
</tr>
<tr>
<td>no DNA</td>
<td>(cw_d)</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>pSP108</td>
<td>(cw15)</td>
<td>-</td>
<td>1.4</td>
</tr>
<tr>
<td>no DNA</td>
<td>(cw15)</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>pSP108</td>
<td>(cw15)</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>no DNA</td>
<td>(cw15)</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>
lower (an average of 1.4/plate) however in the presence of PEG this rate increased 15-fold with an average of 21 transformants/plate being recovered and the greatest number present on a plate being 43. This result suggests that the efficacy of PEG is related to the recipient strain rather than the marker employed. The effect of the addition of 5% PEG to transformation experiments with cw15 as the host is graphically demonstrated in figure 4.1.

Kindle (1990) found that plating transformants in a suspension in soft agar resulted in a higher recovery rate than spreading the cells directly onto agar plates. Recently at meetings different researchers have begun to report that they have experienced the opposite and achieve better rates by directly spreading. To investigate this, ten cw15 transformations using pSP108 were performed in the presence of 5% PEG. Five were plated in soft agar and five spread directly to agar plates. ‘No DNA’ controls were also performed and treated in the same way. The results (presented in table 4.3) show that in my hands plating in soft agar results in approximately twice as many transformants being recovered. This supports the findings of Kindle (1990), that plating in soft agar leads to the recovery of more transformants.

Another parameter investigated was the effect of growth rate on recovering transformants. One of the easiest ways to slow growth in *C. reinhardtii* is to reduce light intensity. Ten transformations were performed introducing pSP108 into cw4 cells. Following plating, five plates were incubated in bright light (45μE/m²/s), the other five were incubated in dim light (2μE/m²/s) for 7 days before being transferred to bright light. The results are given in table 4.4. As can be seen most transformants were recovered from the plates grown in bright light, indicating that actively growing cells provide better conditions for
Figure 4.1 The effect upon transformant recovery for \textit{cw15} cells transformed in the presence and absence of 5\% PEG.
Table 4.3 The effect upon transformant recovery of direct spreading or plating in soft agar of transformed cells.
Table 4.4  The effect of light intensity upon the recovery of transformants.

<table>
<thead>
<tr>
<th>marker</th>
<th>light intensity (μE/m²/s)</th>
<th>average number transformants/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSP108</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>pSP108</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>no DNA</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>no DNA</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
selection of transformants.

4.2.2 Covalent linkage of DNA for transformation.

As previously discussed it is conceivable that in co-transformants expressing the ble gene, extrachromosomal recombination between pSP108 and pARG7.8 has occurred and that this promotes integration within transcriptionally active regions of the genome (perhaps via the repetitive DNA present in the introns and upstream region of ARG7). To test this the ble marker in the form of the 1.4 kb RBCS2/ble EcoRI cassette was cloned into the unique EcoRI site of pARG7.8 to give plasmid pARG7.8/ble (figure 4.2). This construct was transformed into arg7-8 cw^ cells and transformants selected for their ability to grow in the absence of exogenously added arginine. One hundred of these transformants were selected and screened for resistance to phleomycin. Six transformants were recovered: Pm1.16, Pm1.25, Pm1.38, Pm1.55, Pm1.67 and Pm1.95. Whilst this does not represent a tremendous advance on the previous rates of co-transformation achieved (2, 4 and 4 out of three sets of 100 transformants screened) it never the less warranted further investigation of the merits of linking ble to repetitive DNA.

4.2.3 The effect of repetitive DNA

Once a direct selection protocol had been devised it became possible to test the efficacy of including repetitive DNA in the pSP108 plasmid. A 0.55 kb PstI fragment of the ARG7 gene that has been demonstrated to hybridise to DNA throughout the nuclear genome (Debuchy et al., 1989) was cloned into a unique PstI site of pSP108 upstream of the 1.4 kb EcoRI cassette to give plasmid pSP108.55 (figure 4.3). The construct was tested by comparing its transformation
Figure 4.2 Map of plasmid pARG7.8/ble. The 1.4 kb RBCS2/ble cassette from pSP108 is cloned within the unique EcoRI site of pARG7.8
Figure 4.3 Map of plasmid pSP108.55. The 0.55 kb PstI fragment of pARG7.8 is cloned within the unique PstI site of pSP108 upstream of the hybrid RBCS2/ble gene.
efficiency to that of pSP108. Five transformations using pSP108 and five using pSP108.55 were performed using cw15 as the host. ‘No DNA’ controls were also performed. The inclusion of the repetitive DNA was found to have an adverse effect on the transformation rate with an average of only 0.8 transformants/plate recovered compared to an average of 5 transformants/plate recovered using pSP108.

4.2.4 The effect of increasing promoter sequence.

As the RBCS2 promoter has been used to efficiently express other C. reinhardtii genes (e.g. Kozminski et al., 1993) the possibility was addressed that insufficient promoter sequence from the RBCS2 had been included in pSP108. Of the cloned RBCS2 promoter (Goldschmidt-Clermont & Rahire, 1986) all but the first ~300 bp had been included on pSP108. To investigate whether some important distal element had been omitted from pSP108 this additional sequence was cloned into pSP108. A ~400 bp HindIII, BstXI fragment was excised from the RBCS2 genomic clone p1.03 (Goldschmidt-Clermont & Rahire, 1986). A 70 bp HindIII, BstXI fragment was deleted from pSP108 and replaced with the fragment from p1.03 to create pSP110 (figure 4.4). This construct was tested by co-transforming arg7-8 cwd cells with pARG7.8 and pSP110. Of 100 transformants to arginine biosynthesis only one (Pm3.9) exhibited a PmR phenotype, less than had been achieved with pSP108.

4.2.5 The effect of a scaffold attachment region.

The addition of a scaffold attachment region (SAR) to a transgene has been shown to increase reporter gene expression (e.g. Allen et al., 1993) and it has been suggested that the use of an SAR may improve both foreign and endogenous gene expression in Chlamydomonas (Kindle & Sodeinde, 1994). An
Figure 4.4 Map of plasmid pSP110. An additional ~400 bp fragment of RBCS2 5' UTR DNA is cloned between the HindIII and BstXI sites of pSP108.
SAR from pea contained within a ~ 200bp *HindIII* fragment of plasmid pH268.7 (Pwee & Gray, 1993) was cloned into the unique *HindIII* site of pSP108 to give pSP113 (figure 4.5). This construct was tested by comparing its transformation efficiency to that of pSP108. Five transformations using pSP108 and five using pSP113 were performed using *cvl5* as the host. 'No DNA' controls were also performed. The inclusion of the SAR was found to have an adverse effect on the transformation rate with only one transformant being recovered from all five transformations compared to an average of 5 transformants/plate recovered using pSP108.

4.2.6 The effect of introns

As discussed previously various evidence points to the importance of introns for the efficient expression of nuclear genes in *Chlamydomonas*. In an attempt to investigate this two approaches were taken; firstly a construct was made in which the *ble* gene was interrupted by an intron, secondly the cDNA for the *OEE1* gene was cloned into the *C. reinhardtii* expression vector pSP105.

The vector pARG7-X had previously been constructed by Saul Purton (unpublished). This construct takes advantage of the fact that the first intron of the *ARG7* gene occurs after just the initiation methionine and a threonine codon (Purton & Rochaix, 1995). In pARG7-X the 5’ UTR, the first two codons and the first intron of *ARG7*, together with the 3’ UTR from the *RBCS2* gene have been cloned into pBluescript SK- (Stratagene) such that they are separated by a multiple cloning site. This allows the cloning of genes in which the cloned gene would be expressed as a fusion with the first two codons of *ARG7* and any codons remaining from the polylinker (figure 4.6). The *ble* gene (present as a PCR product in pSK-) was excised using *ClaI* and cloned into the *ClaI* site of pARG.
Figure 4.5 Map of plasmid pSP113. An ~200 bp HindIII fragment of plasmid pH268.7 containing an SAR from pea is cloned into the unique HindIII site of pSP108.
Figure 4.6 Map of plasmid pARG7-X. The construct contains the 5' UTR, the first two codons and the first intron of ARG7, together with the 3' UTR from the RBCS2 gene cloned into pBluescript SK- (Stratagene) and separated by a multiple cloning site. * Indicates site not unique.
7-X to give pARG7-X/ble (figure 4.7). This construct was introduced into arg7-8 cwd cells as a co-transformation with pARG7.8. One hundred transformants rescued to arginine biosynthesis were screened for PmR however none grew. The construct was also used to directly select transformants of cw15. Five sets of transformations were performed using pSP108 and five using pARG7-X/ble plus ‘no DNA’ controls. An average of 12.6 transformants/plate were recovered when using pSP108 but only 0.8/plate when using pARG7-X/ble. Whilst pARG7-X/ble does transform cells to PmR the efficiency is lower than pSP108. Conclusions about the efficacy of an intron can not be drawn from this result however as the ble gene on pARG7-X/ble is under the control of a different (weaker) promoter and also would be expressed as a fusion protein with an additional seven amino acids at the amino terminus that could affect biological activity.

Another approach was also employed to investigate the importance of an intron. A genomic copy of the OEE1 gene (also known as psbO in the wider plant community) that encodes the 33 kDa subunit of the water splitting complex of photosystem II, has previously been used to transform oee1- cells to photoautotrophic growth (Mayfield & Kindle, 1990). To investigate whether the cDNA could be similarly used, the OEE1 cDNA from plasmid pPII-12P4 (Mayfield et al. 1989) was cloned into the expression vector pSP105. The OEE1 cDNA was amplified from pPII-12P4 by PCR using the 25mer GCAAAAGATGCCCCGCTGCCC (5’ sense oligo) and the 35mer ACGAATTCTAGACTCGAGTTTTTTTTT (3’ antisense oligo). An initial denaturation step at 95°C for 3 minutes was followed by 12 cycles of; denaturing 95°C - 1 minute, primer annealing 50°C - 1 minute and primer extension 72°C - 2 minutes. A 1.6 kb product containing two MscI restriction sites; one (underlined) at
Figure 4.7 Map of plasmid pARG7-X/ble. A Clal fragment containing the ble gene was cloned into the Clal site of pARG7-X to give pARG7-X/ble.
the start codon (bold), and another 120 bp downstream and a unique XbaI site (underlined) was blunt cloned into the EcoRV site of pSK-. The 1.5 kb MscI, XbaI fragment was cloned into the MscI and XbaI sites of pSP105 to create pSP111. The 120 bp MscI fragment was then cloned into pSP111 to give pSP112 (figure 4.8). The 120 bp MscI fragment was confirmed to be in the right orientation and the RBCS2-OEE1 junction was confirmed to be in frame by sequencing through the junction using the following 19mer CCCTCCTGATAAACCGGCC (5’ sense oligo to the 5’ UTR RBCS2 sequence).

The cell-wall deficient, oee1- strain FuD44 cwΔ (in which the OEE1 gene is disrupted by the integration of a transposon (Day et al., 1988)) was transformed with pSP112. Ten separate transformations using pSP112 and ten 'no DNA' controls were performed. Five of each kind were plated directly to Tris-minimal medium to select for transformants capable of photoautotrophic growth and the remaining five of each kind were grown overnight in TAP to allow expression of the OEE1 gene prior to plating to selective medium the following day. No transformants were recovered and repeating the experiment yielded the same result. This suggests that the presence of an intron is important. When the genomic copy of the OEE1 gene was used in the biolistic transformation of FuD44 cells many revertants were isolated in which the disrupting transposon had excised. Interestingly no revertants arose as with the biolistic transformation of an oee1- strain (Mayfield & Kindle, 1990) suggesting that the glass bead method (unlike particle bombardment) does not induce transposon excision.

4.2.7 Use of the ble marker as an insertional mutagen.

One use of selectable markers employed in transformation is to create
Figure 4.8 Map of plasmid pSP112. A cDNA copy of the *C. reinhardtii* OEE1 gene was cloned between the *MscI* and *XbaI* sites of pSP105 creating a translational fusion between *RBCS2* and *OEE1*.
mutants by insertional mutagenesis. This is a process by which transforming DNA that has integrated into the nuclear genome sometimes disrupts a gene with a scorable phenotype, thus creating a mutant and ‘tagging’ the gene in one operation. To investigate the use of the ble marker as an insertional mutagen, transformants generated in other experiments were screened for any aberrant phenotypes that were readily scorable. Many swimming mutants were observed in which the cells either had paralysed flagella or in which the flagella were absent. In addition mutants with unusual colony colour were also observed. One of these such colour mutants was selected for further analysis and some preliminary characterization has been performed.

The mutant selected came from a direct selection experiment in which pSP108 was introduced to cw\textsubscript{d} cells. It was selected from a plate that had two transformants; one of which had the usual (green) colony colour and the mutant which was yellow/green in colour. It was considered that this mutant was in some way aberrant in chlorophyll biosynthesis. To test this, the chlorophyll from 1 ml of a culture of the mutant and 1 ml of a culture of untransformed cw\textsubscript{d} cells was extracted with 80% (v/v) acetone. The absorbance spectra between 640 nm and 665 nm was determined for both samples. The spectra are presented in figure 4.9.

The ratio of chlorophyll \textit{a} and chlorophyll \textit{b} in a sample can be determined by a method described by Arnon (1949) using the extinction coefficients for chlorophyll extracted in 80% (v/v) acetone. The contributions to absorbance by each chlorophyll at 663 and 645 nm is given by:

\begin{align*}
A_{663} &= 82.04 \text{ chl } a + 9.27 \text{ chl } b \\
A_{645} &= 16.75 \text{ chl } a + 45.6 \text{ chl } b
\end{align*}

where: A = absorbance, chl = chlorophyll concentration in mg/ml.
Figure 4.9 Absorbance spectra of $cw_d$ (panel A) and $cbd_1$ (panel B) cell extracts. Cells from 1 ml of culture were extracted with 80% (v/v) acetone and absorbance between 640 nm and 665 nm determined.
When the simultaneous equations are solved;

\[ \text{chl } a = 0.0127 \ A_{663} - 0.00259 \ A_{645} \]
\[ \text{chl } b = 0.0229 \ A_{645} - 0.00467 \ A_{663} \]

The \textit{cwd} sample had absorbance values at \( A_{645} \) and \( A_{663} \) of 0.084 and 0.345, respectively, the values for the mutant sample were 0.204 and 0.580. By using the above formula the chlorophyll \( a: b \) ratios were determined as being 3.5:1 for \textit{cwd} but 13.5:1 for the mutant, indicating a marked reduction in the amount of chlorophyll \( b \) (see figure 4.10). It can be observed from this figure that the aberrant colony colour of the mutant can be ascribed to a low level of chlorophyll \( b \) in relation to chlorophyll \( a \). The mutant has therefore been named \textit{cbd1} (chlorophyll \( b \) deficient 1). The ability of \textit{cbd1} to grow photoautotrophically was tested by spotting cultures to minimal medium plates. It was found that \textit{cbd1} was capable of phototrophic growth as previously described for other chlorophyll \( b \) deficient mutants (e.g. Eichenberger \textit{et al.}, 1986). From this result it is possible to conclude that the reaction centres (which contain chlorophyll \( a \), but no chlorophyll \( b \)) are unaffected in the mutant.

Chlorophyll samples for both \textit{cwd} and \textit{cbd1} cells were adjusted to approximately the same concentration and absorbance spectra between 350 and 700 nm were determined. These are presented as figure 4.11. On these spectra chlorophyll absorbance is responsible for peaks 1 and 4 and peaks 2 and 3 are due to other pigments \( e.g. \) carotenoids. In \textit{cwd} cells peak 2 is present as a shoulder on peak 1, however on the spectra for the \textit{cbd1} cells 2 is present as a discrete peak. This is due to a narrowing of peak 1; further evidence for a reduced amount of chlorophyll in the sample.

In order to establish that \textit{cbd1} is harbouring the \textit{ble} marker, DNA from
Figure 4.10 Morphology of cwt and cbdl cells and their respective chlorophyll a:b ratios.
Figure 4.11 Absorbance spectra of \( c_{\text{wd}} \) (panel A) and \( c_{\text{bd}1} \) (panel B) cell extracts. Cells from 1 ml of culture were extracted with 80% (v/v) acetone and adjusted to approximately equal concentrations. The absorbance spectra between 350 nm and 700 nm were determined.
cbd1, untransformed cw4 host cells and the other transformant from the same plate as cbd1, henceforth referred to as transformant 1(Tr1), was subjected to Southern analysis using a ble specific probe. As shown in figure 4.12 the ble marker is present in cbd1 and Tr1.

Further analysis would be required to demonstrate conclusively that the integration of the ble marker is responsible for the aberrant phenotype of cbd1. However, once established, the isolation of the tagged gene (believed to be involved in the synthesis of chlorophyll b, its turnover or its assembly into the light harvesting complex) should be facilitated by the advantages of the ble marker as previously discussed in chapter 3.
Figure 4.12 Southern analysis of DNA extracted from Tr 1, *cbd* 1 and *cwd* cells. DNA was digested with *Eco*RI and hybridised with a probe specific for the coding region of *ble*. The positive (+ve) control is the 1.4 kb *Eco*RI fragment of pSP108. Size markers are in kilobase pairs.
4.3 Discussion

With the exception of the use of PEG none of the adaptations to the glass bead protocol had a beneficial effect. The results obtained from using PEG suggest that the efficacy of the treatment is related to the status of the residual cell wall of the recipient strain rather than the marker used. Hyams & Davies (1971) generated a number of cell wall mutants of *C. reinhardtii* and characterised them as belonging to three groups according to their morphology; Group A - in which the walls are produced in more or less normal amounts but are not attached to the plasmalemma, Group B - in which the walls are produced in normal amounts and are attached to the plasmalemma and Group C - in which the walls are produced in minute quantities or are absent. The strain used in which PEG had a beneficial effect (cw15) was generated by Hyams & Davies (1971) and belongs to group C, the other strain cw_d is uncharacterized with respect to this criterion. This finding explains the apparent contradiction whereby transformations with the NIT1 marker benefit from the inclusion of PEG (Kindle, 1990) but no difference is observed with the ARG7 marker (Purton & Rochaix, 1995). The recipient strain used by Kindle is based on the cw15 mutation whereas Purton & Rochaix used cw_d. Further work in our laboratory suggests that transformations of other strains in group C benefit from the inclusion of PEG (M. Turner personal communication).

My findings regarding plating transformants in soft agar versus spreading directly to agar support those of Kindle (1990). However as already discussed other researchers have observed the opposite. It is possible that the temperature of the soft agar may explain this inconsistency, it should be as cool as possible without setting when used otherwise cell death may occur. Alternatively this too
may be related to the status of the cell wall with some strains representing group C being more susceptible to mechanical damage due to the shear forces encountered during spreading.

The use of constructs based on pSP108 but that included additional sequences; pSP108.55 (repetitive DNA), pSP110 (extra 5’ UTR) and pSP113 (SAR) all resulted in the recovery of fewer transformants than pSP108. Whilst it is accepted that these modifications may not improve transformation efficiency it is difficult to see why (particularly in the case of the simple addition of more 5’ UTR from RBCS2) an apparently deleterious effect is promoted. It should be noted however that all of these constructs represent different DNA preparations and it is conceivable that differences in the quality of the DNA between batches influences transformation rates.

The work with the use of introns is inconclusive. The cDNA copy of OEE1 failed to transform FuD cw^ cells and this is in agreement with the findings that introns are required for the expression of endogenous genes in the related alga Volvox carteri (Gruber & Schmidt - unpublished). However the ble gene present on pSP108 does not contain an intron and yet is expressed (albeit at a low level). The inclusion of an intron within ble as pARG7-X/ble results in the recovery of fewer transformants than obtained with pSP108 but, as previously noted, it is under the control of a different promoter. Furthermore, due to the way in which the construct is made the gene product would be a fusion protein with an additional seven amino acids at the amino terminus thus making a direct comparison with pSP108 impossible. To investigate the effect of an intron in the ble gene a better approach may be to artificially introduce an intron within the ble gene on pSP108.
CHAPTER 5

The L41 ribosomal protein gene of *Chlamydomonas reinhardtii*. 
5.1 Introduction

*Chlamydomonas reinhardtii* is sensitive to the translation inhibitor cycloheximide (Cyh) which exerts its effect on cytoplasmic 80S ribosomes and inhibits eukaryotic protein synthesis (Hoober & Blobel, 1969). It has been proposed that cycloheximide inhibits translation during initiation, elongation and termination. It is believed that the step inhibited depends upon the concentration of the drug (Dawson *et al.*, 1986). Nuclear mutations resulting in cycloheximide resistant strains of *C. reinhardtii* have been described; act-1 which maps to linkage group II (Sager & Tsubo, 1961) and act-2 which maps to linkage group VI (Smyth *et al.*, 1975). A separate nuclear mutation resulting in a cycloheximide strain, cyr-1, has also been described (Goodenough *et al.* 1981) however Flemming *et al.* (1987) report that cyr-1 is closely linked or allelic with act-2. The molecular basis of these mutations has not been determined. The L38 ribosomal protein of act-2 was shown to have an altered charge as demonstrated in 2-D electrophoresis but crosses revealed that this mutant form of the L38 protein did not co-segregate with cycloheximide resistance (Fleming *et al.* 1987).

In several CyhR species of yeast including *Schwanniomyces occidentalis* (Del Pozo *et al.*, 1993), *Kluyveromyces lactis* (Dehoux *et al.*, 1993), *K. fragilis*, *Candida maltosa* and *C. tropicalis* (Kawai *et al.*, 1992) the molecular basis of the CyhR phenotype was found to be a substitution within the ribosomal L41 subunit of a proline residue found in sensitive species *e.g.* *S. cerevisiae* (Kawai *et al.*, 1992) with a glutamine residue in resistant species (Del Pozo *et al.*, 1993). It is believed that the mutation abolishes binding of Cyh to the 60S subunit of the cytoplasmic ribosome. Mutations in the ribosomal protein subunits L23 (Ishiguro,
1985) and L29 (Fried & Warner, 1982; Stocklein & Peipersberg, 1980) have also been demonstrated to confer resistance to Cyh. The L41 proline to glutamine substitution observed in certain yeasts involves the substitution of a non-polar (hydrophobic) residue with a polar (hydrophillic) one and this is likely to alter the architecture of the L41 protein that may in turn affect the ability of Cyh to bind. Dehoux et al. (1993) raise the possibility that L23, L29 and L41 are closely associated and create a Cyh binding domain. This, for the time being, remains conjecture. However, attempts are underway to characterize the structure of the eukaryotic ribosome in rat (Suzuki & Wool, 1993).

Given the convincing evidence that a P -> Q substitution within the L41 protein of yeast confers Cyh resistance, there is a possibility that a mutated form of the endogenous L41 gene could be used as to select for nuclear transformants in C. reinhardtii. This has already been demonstrated in yeast. A mutant copy of the L41 encoding the glutamine residue was introduced by transformation into a Cyh\(^{S}\) strain of S. cerivisiae resulting in the acquisition of a Cyh\(^{R}\) phenotype (Kawai et al., 1992). A similar approach has already been used in Chlamydomonas. A mutant form of the CRY1 gene encoding ribosomal protein S14 has been used to select for cryptopleurine and emetine resistant transformants (Nelson et al., 1994). As an initial step towards developing a marker based on Cyh\(^{R}\) the ribosomal protein L41 gene (RPL41) of C. reinhardtii has been cloned and sequenced.
5.2 Results

5.2.1 Isolation and sub cloning of an L41 cDNA.

A cDNA library of *C. reinhardtii* genes in λgt10 prepared by L-G Franzén (Stockholm) was screened by A. Atteia (Stockholm) using the following oligonucleotides I designed to conserved regions of previously published L41 sequences: The 23mer L41 5’ ATGGTG/cAACGTG/cCCCAAGACCCG (5’ sense oligo), the 24mer L41 3’ CTTCTTGGTGGTCTTG/cGCCTTCTT (3’ antisense oligo) and the 35mer L41#3 CAGGGCAAGCGCCGCTACGACCGCAAGCAGTCG/cGG (sense oligo to an internal sequence). Ten tertiary screen clones that hybridised to all three oligos were selected for analysis. DNA was prepared from all ten phage clones by the plate lysate method as described in section 2.5.3 and the presence of an insert checked by digestion with *Eco*RI and agarose gel electrophoresis. One clone (λgt10 L41#7) with an insert of ~650 bp was selected. To establish that the insert was an authentic L41 clone and to determine its orientation within λgt10, diagnostic PCR reactions were performed. The insert from this clone was amplified by PCR using λgt10 forward: AGCAAGTTCAGCCTGGTTAAG and λgt10 reverse: CTTATGAGTATTTCTTCCAGGGTA primers and also the L41 3’ primer detailed above with either λgt10 forward or λgt10 reverse primers. An initial denaturation step at 95°C for 4 minutes was followed by 15 cycles of; denaturing 95°C - 1 minute, primer annealing 50°C - 1 minute and primer extension 72°C - 1 minute. A product of ~700 bp was obtained using the λgt10 forward and λgt10 reverse primers and a product of ~500 bp was obtained using
the λgt10 forward and L41 3' primers. No product was obtained using the λgt10 reverse and L41 3' primers. These results indicated that the L41 gene was in the same orientation as the λgt10 forward primer (figure 5.1). The ~700 bp PCR product generated with the λgt10 forward and reverse primers was blunt cloned into the EcoRV site of pBluescript pKS- (Stratagene) to give pKS.L41#7.

5.2.2 Nucleotide sequence of the C.reinhardtii L41 ribosomal protein gene.

The sequence of RPL41 was determined on both strands by using the λgt10 forward and reverse primers, the L41 5', L41 3' and L41#3 primers and the T3 (ATTAAACCCTCACTAAAG) and T7 (AATACGACTCACTATAG) primers to pKS-, using the plasmid DNA sequencing protocol as detailed in section 2.12. The L41 5' primer failed to prime sequencing reactions and analysis of sequence generated from the L41 3' primer revealed that the two bases at the 3' terminus of the L41 5' primer were mismatched. Therefore, the following primers were raised to generated sequence in order to complete the sequence on the second strand of the L41 coding region and to sequence back towards the T7 and λgt10 forward primers ; the 21mer L41.5'Δ: ATGGTGAAACGTTCTCGGAG (a 5' sense oligo) and the 21mer L41.5'Δα: GGCTTACGAACGTTTTTGTGTT (a 5' antisense oligo). In addition, a further oligo was raised to internal L41 sequence to enable sequencing through the start codon region; the 22mer L41#6: GCCCTGCGCGTAGAGGGAGGCC (an antisense oligo to internal sequence). The sequencing strategy is illustrated in figure 5.2. As can be seen from this figure the clone λgt10 L41#7 contained RPL41 but also some additional sequence and a polyA tail ligated head to head with RPL41. This is believed to be an artefact of library construction. In addition the polyA tail from the L41 sequence is absent.

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Figure 5.1 Orientation of L41#7 in λgt10. PCR reactions using combinations of: λgt10 forward, λgt10 reverse and L413' primers (single head arrows) gave products (double headed arrows) only between λgt10 forward/λgt10 reverse and between λgt10 forward/L413' pairs of primers, indicating the orientation of the L41#7 cDNA insert to be as indicated.
Figure 5.2 Sequencing strategy for pKSL41#7. Primers used are indicated by solid arrows and the sequence raised from them indicated by broken arrows. Scale is indicated by the size bar (100bp).
The nucleotide sequence of RPL41 coding region and the predicted amino acid sequence of the L41 ribosomal protein is presented as figure 5.3. The RPL41 gene has a GC content of 57.5%. This is in agreement with the overall GC content of Chlamydomonas reinhardtii nuclear genome (61.6%) (Jarvis et al. 1992). The high GC content of C. reinhardtii nuclear genes is reflected in the pattern of codon usage (Harris, 1989). Although the sequencing of a number of C. reinhardtii nuclear genes has demonstrated that all codons are used, some are extremely rare with codons that have an A in the third position being used hardly at all (Sharpe & Day, 1993). This codon bias is also reflected in the sequence for RPL41 (table 5.1). With the exception of the TAA stop codon only one codon (CAA encoding glutamine) has an A in the third position. Because of the cloning of RPL41 head to head with other sequence in the λgt10#7 clone and the absence of much 3' untranslated region (figure 5.2) very little can be observed from untranslated flanking sequences particularly at the 5' end where the junction between the two sequences is unclear. It can however be observed that the consensus tetranucleotide AAAA is present immediately upstream of the initiator methionine codon (ATG) at positions -1 to -4 (figure 5.3). This fits exactly with the known consensus for C. reinhardtii genes (Merchant et al., 1990).

5.2.3 The L41 ribosomal protein

From the determined cDNA sequence of RPL41, the L41 ribosomal protein of C. reinhardtii is predicted to be a polypeptide of 99 amino acids with a molecular weight of 11421.5 Da (including the initiating methionine). Inspection of the sequence confirms that residue 56 is proline and not glutamine which occurs in the CyhR yeast strains and this corresponds with the CyhS phenotype
Figure 5.3 cDNA sequence and derived amino acid sequence of the ribosomal protein L41 subunit gene of *Chlamydomonas reinhardtii*. Amino acid residues are positioned below their corresponding codon. The stop codon is marked with a star and the ‘Kozak’ consensus sequence is underlined.
### Table 5.1 Codon usage in the Chlamydomonas reinhardtii RPL41 gene.

Numbers next to each codon indicate the number of times it occurs within the gene. The most frequently used codon for each amino acid and stop codon in *C. reinhardtii* (as determined by analysis of codon usage in previously sequenced nuclear genes) is indicated by bold type.
of wild type *C. reinhardtii* cells. The protein contains 17 hydrophobic amino acids (A, I, L, W and V) and 28 polar amino acids (N, C, Q, S, T and Y). There are four strongly acidic (−) amino acids (D and E) and 29 strongly basic (+) residues (K and R). The protein is predicted to have an isoelectric point of pH 10.3 and will therefore have a net positive charge at physiological pH (+25.2 at pH 7.0).

Sequence alignment with other previously published L41 amino acid sequences demonstrates a high level of similarity (figure 5.4). The most noticeable difference in the *C. reinhardtii* L41 protein sequence is a run of three methionine residues from position 21. Although there is no consensus in this region these three hydrophobic residues are in the place of three which have predominantly hydrophilic residues in the first and third positions with the third residue being a histidine in all but one of the other published L41 protein sequences. The other major difference between *C. reinhardtii* L41 and the others is that the *Chlamydomonas* protein is truncated at the amino terminus being shorter, on average, by five residues.

5.2.4 Southern analysis

To investigate whether or not *RPL41* is a single copy nuclear gene DNA from wild type cells prepared by the miniprep method (section 2.5.1) was digested with various restriction enzymes and separated on a 1% agarose gel. The DNA was transferred to a nylon membrane (section 2.7.1) and hybridised to a probe specific for *RPL41* synthesised from a PCR product generated using the L41#3 and λgt10 reverse primers (figure 5.5). The banding pattern on the Southern blot demonstrates that *RPL41* is most probably a single copy nuclear gene with most restriction enzyme digestions giving single, high molecular weight bands. Interestingly the *PstI* digested DNA reveals two, low molecular weight bands.
Figure 5.4 Alignment of the amino acid sequence of *Chlamydomonas reinhardtii* ribosomal protein L41 with those previously published from other organisms. Cycloheximide sensitive species have a proline residue at position 56 (marked with a star) whereas cycloheximide resistant species have a glutamine residue at this position.
Figure 5.5 Southern analysis of the \textit{RPL41} gene from wild type \textit{Chlamydomonas reinhardtii} cells. DNA was extracted from wild type cells and digested with \textit{BamHI} (B), \textit{EcoRI} (E), \textit{HindIII} (H) and \textit{PstI} (P) and hybridised with a probe specific for \textit{RPL41} synthesised from a PCR product generated using the L41#3 and \textit{\lambda}gt10 reverse primers. The positive (+ve) control is the \textit{EcoRI} insert from \textit{\lambda}gt10#7 clone and the sizes of markers are in kilo\textit{b}ase pairs.
Nucleotide sequence analysis of the region covered by the probe reveals that no PstI site (CTGCAG) is present. This result indicates therefore that additional sequences are present in the genomic DNA in this region (downstream of the L41#3 primer). The most simple explanation for this observation would be the presence of an intron in this region of the gene.

5.2.5 Analysis of L41 from act1 and act2.

Although wild type C. reinhardtii are sensitive to cycloheximide, two CyhR mutants have been isolated and these exhibit resistance to at least ten-fold the concentration of Cyh required to kill wild type cells. To confirm that the act-1 and act-2 strains obtained from the Chlamydomonas Genetics Center were indeed resistant to Cycloheximide, spot tests with cultures of wild type, act-1 and act-2 were performed. Concentrations of Cyh greater than 1µg/ml were sufficient to kill wild type cells but act-1 and act-2 strains remained viable at a concentration of 20µg/ml (see figure 5.6). The molecular basis of cycloheximide resistance in the two C. reinhardtii CyhR strains (act-1 and act-2) has not been determined. In order to investigate whether a proline to glutamine substitution at amino acid 56 is responsible for one of these CyhR phenotypes attempts were made to clone this region of RPL41 from both act-1 and act-2. Because the proline residue at position 56 occurs between the L41#3 and L41 3' primers these were used to amplify the region from act-1 and act-2 miniprep DNA using PCR. Reactions were as described in section 2.11; an initial denaturation step at 95°C for 4 minutes was followed by 4 cycles of; denaturing 95°C - 1 minute, primer annealing 37°C - 1 minute and primer extension 72°C - 1 minute and by 15 cycles of; denaturing 95°C - 1 minute, primer annealing 60°C - 1 minute and primer
Figure 5.6 Sensitivity of wild type (WT), act-1 and act-2 strains of Chlamydomonas reinhardtii to cycloheximide. Cultures of each strain were spotted to TAP-agar plates supplemented with cycloheximide at the concentrations indicated and allowed to grow at 250°C in the light for seven days.
extension 72°C - 1 minute. PCR products that appeared to be the right size were purified by the ‘gene clean’ method and blunt cloned into the EcoRV site of pBluescript KS- (Stratagene). Sequencing of these inserts however revealed them to be ‘primer-dimer’ artefacts. The combined length of the two primers (59 bp) and the short distance separating them (31 bp) would have given an authentic product of 90 bp. Practically there are often difficulties encountered in accurately sizing fragments of such a small size on agarose gels where the GC content of the molecule can make a marked difference in its migration properties. The major PCR product on the gel (that appeared to be of the correct size) when cloned and sequenced was, as stated above, an artefact generated by the mutual hybridisation to and amplification of the L41#3 and L41 3’ primers.

To circumvent this problem, attempts were made to amplify a larger fragment of the L41 gene by using the L41 5’Δ and L41 3’ oligos as primers for PCR. Reactions were as described in section 2.11: an initial denaturation step at 95°C for 4 minutes was followed by 4 cycles of; denaturing 95°C - 1 minute, primer annealing 37°C - 1 minute and primer extension 72°C - 1 minute and by 15 cycles of; denaturing 95°C - 1 minute, primer annealing 55°C - 1 minute and primer extension 72°C - 1 minute. The predicted PCR product from the cDNA is 206 bp. However the size of the major PCR product from these reactions was estimated from agarose gel electrophoresis to be approximately 250 bp (figure 5.7). This discrepancy in size may be due to the presence of a small intron within this region in genomic DNA. The PCR product was purified by the ‘gene clean’ method. To establish whether it was authentic or an artefact some diagnostic PCR reactions were performed using the isolated fragments as templates. The L41 5’Δ and L41 3’ primers reamplified products from wild type, act-1 and act-2.
Figure 5.7 Amplification of a fragment of the *RPL41* gene from wild type (CC-125), *act-1* and *act-2* DNA by PCR using the L415'Δ and L413' primers. The PCR products were separated on a 2% agarose gel. The major product is indicated with an arrow and the sizes of markers are given in base pairs.
However, nested PCR using the L41#3 and L41 3’ primers only amplified a product from the act-2 isolated fragment (figure 5.8) indicating that the act 2 PCR product alone represented authentic RPL41 sequence. Given that it has been demonstrated that by using this method it is possible to amplify the region of interest of RPL41 from genomic DNA, it should now be possible to clone and sequence this fragment from both act-1 and act-2.
Figure 5.8 Diagnostic PCR screening of the RPL41 genomic PCR products from figure 5.7. PCR reactions (a) were performed using L415’Δ and L413’ primers. ‘Nested’ PCR reactions (b) were performed using L41#3 and L413’ primers. In addition; positive (+ve) control (pKSL41#7) and negative (-ve) control (pBluescript KS-) DNA templates were used for the same PCR reactions. The PCR products were separated on a 2% agarose gel. The products are indicated with arrows: 1 = genomic PCR product between L415’Δ and L413’ primers, 2 = cDNA PCR product between L415’Δ and L413’ primers, 3 = nested PCR product between L41#3 and L413’ primers and 4 = ‘primer-dimer’ artefacts. The sizes of markers are given in base pairs.
5.3 Discussion

In this chapter I have described the sub-cloning and sequencing of a cDNA clone of the gene for *C. reinhardtii* ribosomal protein L41. I have also confirmed the level of Cyh resistance in the mutant strains *act-1* and *act-2*. As reported in section 5.2.2 and illustrated in figure 5.2 the λgt10 clone selected was demonstrated to contain both a truncated cDNA for *RPL41* (5' UTR and polyA tail absent) and some additional sequence with a polyA tail ligated head to head. It is believed that this is an artefact of library construction and that this may be quite common (the ten tertiary screened clones that were positive for all three L41 probes had a range of insert sizes from ~650 bp to ~2.5 kb (data not shown)). The loss of the 5' UTR and polyA tail from the *RPL41* cDNA may be due to the presence of an *EcoRI* site just downstream of the coding region and it is believed that the additional sequence and poly A tail was acquired during the ligation of inserts to λ arms. The additional sequence generated from the region upstream of *RPL41* was used to search data bases but no matches of significant homology were found.

Because of the absence of much 3' UTR downstream of the *RPL41* cDNA coding region and the uncertainty regarding how much of the sequence upstream is authentic 5' UTR, very few conclusions can be drawn about the non-coding regions of *RPL41* represented on this cDNA clone. For example there is no polyadenylation consensus sequence to analyse. However the tetranucleotide 'Kozak' consensus sequence AAAA that appears immediately upstream of the initiation methionine codon in *C. reinhardtii* nuclear genes (Merchant *et al.*, 1990) is present at positions -4 to -1.
The sequence of the \textit{RPL41} coding region is however typical of \textit{C. reinhardtii} nuclear genes in so much as it has a high GC content (57.5\%) - \textit{C. reinhardtii} nuclear genome GC content (61.6\%) (Jarvis \textit{et al.} 1992). This high GC content is reflected in a codon usage that is typical of \textit{C. reinhardtii} nuclear genes (Sharpe & Day, 1993).

The predicted amino acid sequence of the \textit{C. reinhardtii} L41 ribosomal protein gene (figure 5.3) shows a high level of similarity to other L41 proteins (\textit{e.g.} 72\% amino acid identity with the L41 subunit of \textit{S. cerevisiae}) as demonstrated by the alignment in figure 5.4 but there are divergences; notably the methionine tripeptide at positions 21-23 and the truncated amino terminus. However, the vast majority of published sequences for L41 are from different species of yeast and these show very strong homology to each other. When looking at figure 5.4 it is worth considering that most of these sequences do come from very closely related organisms and that the divergence in homology that the \textit{C. reinhardtii} protein shows may not be unusual. Certainly there are more differences when one considers the sequences from human, rat and trypanosome L41. It is unfortunate that there is, as yet, no available L41 sequence from another photosynthetic organism as it would have been interesting to compare the \textit{C. reinhardtii} sequence to that of a species more closely related. The residue at position 56 is a proline and this is consistent with the Cyh\textsuperscript{S} phenotype of WT \textit{C. reinhardtii} cells (this residue is present as a proline in Cyh\textsuperscript{S} organisms but as a glutamine in Cyh\textsuperscript{R} ones).

Despite ongoing research to characterize the structure of the eukaryotic ribosome (Suzuki & Wool, 1993), the function of the L41 subunit is unknown. Data base searches for motifs within the L41 protein revealed no matches of
significant homology. It is likely however, as observed by Dehoux et al. (1993), that as mutations in ribosomal sub-units L23, L29 and L41 can all result in Cyh\textsuperscript{R} that these are closely associated and create a Cyh binding domain.

Although a genomic copy of RPL41 from C. reinhardtii has not been sequenced certain details about the structure of the gene can be inferred from the results that I have presented. My results indicate that there are at least two introns that interrupt the coding region of RPL41 and the positions of these introns can be mapped to certain regions of the gene. The Southern analysis of RPL41 from total genomic DNA (figure 5.5) demonstrates the presence of a PstI site within the region of the probe. However, the region used as a probe (a PCR product amplified with λgt10 reverse and L41#3 primers from pKS.L41#7) contains no PstI sites. This indicates a divergence between the genomic and cDNA sequences between the region of the #3 primer and the 3' end of the coding sequence and is most likely due to the presence of an intron. PCR using L41#3 and L41 3' primers has revealed no difference in the size of product between genomic and cDNA templates (figure 5.8). It follows therefore that the position of the intron must be located between the region of the 3' primer and the 3' end of the coding sequence. The size of the intron is unknown.

The position of a second intron has also been mapped. PCR reactions using L41 5'Δ and L41 3' primers have demonstrated that a larger product is obtained when using genomic DNA rather than the RPL41 cDNA clone as the template (figure 5.8). Again, it is believed that this larger size is due to the presence of an intron. Results discussed above have demonstrated that the region between the L41#3 and L41 3' primers is the same size between cDNA and genomic copies. The position of this second intron must therefore occur between
the 5'Δ and #3 primers. The difference in the size of PCR products estimated from agarose gel electrophoresis indicates that this is a small intron of approximately 50 bp.

Work involving the amplification of specific regions of RPL41 containing amino acid residue 56 by PCR has proved a successful approach at least for the act-2 strain (figure 5.8). There is no reason therefore why this approach may not be used to clone and sequence the P/Q 56 region from act-1 and act-2 to establish whether a mutation corresponding to that observed in many yeast is responsible for the CyhR phenotype of either of these strains.

The work presented in this chapter represents the initial stages of the development of a marker based on CyhR for the nuclear transformation of C. reinhardtii. To construct a marker based on an altered copy of the L41 ribosomal protein a copy of RPL41 would first need to be isolated from a C. reinhardtii genomic library. Three cosmid libraries are currently available (Purton & Rochaix, 1994; Purton & Rochaix, 1995; Zhang et al. 1994) so the isolation of genomic copies using the L41 cDNA as a probe should be routine. Following determination of the genomic copy nucleotide sequence, established molecular biology techniques would be employed to mutate the amino acid 56 proline codon to CAG for glutamine. This work is currently underway.
CHAPTER 6

DISCUSSION
Chlamydomonas has for many years been used as a model system for studying diverse aspects of cell biology, genetics and biochemistry (Harris, 1989). The advent of transformation protocols for this organism (Boynton et al., 1988; Debuchy et al., 1989; Kindle et al., 1989; Kindle, 1990;) has extended the range of these studies to a molecular-genetic level. With the transformation of the mitochondrial genome (Randolph-Anderson et al., 1993), Chlamydomonas is unique in being the only organism for which transformation protocols exist for all three genomes. Nuclear transformation of C. reinhardtii has, until now, relied on the introduction of endogenous genes to rescue an auxotrophic mutant (Debuchy et al., 1989; Kindle et al., 1989) or the introduction of a mutant allele to act as a co-dominant selectable marker (Nelson et al., 1994). Endogenous genes have previously been used as selectable markers because of the difficulties encountered expressing foreign genes in the nucleus of this alga (Day et al., 1990; Blankenship & Kindle, 1992; Kindle & Sodeinde, 1994).

Work presented in this thesis describes the stable expression and inheritance of a bacterial gene, ble, in the nucleus of C. reinhardtii. Parameters affecting the expression of genes introduced during transformation have also been investigated and the suitability of the endogenous ribosomal protein L41 gene to act as an alternative selectable marker has been explored.

Various reasons for the inability to express foreign genes in the nucleus of Chlamydomonas have previously been suggested including: methylation of transgenes (Blankenship & Kindle, 1992); integration at ectopic locations (Kindle & Sodeinde, 1994); the absence or incompatibility of controlling elements or the absence of introns (Stevens & Purton, 1994). Finally, because the nuclear genome of C. reinhardtii has a high GC content (61.6% (Jarvis et al., 1992)) and this is reflected in a biased codon usage (Campbell & Gowri, 1990), it has been
suggested that this codon bias may prevent the efficient expression of heterologous genes containing many rarely used codons. The construction of a marker based on the bacterial phleomycin resistance gene *ble* of *Streptoallotichus hindustanus* has addressed two of these points.

Firstly the genome of *S. hindustanus* is also GC rich (the *ble* gene = 71% GC (Drocourt *et al.*, 1990)). Analysis of the published sequences of *C. reinhardtii* nuclear genes has revealed that although all codons are represented, some (particularly those codons ending in A) are very rare (Sharpe & Day, 1993). Analysis of codon usage in the *ble* gene has demonstrated that none of the codons with the exception of GGA (glycine) end in A, with the vast majority of the codons also corresponding to those most favoured in *C. reinhardtii* nuclear genes. The stop codon UGA present on the *ble* gene is used in only two previously sequenced *Chlamydomonas* genes, however on the pSP108 construct this is replaced with the favoured UAA stop codon. The second factor addressed by pSP108 is that of endogenous controlling elements. The 5' UTR (containing the promoter) and 3' UTR (containing the polyadenylation signal) from the strongly expressed nuclear *RBCS2* gene (Goldschmidt-Clermont & Rahire, 1986) have been linked to the *ble* gene as a translational fusion at the initiating methionine (ATG) and stop (TAA) codons.

Results presented in chapter 3 demonstrate that the introduction of the *RBCS2/ble* gene can result in the acquisition of phleomycin resistance, that this resistance is due to the integrated DNA and that the phenotype is stable in sexual crosses. This is the first example of the stable expression and inheritance of a foreign gene in the nucleus of *Chlamydomonas*. Furthermore I have demonstrated that by modifying the glass bead transformation method (Kindle, 1990) it is possible to use the *RBCS2/ble* construct to select directly for PmR
transformants. Analysis of $Pm^S$ and $Pm^R$ co-transformants has revealed however that whilst the vast majority have integrated the construct (figure 3.4) only ~3% express the $ble$ gene.

Reasons for the silencing of the great majority of the introduced transgenes was investigated. Methylation of an introduced transgene has previously been implicated in lack of expression in *Chlamydomonas* (Blankenship & Kindle, 1992) however no evidence was found to demonstrate differing methylation patterns of the $ble$ gene from $Pm^R$ and $Pm^S$ transformants. Furthermore, use of the hypomethylating agent 5-azacytidine failed to activate silent copies of the gene as has previously been demonstrated in e.g. transgenic tobacco (Zhu *et al*., 1991; Bochardt *et al*., 1992; Palmgreen *et al*., 1993).

An alternative explanation is that the differential pattern in expression is due to some, uncharacterised, positional effect. The expression of eukaryotic genes is regulated by two types of element. The first are in close proximity to the gene, namely the CAAT box and the TATA box (Benoist & Chambon, 1981). Other elements such as enhancers can exert their effect several hundred base pairs from the start of transcription and in an orientation independent manner (e.g. Parslow *et al*., 1987). Furthermore, the higher order structure of eukaryotic DNA as chromatin may effect the expression of genes. Highly condensed chromosomal material or heterochromatin is considered to be transcriptionally inactive (Brown, 1966) whilst the more open euchromatin is believed to contain actively transcribed genes (Callan, 1982). There is evidence to suggest that within euchromatin actively transcribed genes are present in regions that loop out from the nuclear scaffold and are attached to it (and perhaps insulated from neighbouring regions) by nucleotide sequences termed scaffold attachment
regions (SARs) (Gasser & Laemmli, 1987). Certainly there is evidence to suggest that transgenes linked to SARs exhibit a higher level of expression when introduced in transformation in a number of systems (Allen et al., 1993; Stief et al., 1989).

There is a possibility therefore that the \textit{RB}CS\textit{2} promoter elements contained within the pSP108 construct are insufficient in themselves to drive transcription of the \textit{ble} gene and that those copies of the \textit{ble} gene that are expressed have fortuitously integrated close to an enhancer or other \textit{cis} acting element. Alternatively differences in expression may be the result of integration into ‘active’ or ‘silent’ regions of the chromosome.

The fact that the \textit{RB}CS\textit{2} promoter has been used to efficiently express endogenous \textit{Chlamydomonas} genes reintroduced in transformation (e.g. Kozminski, 1993) would suggest that all \textit{cis} acting elements necessary for expression are present. To investigate the possibility that too little of the cloned and available \textit{RB}CS\textit{2} promoter region (Goldschmidt-Clermont & Rahire, 1986) was included on pSP108 the remainder was reintroduced on pSP110. Transformations using this modified construct did not however result in an increased transformation efficiency. To test the possibility that the inclusion of an SAR would promote expression of the \textit{ble} gene an element identified as a scaffold attachment region from pea (Pwee & Gray, 1993) was included on the \textit{RB}CS\textit{2}/\textit{ble} marker. However this construct (pSP113) also failed to increase transformation rates. This result does not however rule out the possibility that SARs would be beneficial in promoting transgene expression in \textit{Chlamydomonas}, it may be that the element chosen is incompatible or that two SARs bounding the transgene are required.

Another factor that may effect the expression of transgenes in the nucleus
of *Chlamydomonas* is the presence of an intron. The inclusion of introns within transgenes has been demonstrated to improve mRNA accumulation and consequent protein production from transgenes introduced into both plants and animals (Kyozuka & Shimamoto, 1995; Webb & Nisbet, 1993). The reason for this enhancing effect has not been established. All *Chlamydomonas* nuclear genes sequenced to date contain introns, for example the argininosuccinate lyase gene (*ARG7*) which contains 11 (Debuchy *et al.*, 1989). It would therefore be reasonable to hypothesise that introns are required for the efficient expression of transgenes in the nucleus of *C. reinhardtii*. Certainly in the related alga *V. carteri* it has been demonstrated that the cDNA for the endogenous nitrate reductase gene is expressed only upon the reintroduction of an intron (Gruber & Schmidt - unpublished). Results that I have presented in chapter 4 demonstrate that introducing a cDNA copy of the *OEE1* gene (Mayfield *et al.*, 1989) linked to *RBCS2* controlling elements fails to rescue an *oee1*- strain to photoautotrophic growth whereas introduction of a genomic copy does (Mayfield & Kindle, 1990). This seems to support the assumption that introns are important. However, there is an apparent paradox in so much as some introduced copies of the *ble* gene are expressed despite the absence of an intron. Attempts to improve the expression of the *ble* gene by linking it to the first two codons and first intron of *ARG7* on the pARG7-X/ble construct resulted in the recovery of fewer transformants. However a direct comparison with the pSP108 construct is not possible as the *ble* gene on pARG7-X/ble is under the control of a different (possibly weaker) promoter and would, furthermore, be expressed as a fusion protein that may adversely affect its biological activity. As I have previously suggested in chapter 4 a better way to investigate the effect of an intron upon the expression of the *ble* gene may prove to be the introduction of an intron within the pSP108
construct thus allowing a direct comparison.

Another way to investigate the effect of introns upon the expression of nuclear genes in *C. reinhardtii* would be to compare the expression of an endogenous gene and its cDNA counterpart under the control of its own promoter. Such a system would be possible with the *OEE1* gene. *OEE1* codes for the oxygen evolving enhancer protein 1 of photosystem II and is essential for photoautotrophic growth. Both genomic (Mayfield & Kindle, 1990) and cDNA (Mayfield, et al., 1989) clones of this gene are available and it has previously been demonstrated that the genomic clone can rescue an *oee1*- strain to photosynthetic growth (Mayfield & Kindle, 1990). Using established molecular biology techniques the coding region of the *OEE1* gene present on the genomic clone could be replaced as a cassette by the intronless coding region from the cDNA copy. The ability of the two constructs to rescue *oee1*- cells to photoautotrophy could be tested by introducing the gene via the simple glass bead transformation method (Kindle, 1990) and the selection of transformants on minimal medium. Assuming a difference between genomic and cDNA copies, the effect of introns could be investigated by their re-introduction (again as cassettes) to the cDNA clone and once more performing comparative transformations.

Of the various methods employed to attempt to increase transformation efficiency with the *ble* marker only the inclusion of PEG had a beneficial effect. Work presented in chapter 4 resolves the apparent dichotomy that surrounded the use of PEG to improve transformation efficiency. Kindle (1990) reported that the inclusion of 5% PEG in glass bead transformations to rescue *nit1*- cells had a marked beneficial effect. Purton & Rochaix (1995) however found that including 5% PEG in transformations with *ARG7* did not alter transformation rates. Work presented here demonstrates that whilst transformation efficiency when using *cwl*
cells as the recipient is not influenced by the addition of PEG, similar experiments using cw15 cells as the host demonstrate an improved transformation efficiency in the order of a ten-fold increase in the recovery of transformants. Recipient cells used by Kindle (1990) were based on the cw15 cell wall-less mutation, whereas those used by Purton & Rochaix (1995) were cw_d. It seems therefore that the differences in the effects of the inclusion of PEG can be attributed to the status of the cell wall mutation rather than the marker used.

Given the fact that a direct selection protocol has been developed, the \textit{RBGS2/ble} construct promises to be an improved marker for nuclear transformation. Transformations using this marker do not rely on the rescue of auxotrophic cells as with \textit{ARG7} (Debuchy \textit{et al.}, 1989) and \textit{NIT1} (Kindle \textit{et al.}, 1989) and, unlike \textit{CRY1-1} (Nelson \textit{et al.}, 1994), the marker is truly dominant (not co-dominant). In contrast to resistance to emetine and cryptopleurine, spontaneous mutants to PmR have not been observed and, furthermore, the protocol for transformation with \textit{ble} is somewhat simpler than that employed with \textit{CRY1-1}. Given that the use of autolysin renders walled cells susceptible to transformation by the glass bead method (Kindle, 1990) the \textit{RBGS2/ble} construct should be suitable for transforming any \textit{C. reinhardtii} strain.

The \textit{ble} marker also benefits from its small size, being markedly smaller than those which are presently used: 9 kb for \textit{NIT1} (Nelson \textit{et al.}, 1994); 7.6 kb for \textit{ARG7} (Purton & Rochaix, 1995) and 4.5 kb for \textit{RBGS2/CRY1-1} (Nelson \textit{et al.}, 1994). The \textit{RBGS2/ble} construct is portable as a 1.4 kb \textit{EcoRI} cassette or as a slightly larger 1.7 kb \textit{PvuII} cassette when some of the flanking vector (pBS) DNA is included. Work in our laboratory has demonstrated that these fragments are sufficient to transform \textit{C. reinhardtii}.
Transformation of *C. reinhardtii* is often accompanied by the loss and rearrangement of genomic DNA flanking the site of integration and by the loss of vector sequences flanking the selectable marker (Tam & Lefebvre, 1993). In the case of ARG7 the presence of extensive repetitive DNA sequences (Debuchy et al., 1989) may also lead to rearrangements and deletions within the marker. These rearrangements can lead to the loss of bacterial marker genes present on the vector thus preventing plasmid rescue. My work has shown that the *RBCS2/ble* construct under the control of the *lac* promoter is expressed in *E. coli* and renders bacterial cells transformed with the construct and grown in the presence of IPTG, resistant to Pm at 5 μg/ml on solid medium. As *C. reinhardtii* Pm*R* transformants are selected on the basis of a functional copy of the ble gene, marker rescue should therefore be facilitated by this construct - thus greatly simplifying the cloning of genes disrupted by insertional mutagenesis. Work presented in chapter 4 demonstrates that this construct is suitable for insertional mutagenesis following the isolation of a mutant deficient in chlorophyll b. In addition, work in our laboratory is currently in progress to characterize and clone the genes for several swimming mutants generated by insertional mutagenesis using this marker (M. Turner, personal communication).

Use of the ble marker should facilitate the development of positive/negative (pos/neg) selection vectors required for gene targeting events. Such pos/neg selection vectors work by cloning a dominant selectable marker within a copy of the target gene to be disrupted and also including at a distal site on the vector a second gene for suicide selection (acquisition of the gene would, in certain circumstances, prove lethal for recipient cells). Vectors of this type can be used to select or enrich for rare homologous recombination / gene targeting
events by selecting transformants under conditions where the dominant selectable marker is required for growth and by also including in the medium a substrate for the suicide selection which would affect only transformants generated by illegitimate recombination. Only homologous recombination events between the target gene and the disrupted copy on the vector result in the loss of the suicide marker and would thus be able to grow on selectable media as described above. Nelson & Lefebvre (1995) have recently employed the CRY1-1 selectable marker cloned within the NIT8 gene as a dominant selectable marker to select for gene targeting events within the NIT8 gene. NIT8+ cells are sensitive to killing by chlorate. Plating transformants in the presence of emetine and chlorate allowed the selection of rare gene targeting events where the endogenous NIT8 gene had been replaced by the disrupted copy.

The targeted disruption of genes with no scorable phenotype should be facilitated by vectors for pos/neg selection using ble as the dominant selectable marker. The small size of the RBCS2/ble marker and the fact that it is portable as an EcoRI cassette should facilitate its cloning within such target genes. The inclusion on the vector of the NIT8 gene would make selection for the disruption of such genes by homologous recombination possible in a nit8- host. This would however limit the use of such a pos/neg vector to a nit8- background. Work in our laboratory is currently underway to develop a negative selection marker based on the cytosine deaminase gene (codA). Cytosine deaminase catalyses the production of 5-fluorouracil from 5-fluorocytosine. 5-fluorouracil is toxic to C. reinhardtii cells whereas 5-fluorocytosine is not. By including 5-fluorocytosine in the selection medium for transformants, illegitimate recombination events that retained the codA gene would result in the production of 5-fluorouracil within
the transformant and thus its suicide selection.

Although the ble marker has many advantages over those previously used to select for nuclear transformants in *C. reinhardtii* there is still a need for other dominant selectable markers. For example, when wishing to reintroduce a modified copy of a gene silenced by disruption or when wishing to rescue with a cosmid library a transformant with a non-selectable phenotype generated by insertional mutagenesis with the ble gene, a different selectable marker would be required. To address this some initial work has been performed to investigate the potential of a mutant form of the *C. reinhardtii* L41 ribosomal protein to act as a selectable marker. A cDNA encoding the L41 ribosomal protein gene has been sequenced. It is believed that a modified version of this gene could act as a selectable marker in nuclear transformations thus extending further the range of selectable markers available for *Chlamydomonas*. Organisms in which amino acid 56 within the L41 ribosomal protein is a glutamine residue rather than a proline exhibit resistance to cycloheximide and the transformation of a Cyh⁺ strain of *S. cerevisiae* with a resistant copy of the gene resulted in the generation of Cyh⁻ transformants (Kawai *et al.*, 1992). The sequence of *RPL41* presented in chapter 5 is typical of *C. reinhardtii* nuclear genes in respect to both GC content (Jarvis *et al.*, 1992) and codon usage (Sharpe & Day, 1993) for nuclear genes of this alga. The predicted polypeptide sequence shows close homology to previously published L41 protein sequences. Amino acid 56 in the *C. reinhardtii* L41 ribosomal protein is a proline residue and this corresponds with the Cyh⁺ phenotype of wild type *C. reinhardtii* cells. There however Cyh⁻ strains of *C. reinhardtii; act-1* and *act-2*. Work to characterize these mutants with respect to residue 56 of their L41 ribosomal proteins is still in progress but, given that
most cases of spontaneous Cyh\(^R\) in eukaryotes are due to the mutation of ribosomal proteins; specifically: the L41, L29 and L23 subunits (Dehoux et al. 1993), a mutation within \(RPL41\) remains a strong candidate for the molecular basis of one of the Cyh\(^R\) phenotypes in the \(act\) mutants. (The \(act\)-1 and \(act\)-2 mutations map to separate linkage groups and, as demonstrated by this work, \(RPL41\) is single copy nuclear gene therefore a mutant form of \(RPL41\) could not be responsible for both Cyh\(^R\) strains). Confirmation that a P to Q substitution in the L41 subunit of one of the mutants is responsible for the Cyh\(^R\) phenotype would reinforce the rationale behind the development of a selectable marker based on such a change. Given the fact that ribosomal proteins are so conserved and that such a selection system has already been developed in yeast (Kawai et al., 1992), there is no reason to assume that such a marker would not work in \(C. reinhardtii\).

Work resulting from the development of transformation systems for \(Chlamydomonas\) has already borne fruit, with experiments previously unavailable in this alga now becoming routine. Mutants have been isolated following insertional mutagenesis with transforming DNA (\(e.g.\) Adam et al., 1993; Pfeifer McHugh & Spanier, 1994) and genes disrupted by such transformations have been cloned (\(e.g.\) Tam & Lefebvre, 1993; Gumpel et al., 1996). Chimeric genes have been constructed for transformation into \(C. reinhardtii\) to act as reporter genes (Davies et al., 1992), to study promoter activity (\(e.g.\) Blankenship & Kindle, 1992; Davies & Grossman, 1994), to investigate mRNA stability (Baker, 1993) and to investigate the role of the acetylation of \(\alpha\)-tubulin (Kozminski et al., 1993). The availability of transformation protocols has also promoted the investigation of homologous recombination in \(C. reinhardtii\) (\(e.g.\) Sodeinde &
Kindle, 1993; Gumpel et al., 1994) and recently gene targeting has been demonstrated (Nelson & Lefebvre, 1995). Furthermore, the construction of cosmid libraries has led to the isolation of genes following the transformation mediated complementation of mutants (Purton & Rochaix, 1994; Zhang et al., 1994).

The development of the dominant selectable ble marker will facilitate further work of this kind for the reasons previously stated: its small size and lack of repetitive DNA, the fact that it is truly dominant and can therefore be used to transform any C. reinhardtii strain and the availability of marker rescue that will exist with all transformants. This marker will also be of use when constructing other vectors, for example those for positive / negative selection as described above. Much exciting work is being undertaken at present using Chlamydomonas as a model system, for example the investigation of the interaction of nuclear and organellar genomes and the control of the expression of chloroplast genes by the nucleus. Molecular-genetic studies in C. reinhardtii are progressing into previously unavailable areas and the next few years promise advances in many fields including gene targeting and replacement. The future heralds much fruitful Chlamydomonas research, it is hoped that the ble marker will facilitate such studies.

Finally, the demonstration that there is no intrinsic block on the expression of foreign genes in C. reinhardtii raises the possibility that once the factors governing the efficient expression of transgenes are understood then the inexpensive production of recombinant eukaryotic proteins in this alga might be achieved.
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