Nuclear Transformation and Gene Expression in *Chlamydomonas reinhardtii.*

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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 *arg7* mutant by co-transformation with the *ARG7* 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 Pm^R cells. Crosses between Pm65 (a Pm^R transformant containing a single copy of the *ble* gene) and wild type cells demonstrated that the Pm^R phenotype segregates in predicted Mendelian ratios in progeny. Southern analysis of these progeny has revealed that the *ble* gene co-segregates with the Pm^r 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 *RPL*41 encoding the ribosomal protein L41 subunit gene is presented. The potential of using a mutated version of *RPL*41 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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ABBREVIATIONS

Ap	ampicillin
ars	autonomously replicating sequence
BSA	bovine serum albumin
bp (kbp)	base pairs (kilobase pairs)
CaMv	cauliflower mosaic virus
cDNA	copy deoxyribonucleic acid
Cyh	cycloheximide
Da (kDa)	dalton (kilodalton)
dATP (A)	2' deoxyadenosine 5'-triphosphate
dCTP (C)	2' deoxycytidine 5'-triphosphate
dGTP (G)	2' deoxyguanosine 5'-triphosphate
dH$_2$O	distilled water
dNTP	2' deoxynucleoside 5'-triphosphate
dTTP (T)	2' deoxythymidine 5'-triphosphate
ddATP	2',3' dideoxyadenosine 5'-triphosphate
ddCTP	2',3' dideoxycytidine 5'-triphosphate
ddH$_2$O	double distilled water
ddGTP	2',3' dideoxyguanosine 5'-triphosphate
ddNTP	2',3' dideoxynucleoside 5'-triphosphate
ddTTP	2',3' dideoxythymidine 5'-triphosphate
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
ds	direct selection
DTT	dithiothreitol
EDTA.Na$_2$	diaminoethanetetra-acetic acid. disodium salt
ES	embryonic stem
EtBr	ethidium bromide
IPTG	isopropylthio-β-galactoside
Kan	kanamycin
LB	Luria broth medium

MBN	mung bean nuclease
MIC	minimum inhibitory concentration
mRNA	messenger ribonucleic acid
mt	mating type
Neo	neomycin
OLB	oligo labelling buffer
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pm	phleomycin
REMI	restriction enzyme-mediated integration
RNA	ribonucleic acid
rpm	revolutions per minute
SAR	scaffold attachment region
SDS	sodium dodecyl sulphate
SSC	salt sodium citrate
Tn	transposon
TAP	tris acetate phosphate medium
TBE	tris borate EDTA
Tc	tetracycline
TE	Tris-EDTA
Tricine	tris(hydroxymethyl)methylglycine
Tris	tris(hydroxymethyl)aminoethane
U	unit (of restriction enzyme)
UTR	untranslated region
v/v	volume for volume
wt	wild type
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
XSO_4	5-bromo-4-chloro-3-indolyl-sulphate
YAC	yeast artificial chromosome
YCp	yeast centromere plasmid
YEp	yeast episomal plasmid
YRp	yeast replicating plasmid
YT	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

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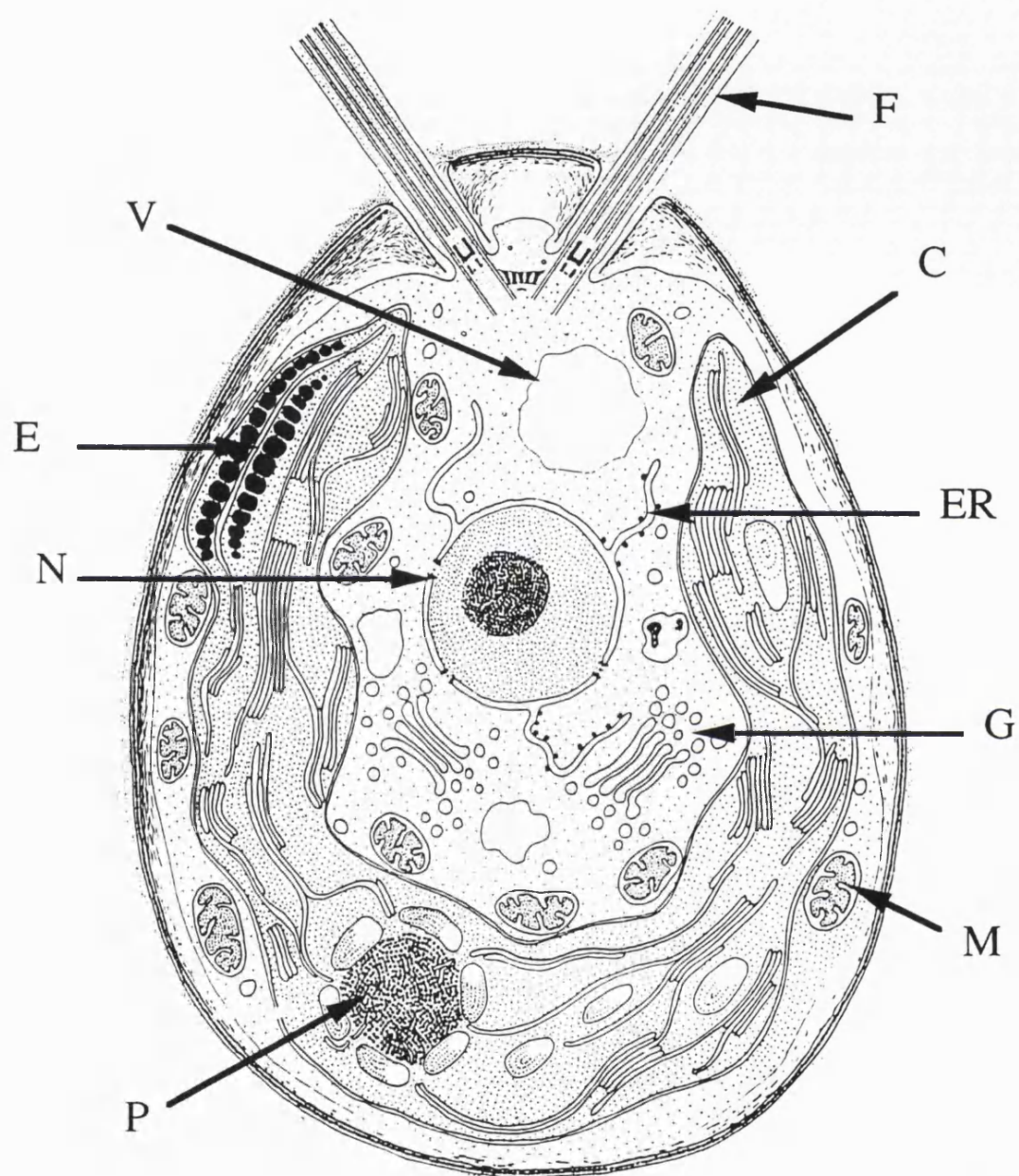
Figure 1.1 Schematic diagram of a *Chlamydomonas reinhardtii* cell. C, chloroplast; E, eyespot; ER, endoplasmic reticulum; F, flagella; G, golgi; M, mitochondria; N, nucleus; P, pyrenoid; V, vacuole. From a drawing by Keith Roberts.

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 world wide (*e.g.* Harris, 1984), containing a broad spectrum of both wild type and well characterised mutant strains.

C. reinhardtii has a small (1×10^5 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

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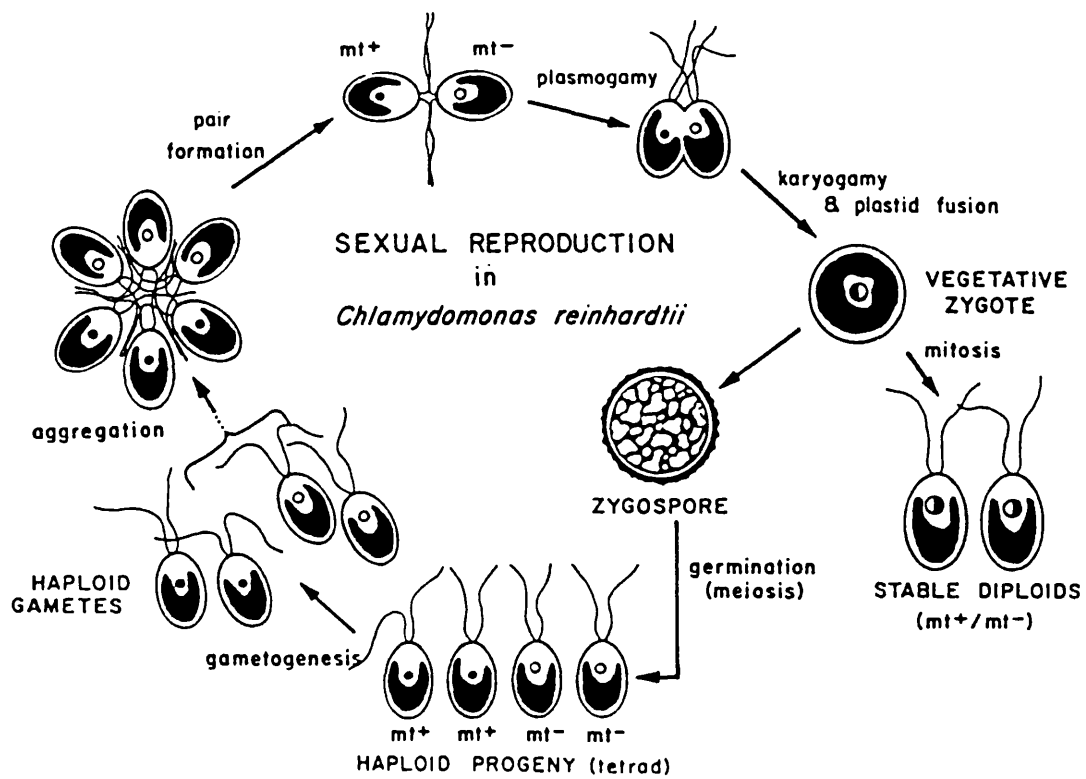
Figure 1.2 Life cycle of *Chlamydomonas reinhardtii*. From a drawing by Karen VanWinkle Swift.

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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

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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 $CaCl_2$ 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

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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 *Sacchromyces cerivisiae* was first transformed by fusing yeast spheroplasts with polyethylene glycol (PEG) in the presence of DNA and $CaCl_2$. 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. cerivisiae* that have the ability to replicate in *E. coli* and which can exist extrachromosomally as circular plasmid molecules in *S. cerivisiae;* The yeast episomal plasmid (YEp) which is based on the naturally occurring $2\mu m$ 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 *Schizosacchromyces 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 (Neo^R) gene from the bacterial transposon Tn5, using a $CaPO_4$ precipitation method (Nellen *et al.*, 1984). Transformation rates were subsequently improved by including on the

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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

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genes such as the bacterial β-glucuronidase (*uid*A) 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

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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 Neo^R 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 Neo^R phenotype. Rare homologous recombinants are then selected from this population by gancyclovir^R 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 psuedopregnant 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, pluropotent 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*

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al., 1978) Rochaix and colleagues presented evidence for the transformation of the *C. reinhardtii* strain *arg7-cw*15 to arginine prototrophy using the corresponding yeast gene *arg*4 (Rochaix & van Dillewijn, 1982; Rochaix *et al.*, 1984). However, the transformation rate was close to the reversion rate of *arg*7 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 (Kan[R]). 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 Kan[R] phenotype. The use of Kan[R] 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 *arg*4 or Neo[R] 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 *ARG7* (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, ~2×10^8 *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 *nit*1 locus indicating non-homologous recombination. Furthermore when a second nonselected DNA species was introduced in a co-transformation with *NIT*1 this DNA was found to be present in some transformants (Kindle *et al.*, 1989). The *OEE*1 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). 4×10^7 or 1×10^8 cells were bombarded with *OEE*1 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 *OEE*1 transcript was present at similar levels to the endogenous *OEE* 1 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 *nit*1-305 *cw*15 lacking both a cell wall and nitrate reductase activity was transformed using this method and

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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 x10^6 cells/ml) resuspended at a concentration of 3 x 10^8 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 $arg7$-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 $NIT1$ DNA into a $nit1$-, $rsp3$- 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).

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

Recently a chimeric gene containing $RBCS2$ promoter sequence fused to a

mutant form of the endogenous *CRY1* gene (encodes ribosomal protein S14) has been used to transform wild type cells (Nelson *et al.*, 1994). The mutated form of the gene (*cry1-1*) 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 sub-unit 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 *et al.*, 1994).

1.4.2 Applications of nuclear transformation in C. reinhardtii

Following the development of the reproducible transformation systems described above, experiments previously impossible in *C. reinhardtii* have now been performed. The observation that a second, unselected DNA species could be introduced along with the selectable marker (Kindle *et al.*, 1989) has led to the rescue of paralysed-flagella mutants both by biolistic transformation (Diener *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 *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 *C. reinhardtii* has also been used to identify the gene for which a known mutant phenotype exists. A motility mutant *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

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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 *NIT*1 and *ARG*7 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 (*CAB*II-1) promoter and the *NIT*1 gene coding region was used to transform *nit*1⁻ cells and it was demonstrated that accumulation of *NIT*1 mRNA was dramatically stimulated by light (Blankenship & Kindle, 1992). This construct was also shown to function as a dominant selectable marker in a *nit*1⁻ *nit*2⁻ strain as the *CAB*II-1/*NIT*1 hybrid is expressed in the presence of ammonium whilst the endogenous *NIT*1 gene is repressed in such an environment. Chimeras of this sort have also been used to study mRNA stability. A chimeric, deflagelation-induced mRNA consisting of the 5' UTR and coding region of the rubisco small sub-unit 2 gene (*RBCS*2) 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 *RBCS*2/α-1 *TUB* chimera in which the acetylable lysine codon had been mutated was transformed into *C. reinhardtii* (Kozminski *et al.*, 1993). The *RBCS*2 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 (*uid*A) under the control of the *CAB*II-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 (XSO_4) 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 XSO_4 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: *NIT*1 is 9 kb (Nelson *et al.,* 1994);*ARG*7 is 7.6 kb (Purton & Rochaix, 1995) and *RBCS2-CRY*1-1 is 4.5 kb (Nelson *et al.,* 1994) In the case of *ARG*7, 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-CRY*1-1 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 *arg*7-*cw*15 to arginine biosynthesis with the corresponding

yeast gene *arg*4 was reported (Rochaix & van Dillewijn, 1982; Rochaix *et al.* 1984), and several laboratories have reported the use of bacterial genes to transform *C. reinhardtii* to kanamycin resistance (Hasnain *et al.*, 1985; Bingham *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 *arg*4 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 *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). 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 *E. coli* flanked by the 35S promoter and 35S polyadenylation signal from cauliflower mosaic virus (CaMv) (Pietrzak *et al.*, 1986) was introduced in a co-transformation but despite the presence of the DNA no co-transformants were resistant to hygromycin (Day *et al.*, 1990). Similarly, attempts to express the bacterial β-glucuronidase gene (*uid*A) under the control of the *Chlamydomonas CAB*II-1 promoter following co-transformation were also unsuccessful (Blankenship & Kindle, 1992). More recently the bacterial neomycin phosphotransferase II (*NPT*II) gene flanked by the Ti plasmid nopaline synthase (*nos*) promoter and polyadenylation signal were used in a co-transformation to transform *C. reinhardtii* to Kan[R] (Hall *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.

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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) *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 cw_d and the cycloheximide resistant strains *act*-1 (Sager and Tsubo, 1961) and *act*-2 (Smyth *et al.* 1975) were obtained from Dr. E. Harris at the Chlamydomonas Genetics Center at Duke University, North Carolina. The cell wall deficient strain cw_d(mt-) (Loppes and Deltour, 1975) and the arginine requiring, cell wall deficient strain *arg7-8cw_d* (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 *C. reinhardtii* are detailed in table 2.1. *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 18^0C and were streaked to fresh plates every 6-8 weeks. Working stocks were maintained at $45\mu E/m^2/s$ at 25^0C 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 25^0C and aerated by shaking at 150 rpm. Starter cultures of 25 ml volume were inoculated from stock cultures and grown to stationary phase (~2×10^7 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.

for 1 litre	TAP Medium	Tris minimal medium
H_2O	975 ml	975 ml
Tris	2.42g	2.42g
4x Beijerink Salts*	25 ml	25 ml
1M (K)PO_4 ph7.0#	1 ml	1 ml
Trace Elements†	1 ml	1 ml
Glacial Acetic Acid	~1 ml to pH 7.0	
Concentrated HCl		~1 ml to pH 7.0
References	Gorman and Levine (1965)	Surzycki (1971)

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* 4x Beijerink Salts
  16g NH_4Cl (omitted in TAP -N media)
  2g $CaCl_2$
  4g $MgSO_4$
  dissolve in 1 litre distilled H_2O

1M (K)PO_4
  250 ml 1M K_2HPO_4
  170 ml 1M KH_2PO_4 (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 H_3BO_4
  22g $ZnSO_4.7H_2O$
  5.06g $MnCl_2.4H_2O$
  4.99g $FeSO_4.7H_2O$
  1.61g $CoCl_2.6H_2O$
  1.57g $CuSO_4.4H_2O$
  1.1g $(NH_4)_6 Mo_7O_{24}.4H2O$
ii. Dissolve 50g EDTA.Na_2 in 250 ml H_2O by heating and add to the above solution. Reheat to 100°C. Cool to 80 - 90°C and adjust to pH6.5 - 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^oC. *E. coli* strains were maintained in the short term by growing overnight at 37^oC on LB media solidified with 1.6% agar (supplemented with appropriate antibiotics were applicable) and then storing at 4^oC. Appropriate aseptic techniques were employed throughout.

Table 2.2 E. coli strains.

Strain	Genotype
JM109	F' *traD36 pro*A+ *pro*B+ *lac*Iq *lacZ*ΔM15/*recA1 end A1 gyr*A96 (Nalr) *thi hsd*R17 *sup*E44 *rel*A1 Δ(*lac-pro*AB) *mcr*A
XL1 Blue	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*-F'[*pro* AB+ *lac*Iq *lacZ*ΔM15 Tn10(tetr)]
C600	*thi*-1 *thr*-1 *leu*B6 *lac*Y1 *ton*A21 *sup*E44 *mcr*A

Table 2.3 E. coli growth media.

For 1 litre	LB	2YT
Bactotryptone	10g	16g
Yeast extract	5g	10g
NaCl	10g	5g
dH$_2$O	to 1 litre	to 1 litre

Media supplemented with the following antibiotics where necessary:
Ampicilin (Ap) 50mg/ml in ddH$_2$O
Tetracycline (Tc) 15mg/ml in 50% ethanol
Phleomycin (Pm) 5mg/ml in ddH$_2$O

Stock solutions were filter-sterilised using a leur lock syringe and a 0.45 μm 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-^{32}P] 2'deoxycytidine 5'triphosphate (dCTP) and [α^{35}S] 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 121°C 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 (ddH$_2$O). Digestions were incubated for 1-2 hours at 37°C. *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.Na$_2$). Samples were mixed at the appropriate ratio with 6x loading buffer: 40% (w/v) glycerol, 0.1%(w/v) SDS, 0.1mM EDTA.Na$_2$ 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. 0.5µg of bacteriophage λ DNA digested with either *Hind*III or *Ava*I and *Bgl*II or 0.5µg of pUC19 plasmid DNA digested with *Hinf*I 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 1 µg/ml ethidium bromide (EtBr), washing in distilled water (dH$_2$O) 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 ddH$_2$O 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

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Table 2.4 Sizes of markers for agarose gel electrophoresis. (Sizes are in bp).

λ: *Hind* III	λ: *Ava* I, *Bgl* II	pUC19:*Hinf* I
23,130	14,866	1,419
9,416	8,778	517
6,557	5,432	396
4,361	4,333	214
2,322	3,780	75
2,027	2,425	65
564	2,215	
125	1,917	
	1,629	
	1,463	
	1,076	
	540	

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 ddH$_2$O. 10μl of 10x reaction buffer and 1μl of alkaline phosphatase were added and the reaction incubated at 55^{0}C for 1 hour and then at 75^{0}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 ddH$_2$O. 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^{0}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:	Restricted vector	1μl
	DNA insert	2μl
	10 x buffer	1μl
	T4 DNA ligase	0.5μl
	ddH$_2$O	5.5μl

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Reaction incubated at 17°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 $MgCl_2$ and incubated on ice for 5 minutes. Cells were repelleted as above and then resuspended in 0.1 volume of cold (4°C) 50mM $CaCl_2$. 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 0.1 μg 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 1×10^7 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.Na$_2$

pH8.0, 20mM Tris-Hcl pH8.0, 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 55°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 pH8.0, 1mM EDTA.Na$_2$ pH8.0) 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 pH8.0, 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

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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.Na$_2$) 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 ddH$_2$O 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 $MgSO_4$ 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 $MgSO_4$) 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 $10,000 \times g$ 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[1]

Diethylaminoethyl (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 17.5μl of a 0.1mg/ml solution of proteinase K and 42.5μl of 10% SDS were added. The contents were mixed and incubated at room temperature for 5 minutes. 173μl of 3M potassium acetate was added (precipitate formed) and the tubes were incubated for 20 minutes at 88°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°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 40μl TE pH8.0.

2.6   Transformation of *C. reinhardtii.*

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 ($2\text{-}4\times10^6$ 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 $3,000\text{x}g$, 4°C for 5 minutes to pellet the cells. The medium was discarded and the

cells resuspended in fresh medium to a concentration of 2×10^8 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 ddH_2O.

To this was added 5μl of oligo labelling buffer[2] (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 ^{32}P-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 ^{32}P-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[3],

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

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OLB was made as detailed below: Solution A; (1ml solution O [1.25M Tris-HCl pH8.0,
0.125M MgCl$_2$ - stored at 40C], 18μl 2-mercaptoethanol, 5μl dATP, 5μl dTTP, 5μl dGTP
each previously dissolved in 3mM Tris-Hcl pH7.0, 0.2mM EDTA.Na$_2$ - stored at -20°C.
Solution B; 2M Hepes titrated to pH6.6 with 4M NaOH - stored at 4°C, 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 -80⁰C 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 - 6×10^6 cells/ml and harvested by centrifugation at 3,000 rpm for 5 minutes at 4⁰C in a Mistral 3000 centrifuge. The cells were rinsed in protein sample buffer (5 mM HEPES pH7.5, 5mM EDTA.Na$_2$, 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 100ºC 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 trans blotter following the manufacturer's protocol. Filters were probed using anti-Sh.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 Pm^R 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,

$1\mu M$ each oligonucleotide primer, 1U Vent DNA polymerase, 1x Vent reaction buffer (Promega) and made up to volume with ddH_2O. Reactions were overlaid with $50\mu l$ 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 $[\alpha^{35}S]dATP$ (Amersham International). $10\mu g$ of plasmid DNA template was made up to a volume of $18\mu l$ with ddH2O, $2\mu l$ of 2M NaOH was added and the mixture heated to 68^0C for 20 minutes to denature the DNA strands. The DNA was precipitated by adding $10\mu l$ of 3M Sodium acetate pH6.0, $100\mu l$ of absolute ethanol and incubating at -20^0C for 30 minutes. The DNA was pelleted in a microcentrifuge, washed with 70% ethanol and dried under a vacuum. The pellet was resuspended in $7\mu l$ of ddH_2O. $1\mu l$ of oligonucleotide primer at $40ng/\mu l$ and $2\mu l$ of 5x Sequenase reaction buffer were added. The mixture was heated to $>65^0C$ in a water bath for 2 minutes and allowed to cool slowly to $<35^0C$ to allow annealing of the primer. Sequencing was then performed according to the manufacturer's protocol. Sequencing reactions were heated for 2 minutes at 80^0C 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 *Streptoallotichus 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 bias 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*.

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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. 1×10^9 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 &

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Phleomycin concentration (µg/ml TAP medium)

	0	0.02	0.04	0.06	0.08	0.1	0.2	0.4	0.6	0.8	1.0	2.0	5.0
WT (liquid)	+	+/-	–	–	–	–	–	–	–	–	–	ND	ND
WT (solid)	+	ND	ND	ND	ND	+	+	+	+/–	–	–	–	–
cw15 (liquid)	+	+/-	–	–	–	–	–	–	–	–	–	ND	ND
cw15 (solid)	+	ND	ND	ND	ND	+	+	+	+/–	–	–	–	–

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 *RBCS2* initiation codon (figure 3.1). The *ble* gene from *S. hindustanus* was cloned into pSP105 by Saul Purton in the following way: the *ble* gene was amplified from pUT430 (Drocourt *et al.*, 1990) using the 28mer GTATCGA<u>TGGCCA</u>AGcTGACCAGcGCCG (5' sense oligo) and the 21mer TTG<u>GTCGaC</u>GTCGGTtAGTCC (3' antisense oligo) an initial denaturation step at 95ºC for 4 minutes was followed by 15 cycles of; denaturing 95ºC - 1 minute, primer annealing 55ºC - 1 minute and primer extension 72ºC - 2 minutes. A product of 395 bp containing a unique *Msc*I restriction site (underlined) at the start codon (bold) and a unique *Sal*I site (underlined) was cloned into the *Msc*I and *Sal*I 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 *C. reinhardtii*. The *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 *RBCS2* sequence. The full sequence of the *RBCS2/ble* hybrid gene is presented (figure 3.3) and is present on pSP108 as a 1.4 kb *Eco*RI cassette.

3.2.3 Co-transformations using pSP108

The *ble* gene was introduced into *C. reinhardtii* cells by co-transformation as described in section 2.6. In two separate sets of experiments, pARG7.8 (Debuchy *et al.*, 1989) and pSP108 were co-transformed into *arg*7-8 *cw*d cells and transformants to arginine biosynthesis were selected by plating to TAP medium that lacked arginine. In each set of experiments 100 independent
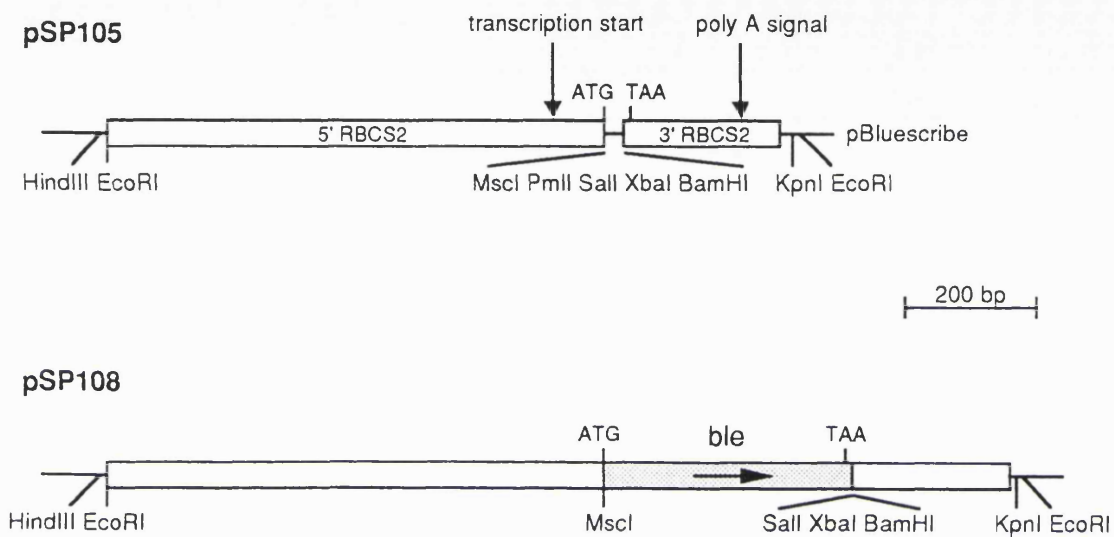
Figure 3.1 Linear maps of the plasmids pSP105 and pSP108. pSP105 contains 5'
and 3' untranslated regions of *RBCS2* cloned into the vector pBluescribe M13+
and separated by a polylinker. pSP108 is derived from pSP105 and contains the
ble gene as a translational fusion with *RBCS2* sequences.

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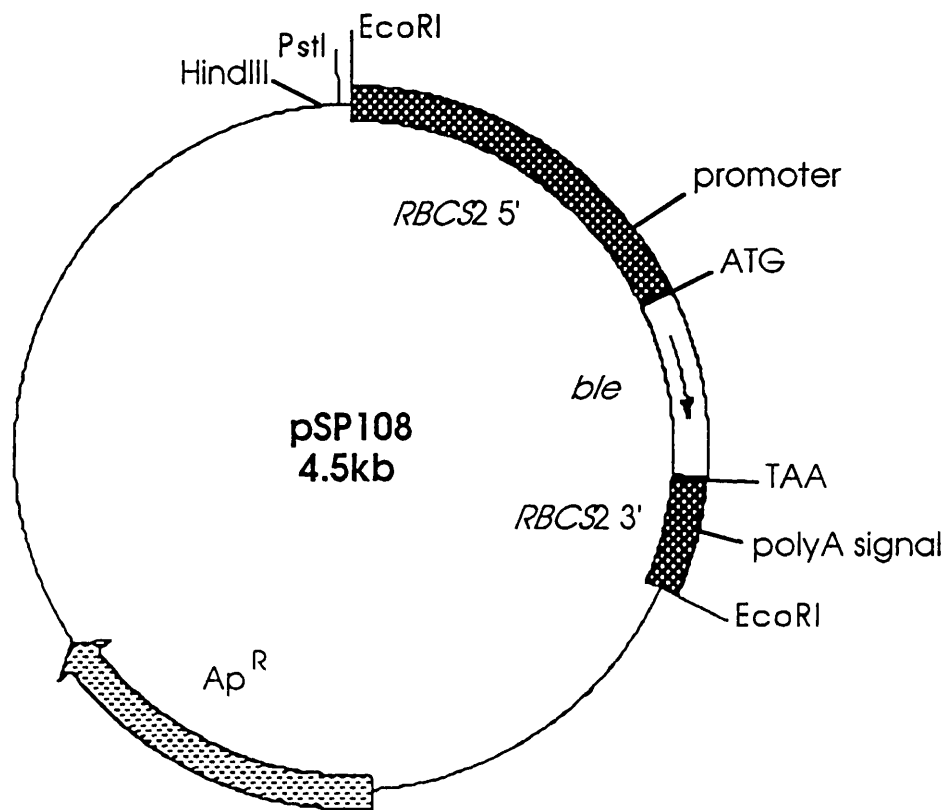
Figure 3.2 Map of plasmid pSP108 showing the *RBCS2/ble* marker present as a 1.4 kb *Eco*RI cassette and unique flanking enzyme sites.

```
gaattcggagtcccctgcacgatggtagtaccgcactgtctcagtgtgtacaaatgatgat
gaacccagtgccccaggggagtggtgaactacgcagcccacgtcaagcaagccgcgaccgt
cggcacaacccggatcgccgcatgcgccggcgcacgggtctatacattcgacgcgagccag
gtaaaactcttccacatacctcttagaggcgacacggcgccagaaacgacgaaaaactgga
caaacggcaggaacattgtctgtttcctagcaacaccgcgagagcggcccagatgccccgc
ctgccgtcctatgatacttcgtgacagatgaaggtaattggcatgctttgcgcgccagccg
gggccgccgcgacgggggcgtatattagttgtgtcacgccacggtttgaactcgcccgcgt
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ttcccgggcgcgccagaaggagcgcagccaaaccaggatgatgtttgatggggtatttgag
cacttgcaacccttatccggaagccccctggcccacaaaggctaggcgccaatgcaagcag
ttcgcatgcagcccctggagcggtgccctcctgataaaccggccaggggggcctatgttctt
tactttttttacaagagaagtcactcaacatcttaaaATGGCCAAGCTGACCAGCGCCGTTC
CGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGACCGGCTCGGGTT
CTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTC
ATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCG
GCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTC
CGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGAC
CCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGACTAACCGACGTCGACccact
ctagaggatccccgctccgtgtaaatggaggcgctcgttgatctgagccttgcccctgac
gaacggcggtggatggaagatactgctctcaagtgctgaagcggtagcttagctccccgtt
tcgtgctgatcagtcttttttcaacacgtaaaaagcggaggagttttgcaattttttgttggtt
gtaacgatcctccgttgattttggcctctttctccatgggcgggctgggcgtatttgaagc
gggtaccgagctcgaattc
```

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 Pm^R colonies and the second hundred two Pm^R 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 Pm^R 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 Pm^R transformants, nine randomly chosen Pm^S

transformants and the untransformed host $arg7$-8 cw_d prepared by the miniprep

method (section 2.5.1) was digested with EcoRI and separated on a 1% agarose

0.5×TBE 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 Pm^R transformants and,

interestingly, that it is also present in eight out of the nine Pm^S clones analysed.

The RBCS2/ble construct is present on pSP108 as a 1.4 kb EcoRI cassette and in

four of the Pm^R clones this cassette appears to be retained intact. In two of the

Pm^R 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 Pm^R clones that have

retained intact EcoRI cassettes (Pm 16, Pm 21 and Pm71) have also integrated
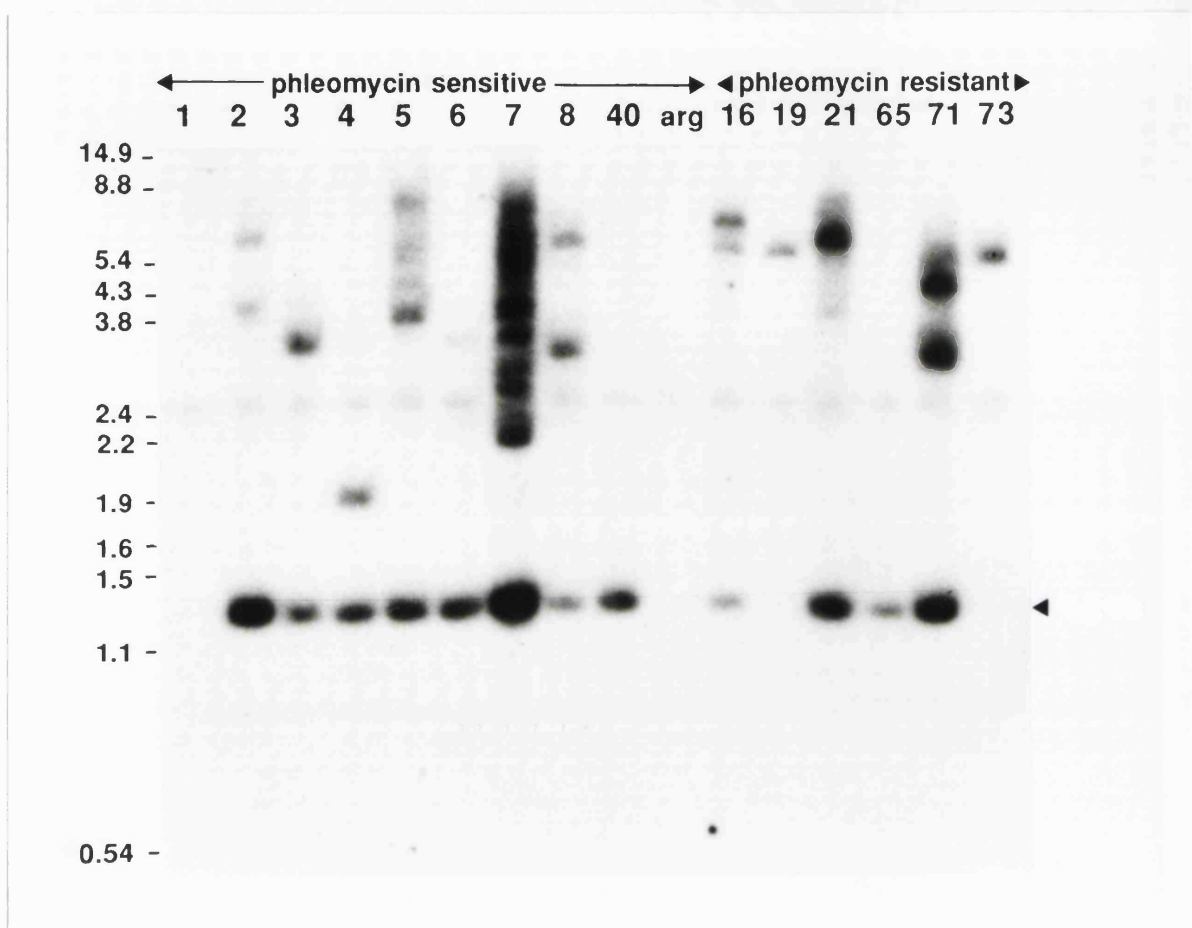
Figure 3.4 Southern analysis of DNA extracted from Pm^S and Pm^R cotransformants and the untransformed host strain *arg*7-8*cw*_d (arg). DNA was digested with *Eco*RI and hybridised with a probe specific for the coding region of *ble*. The 1.4 kb *Eco*RI fragment corresponding to the cassette in figure 3.2 is indicated. Size markers are in kilobase pairs.

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further copies of the *RBCS2/ble* gene in which one or both of the *Eco*RI sites have been lost during integration. Further Southern analysis has shown that Pm65 (the only PmR clone that retains just the *Eco*RI cassette) has integrated only a single copy. All of the PmS clones that contain the *ble* gene harbour an intact *Eco*RI cassette. In addition, six of these (Pms2, Pms3, Pms4, Pms5, Pms7 and Pms8) also contain further copies that have lost one or both *Eco*RI sites.

As the *ble* marker is flanked by 5' and 3' sequences from the *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 *RBCS2* (a 0.65 kb *Bam*HI, *Nco*I fragment of plasmid p1.03; Goldschmidt-Clermont & Rahire 1986). Figure 3.5 shows that the probe detects intact copies of *RBCS2* and the homologous *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 *C. reinhardtii* (Sodeinde & Kindle, 1993; Gumpel *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 *uid*A gene from the *C. reinhardtii NIT*1 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 *ble* gene present as an intact *Eco*RI cassette) and PmS40 (a PmS transformant that also contained an intact *Eco*RI cassette) was digested with the methyl sensitive isoschizomers *Hpa*II and *Msp*I. Blots of these digests probed with the *ble* DNA revealed no difference. Furthermore growing PmS co-
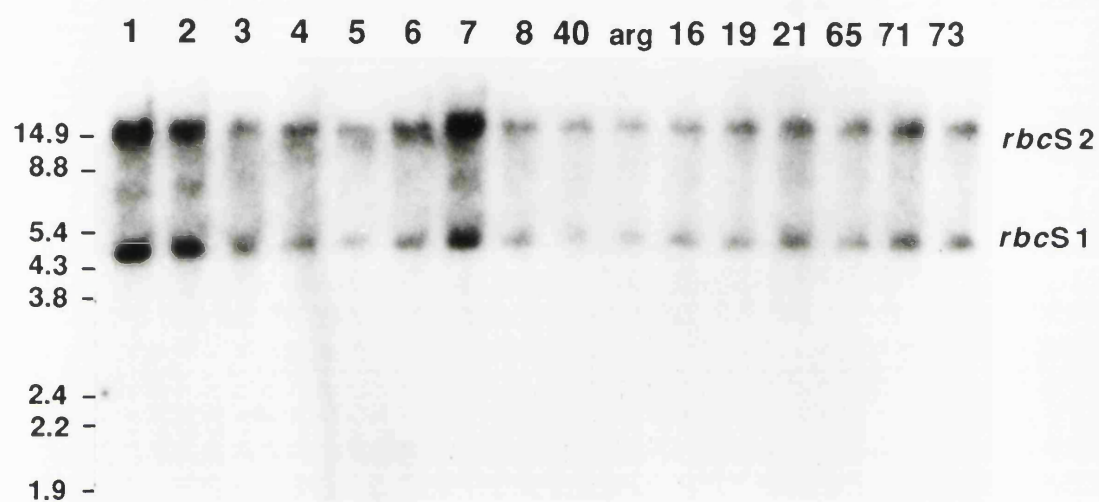
Figure 3.5 Southern analysis of DNA extracted from Pm^S and Pm^R co-transformants and the untransformed host strain *arg*7-8*cw*$_d$ (arg). The blot shown in figure 3.4 was stripped and re-hybridised to a probe for the coding region of *RBCS*2. The *RBCS*2 and *RBCS*1 bands are indicated. Size markers 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 Pm^R transformants and the majority of the Pm^S transformants contain *ble* DNA. Both populations contain transformants that have integrated intact *Eco*RI cassettes alone and transformants that have also integrated copies where one or more of the *Eco*RI sites has been lost. In addition the Pm^R transformants also contain a class of transformant in which no intact *Eco*RI cassettes are present. There is therefore no immediately apparent difference between Pm^R and Pm^S transformants, with most classes of transformant being represented in each population. In these circumstances it is essential to demonstrate that the Pm^R 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 Pm^R 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
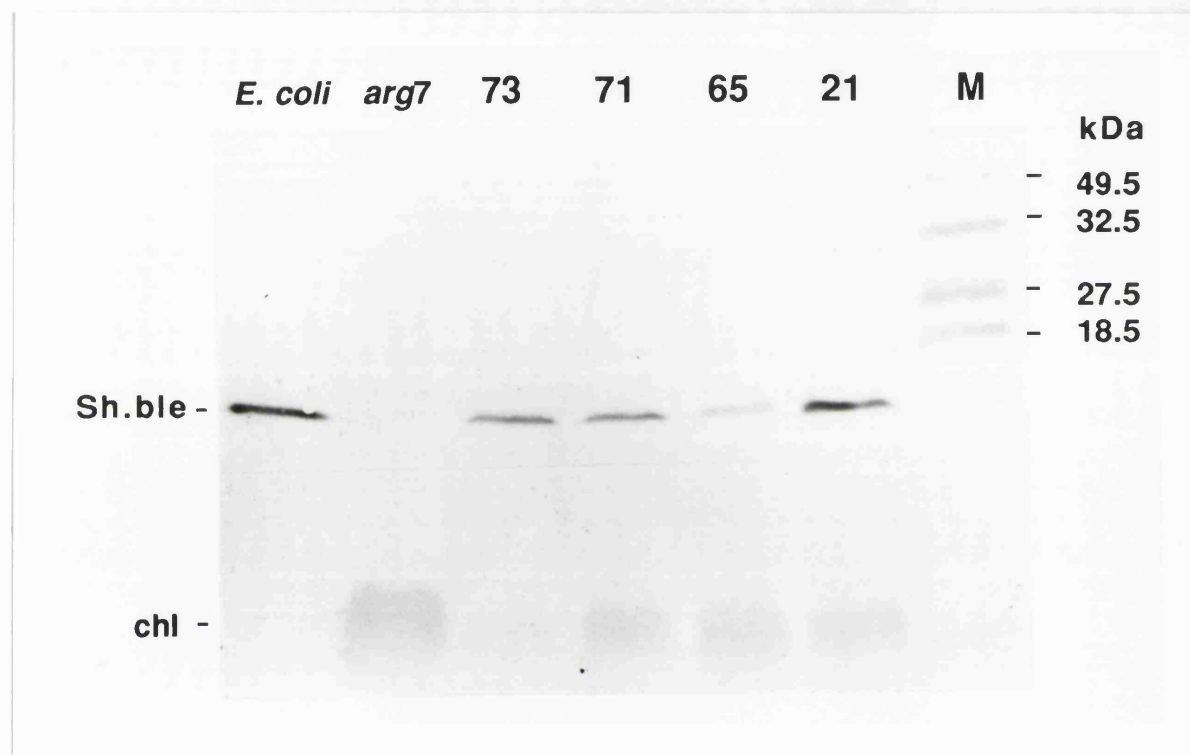
Figure 3.6 Western analysis of four Pm^R *C. reinhardtii* transformants, the untransformed host strain *arg*7-8*cw*$_d$ (arg7) and *E. coli* transformed with pSP108 and grown in the presence of IPTG. Total soluble protein was separated on a 17.5% denaturing polyacrylamide gel, transferred to nitrocellulose and probed using anti-Sh.Ble antibodies. Free chlorophyll (chl) and pre-stained markers (M) are indicated.
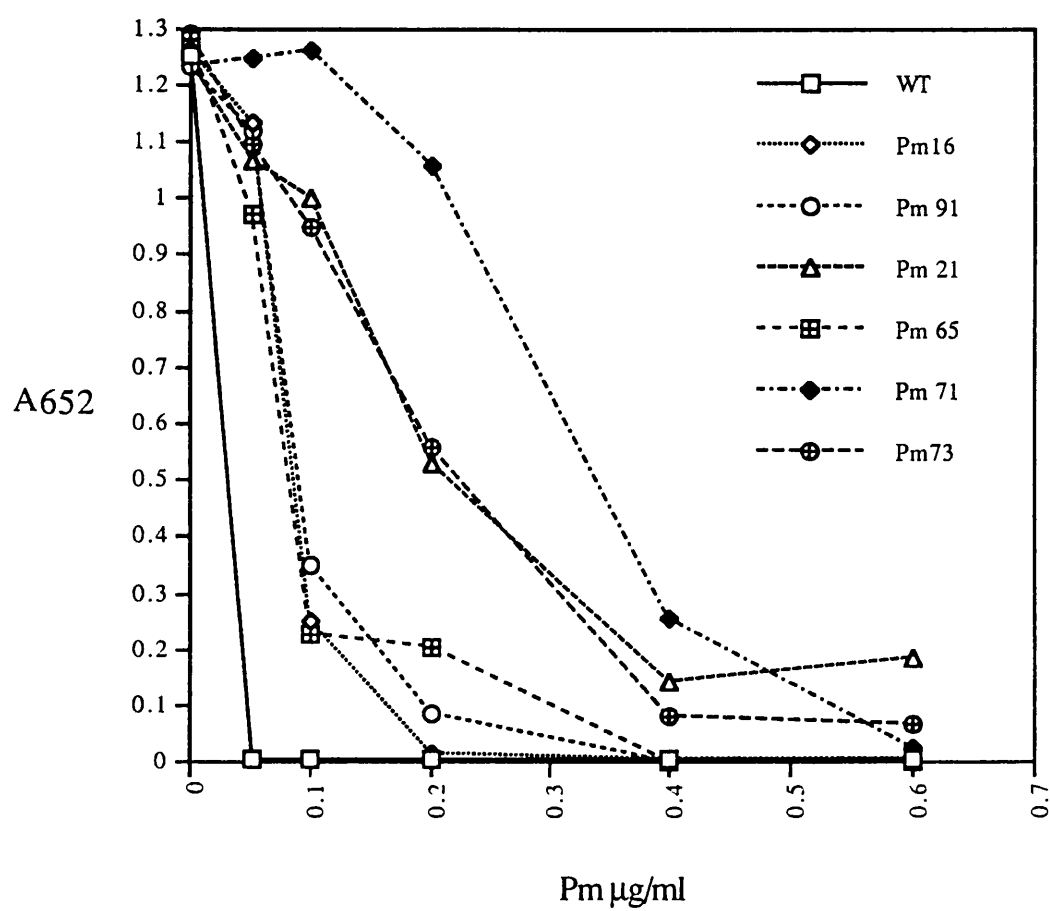
Figure 3.7 Resistance of WT cells and Pm^R transformants to increasing levels of Pm. The amount of chlorophyll in 1 ml of culture is expressed as absorbance units measured at 652 nm.

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 Pm 65, 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.

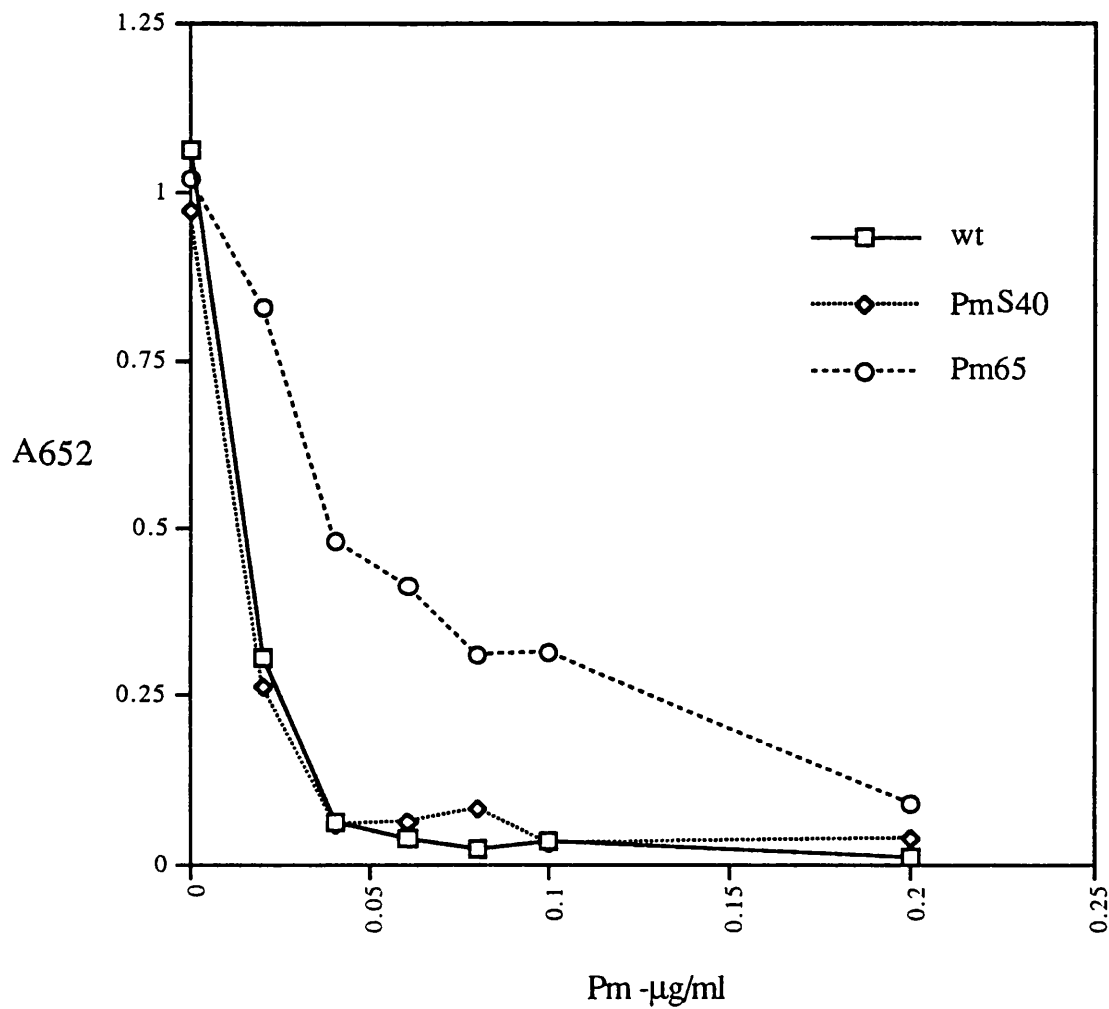*Figure 3.8* Resistance of Pm65, PmS40 and WT cells to increasing levels of Pm. The amount of chlorophyll in 1 ml of culture is expressed as absorbance units measured at 652 nm.

3.2.7 Genetic analysis of Pm^R inheritance

Whilst a Pm^R phenotype, the presence of the *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 *C. reinhardtii* through sexual crosses and tetrad analysis. The inheritance of the *ble* gene was investigated by mating Pm65 (mt-) which contains a single copy of the *ble* gene with WT cells (either CC-125(mt+) or CC-1021(mt+)). It has recently been observed that the host used for transformation (*arg7-8 cw$_d$*) contains one or more uncharacterized mutations that inhibit mating and in addition prevents the recovery of complete tetrads (Gumpel *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 *ble* gene and the Pm^R phenotype. All thirteen of the daughter cells were grown up in liquid culture and spotted in duplicate to TAP agar +/- Pm @2μg/ml to test for the transmission of the phenotype. Furthermore, DNA from these progeny was isolated by the miniprep method, digested with *Eco*RI and, following electrophoretic separation, blotted to a nylon membrane and hybridised to a probe made from the entire pSP108 *Eco*RI cassette. This 1.4 kb fragment contains both the *ble* gene and 3' and 5' UTR sequences from *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
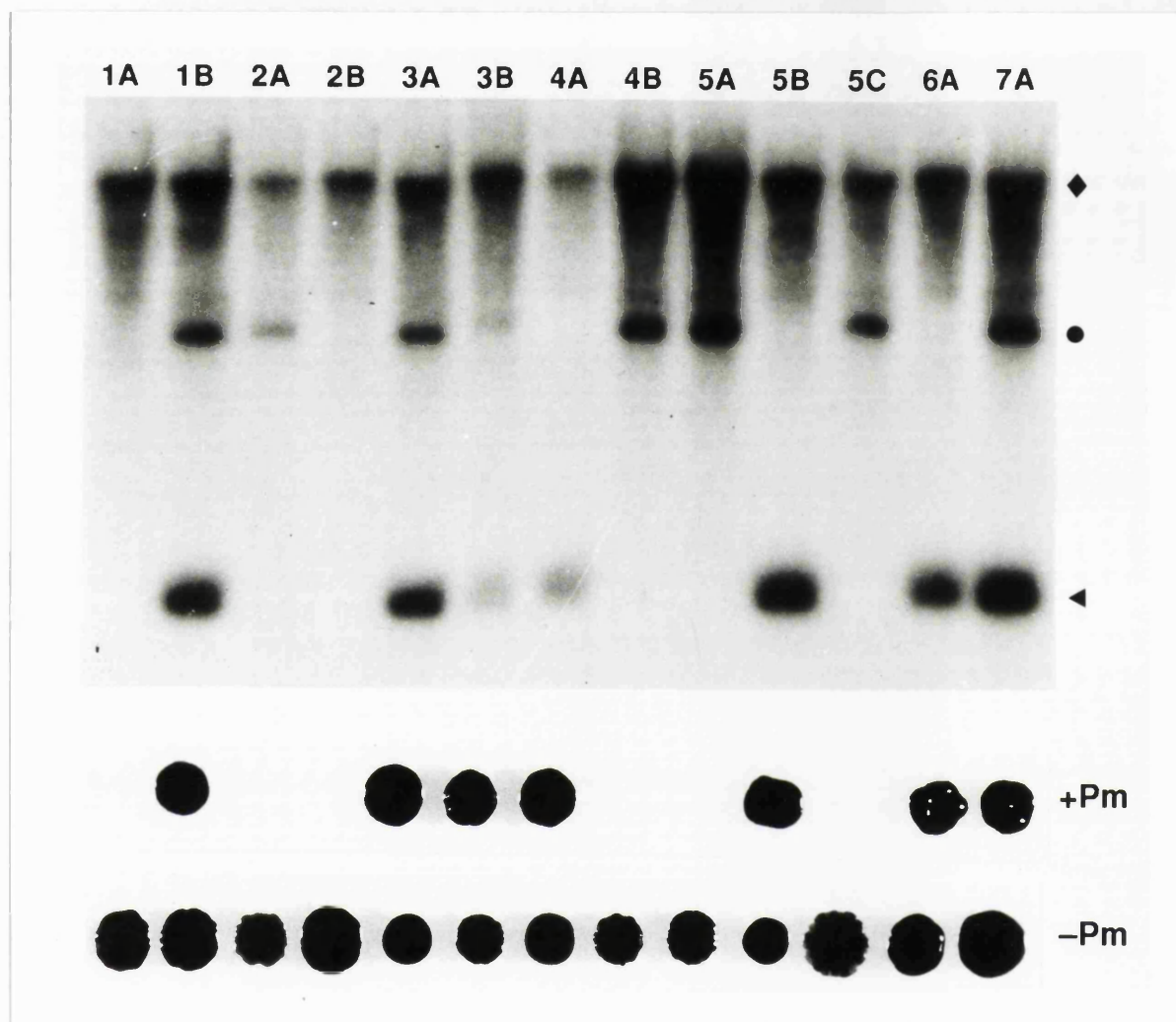
Figure 3.9 The co-segregation of *ble* and the Pm^R phenotype in progeny from a
Pm65(mt-) x WT(mt+) cross. DNA from the 13 progeny was digested with *Eco*RI
and hybridised to the 1.4 kb *Eco*RI *RBCS2/ble* cassette (upper panel). The
endogenous *RBCS2* gene present in all the progeny is marked with a diamond.
The *RBCS2/ble* transgene derived from Pm65 is marked with a triangle. The
presence of this transgene correlates with a Pm^R phenotype as confirmed by spot
tests on to solid medium +/- Pm (lower panel). An additional fragment of *RBCS2*
(marked with a circle) which is derived from Pm65 does not co-segregate with the
Pm^R 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 *arg*7-8 *cw*d 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.

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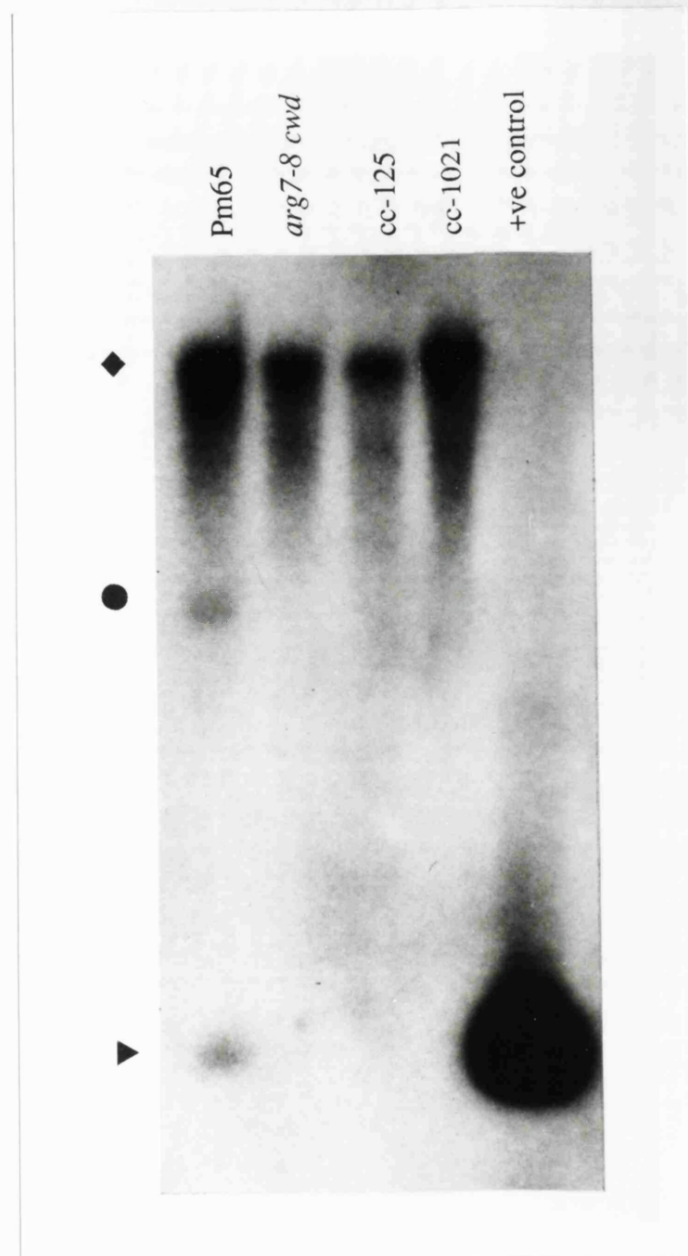
Figure 3.10 Southern analysis of ectopic integration of pSP108. DNA extracted from Pm65, untransformed host $arg7$-$8cw_d$ and WT (CC-1021 & CC-125) cells. DNA was digested with *Eco*RI and hybridised to the 1.4 kb *Eco*RI *RBCS2/ble* cassette. The endogenous *RBCS2* present in all cell types is marked 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 *Eco*RI fragment of pSP108.

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strain	Pm	cw	arg	mt
WT	S	+	+	+
Pm65	R	−	+	−
1A	S	+	+	+
1B	R	−	+	+
2A	S	+	−	−
2B	S	+	+	+
3A	R	−	+	+
3B	R	+	+	−
4A	R	−	+	+
4B	S	+	+	−
5A	S	+	+	+
5B	R	−	+	+
5C	S	+	−	−
6A	R	+	+	−
7A	R	−	+	ND

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 *arg*7-8 mutation present in Pm65 but which is masked by complementation with the introduced *ARG*7 gene); mating type (mt).

3.2.8 Direct selection of Pm^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

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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 $arg7$-8 cw_d as the host and maintaining arginine in the medium throughout. Cells were plated to medium supplemented with Pm @ 2μg/ml in soft agar (also supplemented with Pm @ 2μg/ml). Pm^R colonies appeared after 7-9 days and typically 5-10 transformants per plate were recovered. The cell wall deficient strain cw_d was also transformed in this way and again 5-10 Pm^R colonies were recovered per plate. In these transformation experiments 'no DNA' controls were routinely performed in parallel and 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 Pm @ 2μg/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 EcoRI. Following electrophoretic separation, the DNA was blotted to a nylon membrane and hybridised to the 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 ble gene. As with the co-transformants there are clones that contain intact EcoRI cassettes, clones in which one or both of the 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 ble gene although D2 has at least three. This has implications for gene tagging where it is important that

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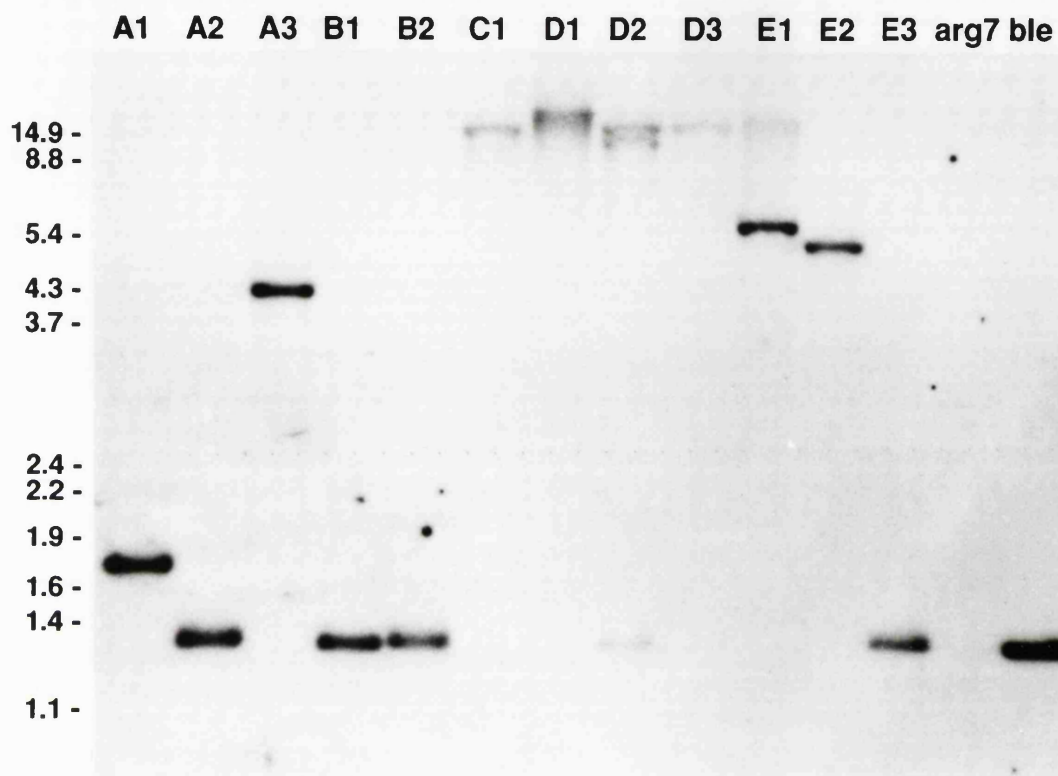
Figure 3.11 Southern analysis of DNA extracted from directly selected PmR transformants (A1 - E3) and the untransformed host strain *arg7-8cw*d (arg7). DNA was restricted with *Eco*RI and hybridised with a probe specific for the coding region of *ble*. Size markers are in kilobase pairs.

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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 Pm^R transformants but is however also present in ~90% of Pm^S 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 Pm^S 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 Pm^R 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*

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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, Pm^R 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 Pm^R 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 Pm^R 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 Pm^R 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 Pm^R 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

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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 *nit*1-, *rsp3*-host. Of 21 *NIT*1+ 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 Pm^S or Pm^R 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 Pm^S and Pm^R 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 Pm^S 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 Pm^S co-transformants would be required to test this possibility. Alternatively the observed difference in expression between Pm^R and Pm^S clones may be due to 'positional' effects. The ble gene in Pm^S clones may have integrated into transcriptionally inactive regions of the genome or, in the clones where the gene is being expressed, the integration

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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 *RBCS2* 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 *RBCS2-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 *NIT*1 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 Pm^R and Pm^S transformants harbouring the *RBCS2/ble* gene may be related to recombination between homologous plasmid sequences. Extrachromasomal 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 *Bam*HI was introduced into *arg*7-8 cw_d cells both with and without 100U of *Bam*HI. Five separate transformations with *Bam*HI, 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 *NIT*1 marker (Kindle, 1990). However the use of PEG with the *ARG*7 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

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marker	100U *Bam* HI	average number transformants/plate
pARG7.8 : *Bam* HI	+	9
pARG7.8 : *Bam* HI	-	104
no DNA	-	-

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.

marker	host	5% PEG	average number transformants/plate
pSP108	cw_d	–	12.6
no DNA	cw_d	–	0
pSP108	cw_d	+	11
no DNA	cw_d	+	0
pSP108	$cw15$	–	1.4
no DNA	$cw15$	–	0
pSP108	$cw15$	+	21
no DNA	$cw15$	+	0

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.

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 cw_d cells. Following plating, five plates were incubated in bright light ($45\mu E/m^2/s$) , the other five were incubated in dim light ($2\mu E/m^2/s$) 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
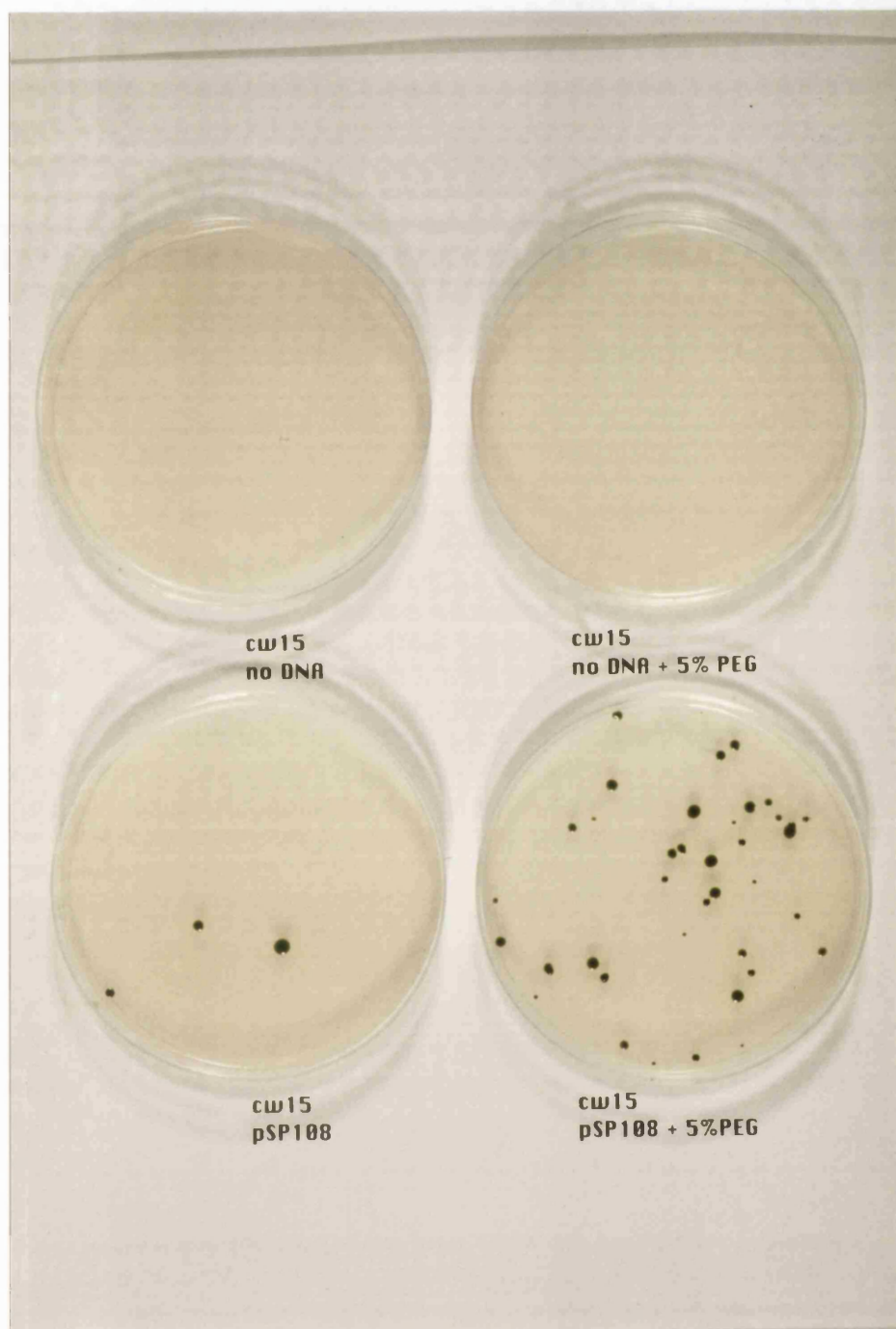
Figure 4.1 The effect upon transformant recovery for *cw*15 cells transformed in the presence and absence of 5% PEG.

marker	treatment	average number transformants/plate
pSP108	soft agar	12.6
no DNA	soft agar	0
pSP108	spread	6.2
no DNA	spread	0

Table 4.3 The effect upon transformant recovery of direct spreading or plating in soft agar of transformed cells.

marker	light intensity (μE/m^2/s)	average number transformants/plate
pSP108	45	5
pSP108	2	2
no DNA	45	0
no DNA	2	0

Table 4.4 The effect of light intensity upon the recovery of transformants.

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, extrachromasomal 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* *Eco*RI cassette was cloned into the unique *Eco*RI site of pARG7.8 to give plasmid pARG7.8/*ble* (figure 4.2). This construct was transformed into *arg*7-8 *cw*$_d$ 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 *Pst*I 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 *Pst*I site of pSP108 upstream of the 1.4 kb *Eco*RI cassette to give plasmid pSP108.55 (figure 4.3). The construct was tested by comparing its transformation

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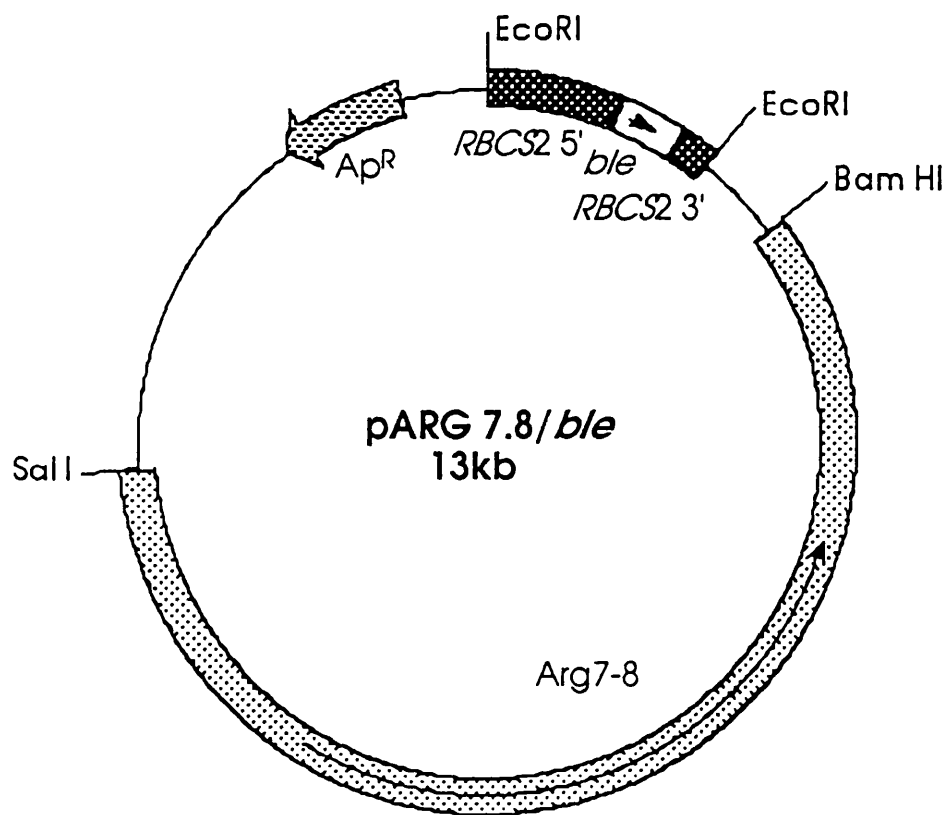
Figure 4.2 Map of plasmid pARG7.8/*ble*. The 1.4 kb *RBCS2/ble* cassette from pSP108 is cloned within the unique *Eco*RI site of pARG7.8
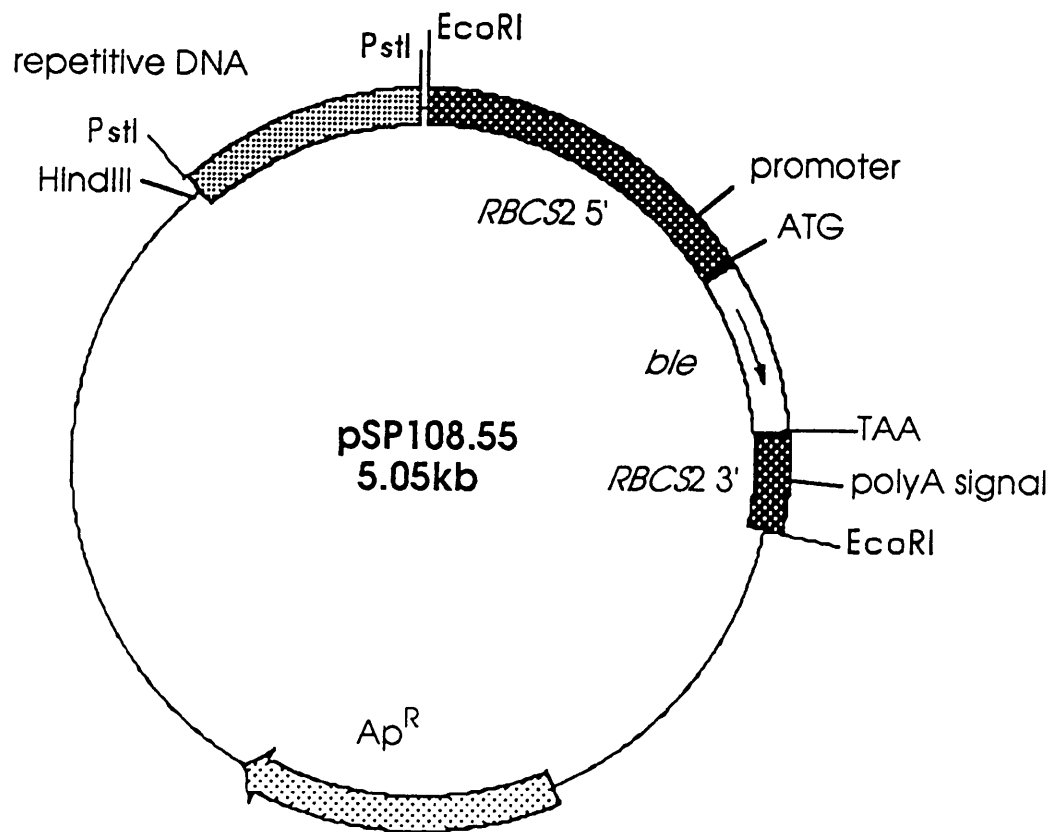
Figure 4.3 Map of plasmid pSP108.55. The 0.55 kb *Pst*I fragment of pARG7.8 is cloned within the unique *Pst*I site of pSP108 upstream of the hybrid *RBCS2/ble* gene.

efficiency to that of pSP108. Five transformations using pSP108 and five using pSP108.55 were performed using *cw*15 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 *RBCS*2 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 *RBCS*2 had been included in pSP108. Of the cloned *RBCS*2 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 *Hin*dIII, *Bst*XI fragment was excised from the *RBCS*2 genomic clone p1.03 (Goldschmidt-Clermont & Rahire, 1986). A 70 bp *Hin*dIII, *Bst*XI 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 *arg*7-8 *cw*d cells with pARG7.8 and pSP110. Of 100 transformants to arginine biosynthesis only one (Pm3.9) exhibited a Pm^R 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 *Hin*dIII and *Bst*XI sites of pSP108.

SAR from pea contained within a ~ 200bp *Hind*III fragment of plasmid pH268.7 (Pwee & Gray, 1993) was cloned into the unique *Hind*III site of pSP108 to give pSP113 (figure 4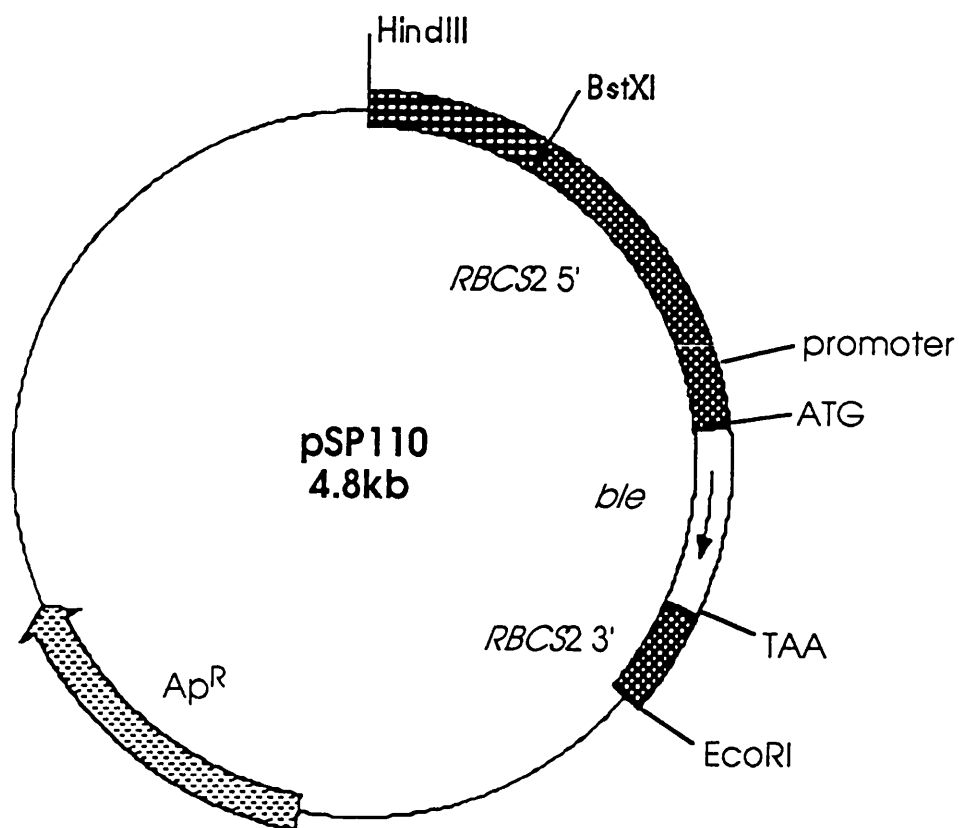.5). This construct was tested by comparing its transformation efficiency to that of pSP108. Five transformations using pSP108 and five using pSP113 were performed using *cw*15 as the host. 'No DNA' controls were also performed. The inclusion of the SAR was found to have an adverse effect on the transformation 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 *OEE*1 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 *ARG*7 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 *ARG*7, together with the 3' UTR from the *RBCS*2 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 *ARG*7 and any codons remaining from the polylinker (figure 4.6). The *ble* gene (present as a PCR product in pSK-) was excised using *Cla*I and cloned into the *Cla*I site of pARG
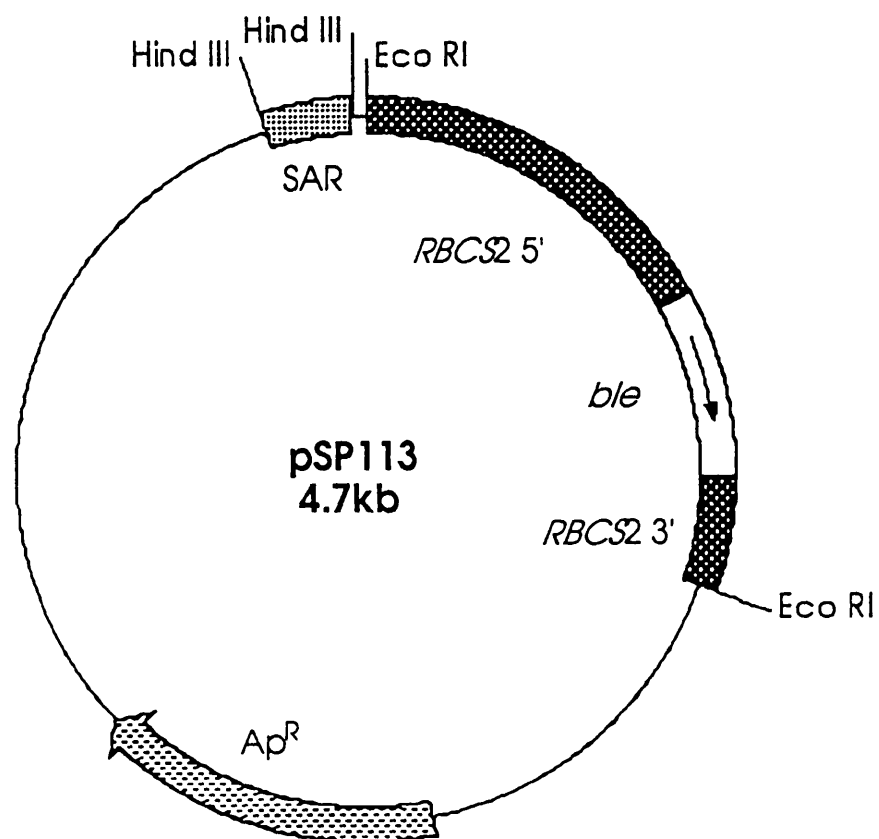
Figure 4.5 Map of plasmid pSP113. An ~200 bp *Hind*III fragment of plasmid pH268.7 containing an SAR from pea is cloned into the unique *Hind*III site of pSP108.
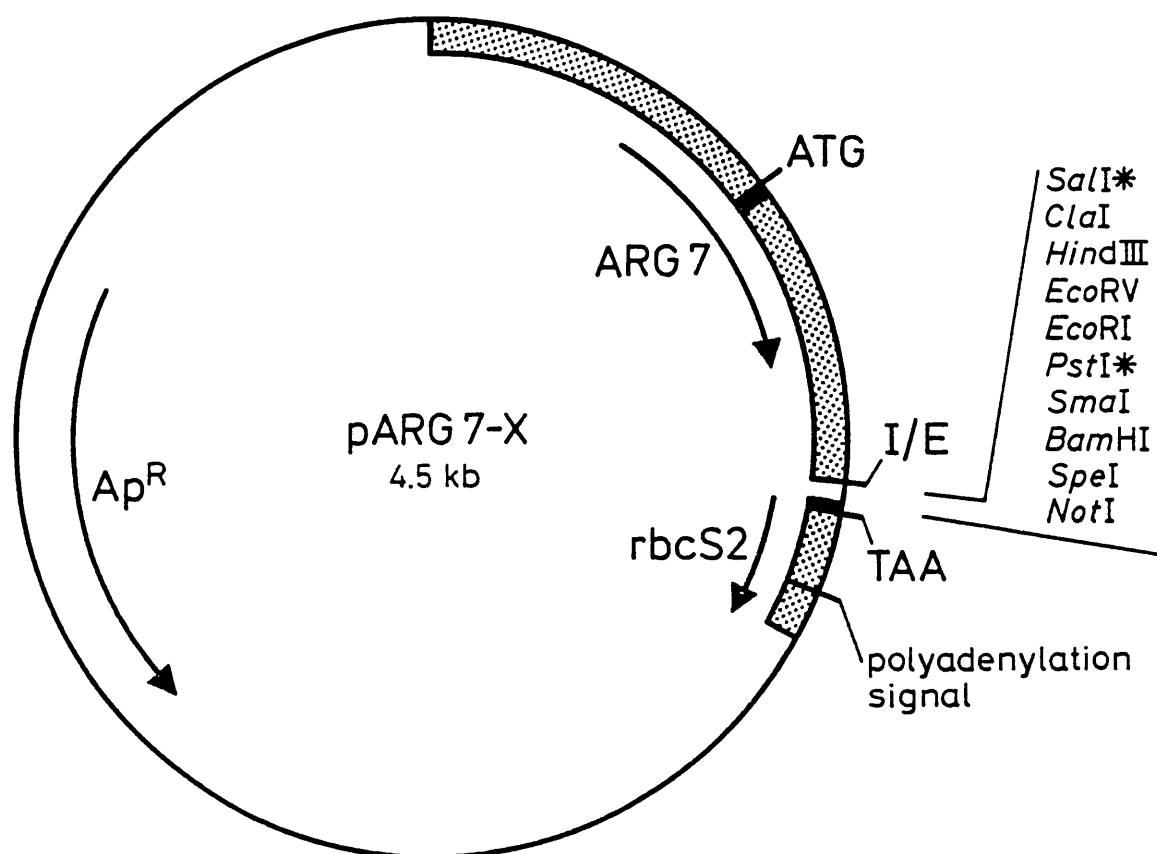
Figure 4.6 Map of plasmid pARG7-X. The construct contains the 5' UTR, the first two codons and the first intron of *ARG7*, together with the 3' UTR from the *RBCS2* gene cloned into pBluescript SK- (Stratagene) and separated by a multiple cloning site. * Indicates site not unique.

7-X to give pARG7-X/*ble* (figure 4.7). This construct was introduced into *arg*7-8 *cw*d cells as a co-transformation with pARG7.8. One hundred transformants rescued to arginine biosynthesis were screened for Pm^R however none grew. The construct was also used to directly select transformants of *cw*15. 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 Pm^R 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 *OEE*1 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 *oee*1- cells to photoautotrophic growth (Mayfield & Kindle, 1990). To investigate whether the cDNA could be similarly used, the *OEE*1 cDNA from plasmid pPII-12P4 (Mayfield *et al.* 1989) was cloned into the expression vector pSP105. The *OEE*1 cDNA was amplified from pPII-12P4 by PCR using the 25mer GCAAAGA<u>TGGCCA</u>CCCGCGCTGCCC (5' sense oligo) and the 35mer ACGAAT<u>TCTAGA</u>CTCGAGTTTTTTTTTTTTTTTTTTT (3' antisense oligo). An initial denaturation step at 95^{0}C for 3 minutes was followed by 12 cycles of; denaturing 95^{0}C - 1 minute, primer annealing 50^{0}C - 1 minute and primer extension 72^{0}C - 2 minutes. A 1.6 kb product containing two *Msc*I restriction sites; one (underlined) at
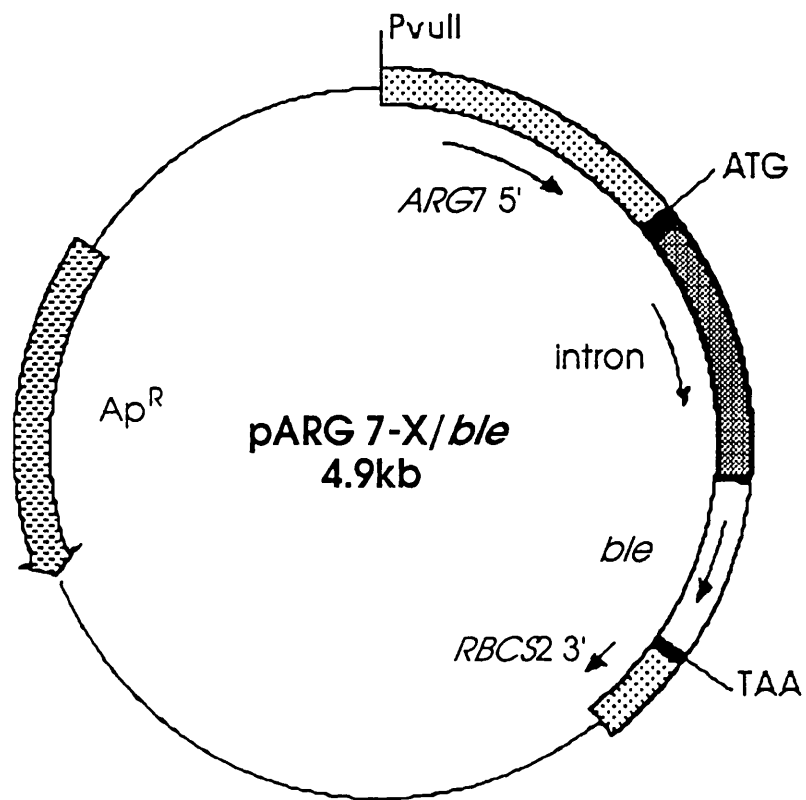
Figure 4.7 Map of plasmid pARG7-X/*ble*. A *Cla*I fragment containing the *ble* gene was cloned into the *Cla*I site of pARG7-X to give pARG7-X/*ble*.

the start codon (bold), and another 120 bp downstream and a unique *Xba*I site

(underlined) was blunt cloned into the *Eco*RV site of pSK-. The 1.5 kb *Msc*I, *Xba*I

fragment was cloned into the *Msc*I and *Xba*I sites of pSP105 to create pSP111.

The 120 bp *Msc*I fragment was then cloned into pSP111 to give pSP112 (figure

4.8). The 120 bp *Msc*I 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, *oee*1- strain FuD44 *cw*d (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 *OEE*1 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 *OEE*1 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 *oee*1- 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
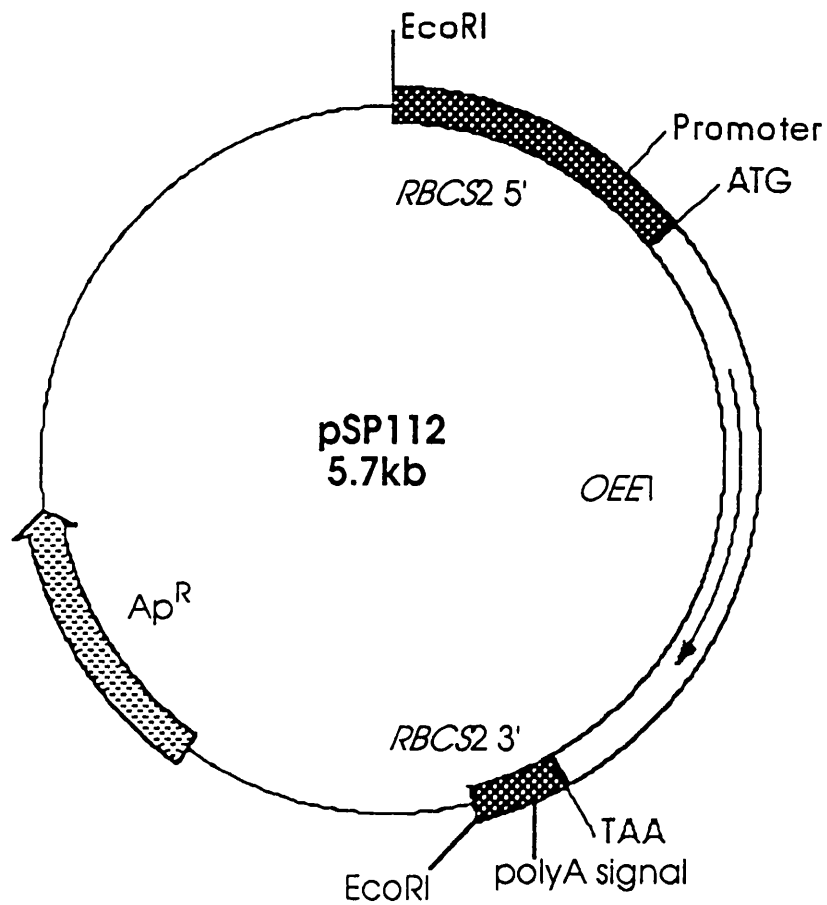
Figure 4.8 Map of plasmid pSP112. A cDNA copy of the *C. reinhardtii OEE*1 gene was cloned between the *Msc*I and *Xba*I sites of pSP105 creating a translational fusion between *RBCS*2 and *OEE*1.

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_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_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 *a* and chlorophyll *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:

$$A_{663} = 82.04 \text{ chl } a + 9.27 \text{ chl } b$$

$$A_{645} = 16.75 \text{ chl } a + 45.6 \text{ chl } b$$

where: A = absorbance, chl = chlorophyll concentration in mg/ml.

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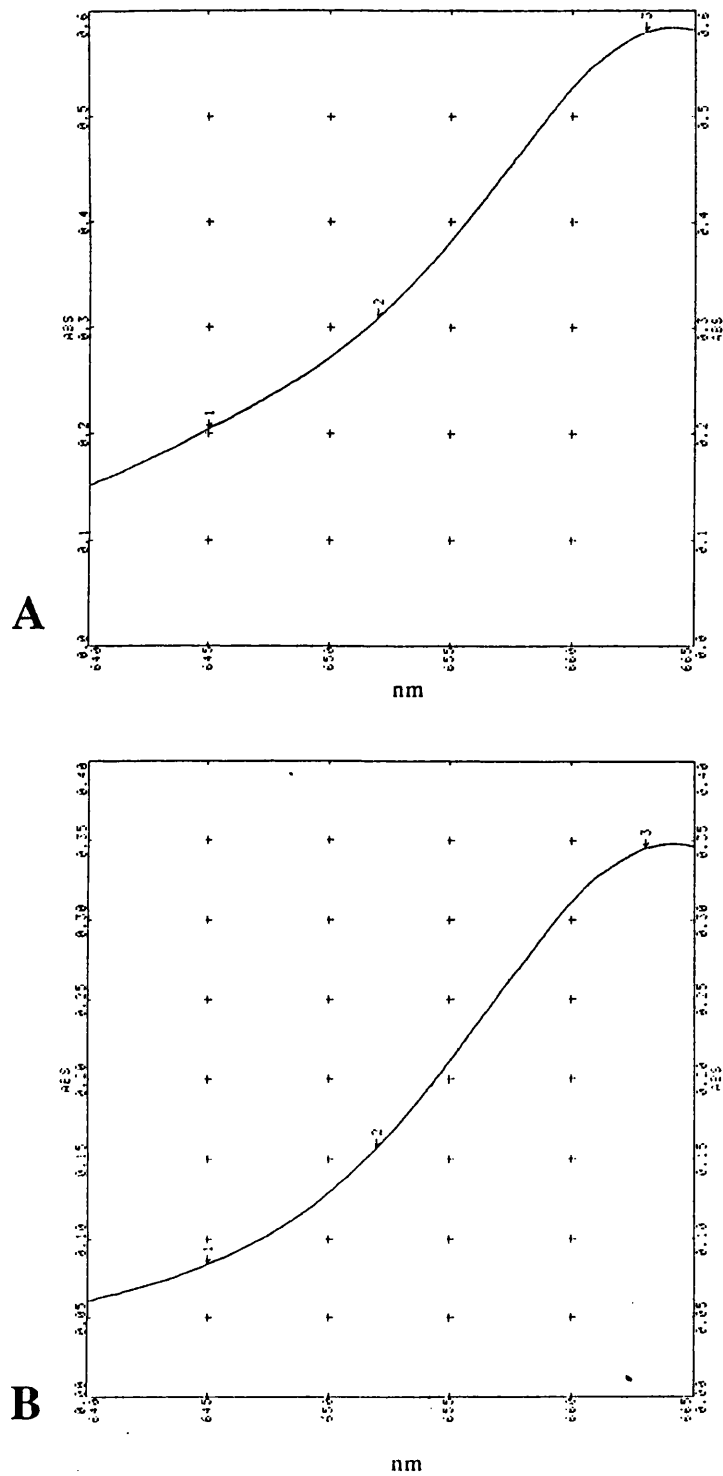
Figure 4.9 Absorbance spectra of cw_d (panel A) and $cbd1$ (panel B) cell extracts. Cells from 1 ml of culture were extracted with 80% (v/v) acetone and absorbance between 640 nm and 665 nm determined.

When the simultaneous equations are solved;

$$chl\ a = 0.0127\ A_{663} - 0.00259\ A_{645}$$

$$chl\ b = 0.0229\ A_{645} - 0.00467\ A_{663}$$

The cw_d 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 cw_d 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 $cbd1$ (chlorophyll b deficient 1). The ability of $cbd1$ to grow photoautotrophically was tested by spotting cultures to minimal medium plates. It was found that $cbd1$ was capable of phototrphic growth as previously described for other chlorophyll b deficient mutants (e.g. Eichenberger 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 cw_d and $cbd1$ 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 cw_d cells peak 2 is present as a shoulder on peak 1, however on the spectra for the $cbd1$ 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 $cbd1$ is harbouring the ble marker, DNA from
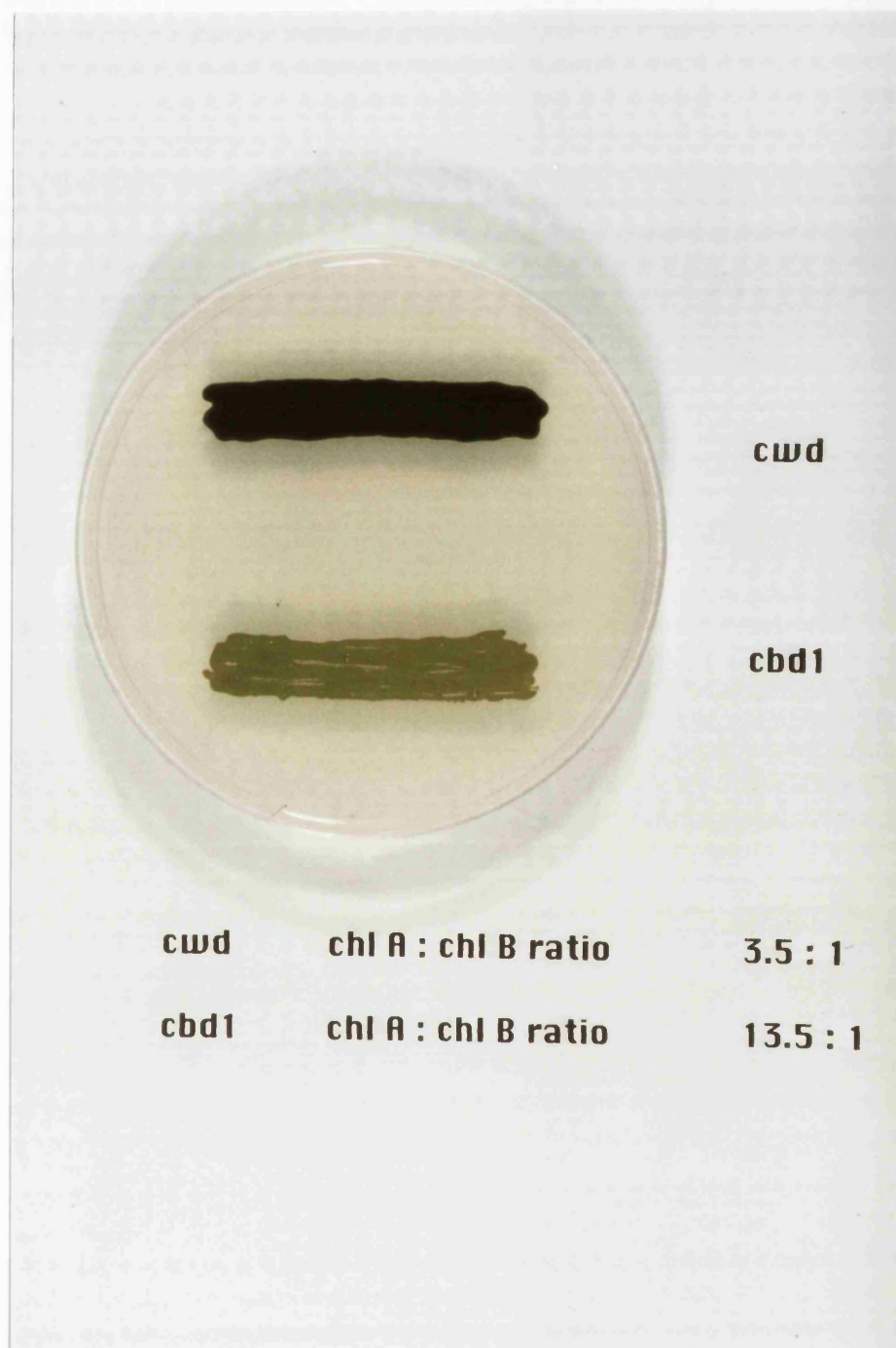
Figure 4.10 Morphology of cw_d and *cbd*1 cells and their respective chlorophyll *a:b* ratios.
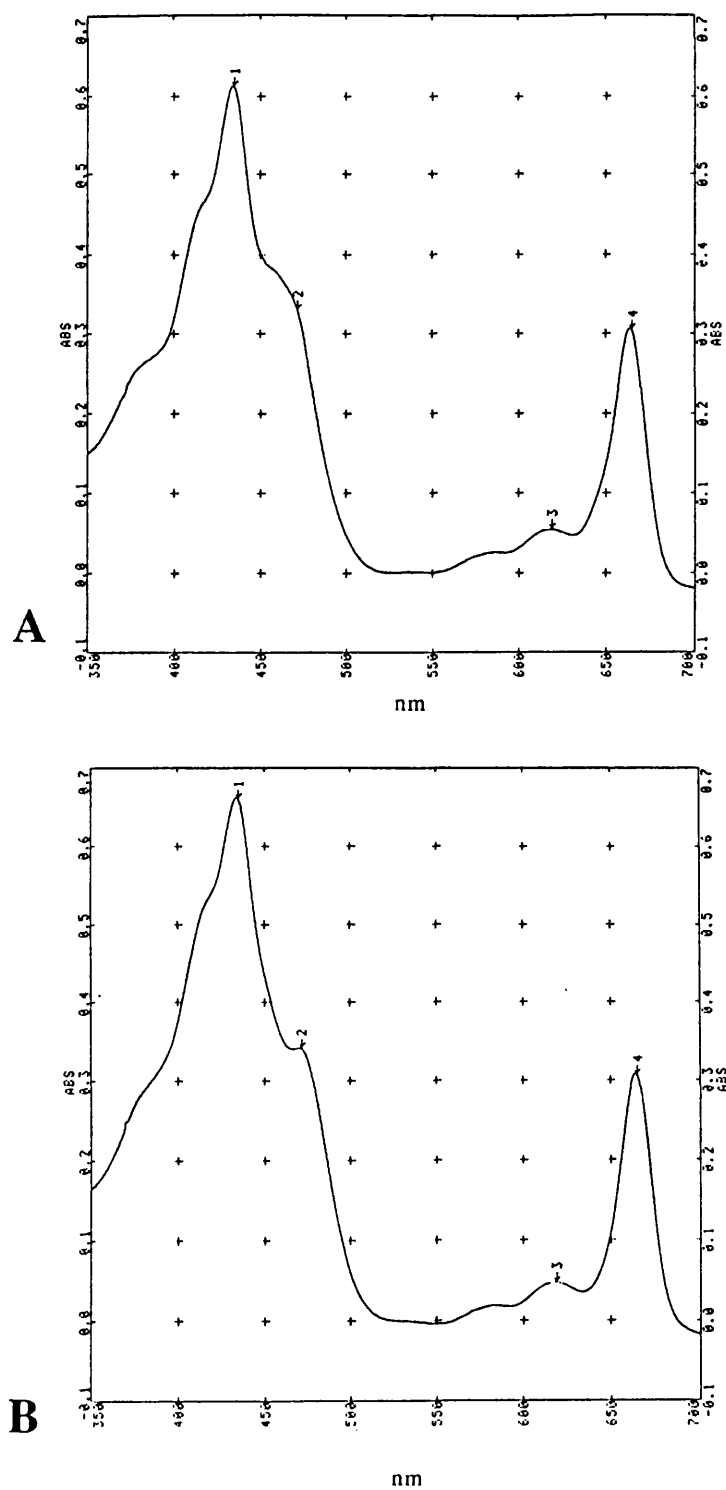
Figure 4.11 Absorbance spectra of cw_d (panel A) and $cbd1$ (panel B) cell extracts. Cells from 1 ml of culture were extracted with 80% (v/v) acetone and adjusted to approximately equal concentrations. The absorbance spectra between 350 nm and 700 nm were determined.

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*cbd*1, untransformed *cw*$_d$ host cells and the other transformant from the same plate as *cbd*1, 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 *cbd*1. 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.
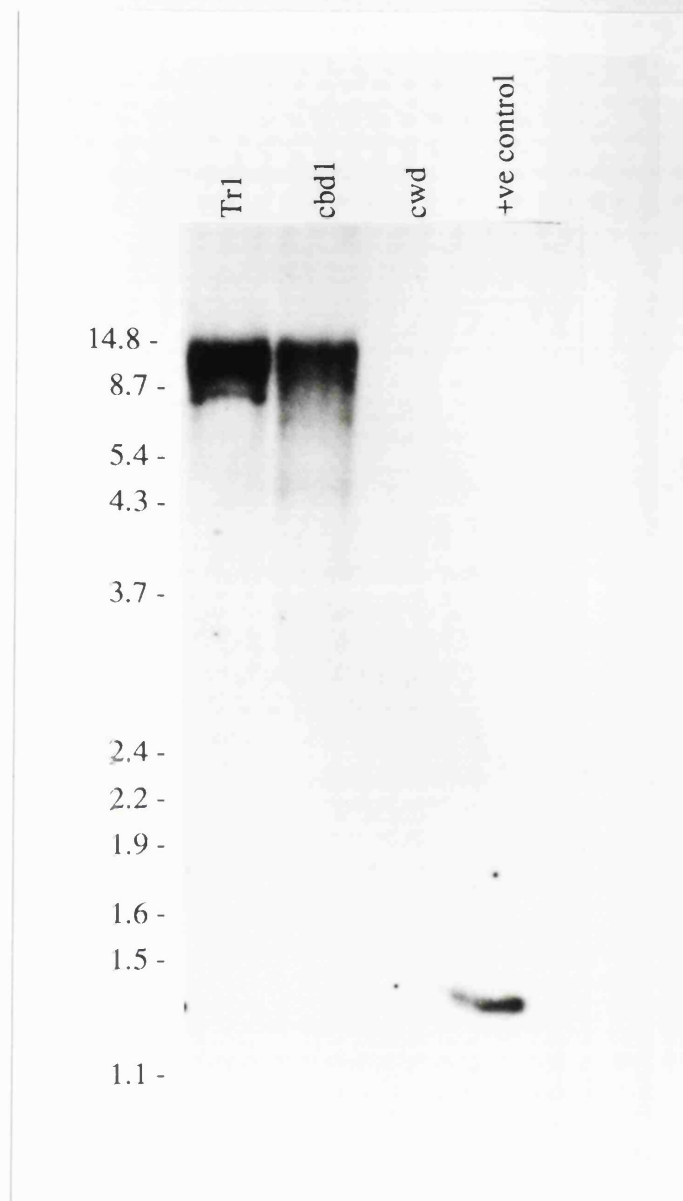
Figure 4.12 Southern analysis of DNA extracted from Tr 1, *cbd*1 and *cw*d cells. DNA was digested with *Eco*RI and hybridised with a probe specific for the coding region of *ble*. The positive (+ve) control is the 1.4 kb *Eco*RI fragment of pSP108. Size markers are in kilobase pairs.

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 *NIT*1 marker benefit from the inclusion of PEG (Kindle, 1990) but no difference is observed with the *ARG*7 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

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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 *OEE*1 failed to transform FuD cw_d 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 Cyh^R 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 Cyh^R phenotype was found to be a substitution within the ribosomal L41 subunit of a proline residue found in sensitive species *e.g. S. cerivisiae* (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,

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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 *CRY*1 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 (*RPL*41) 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 oligogucleotides I designed to conserved regions of previously published L41 sequences: The 23mer L41 5' ATGGT^G/$_C$AACGT^G/$_C$CCCAAGACCCG (5' sense oligo), the 24mer L41 3' CTTCTTGGTGGTCTT^G/$_A$GCCTTCTT (3' antisense oligo) and the 35mer L41#3 CAGGGCAAGCGCCGCTACGACCGCAAGCAGTC^G/$_C$GG (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 (ATTAACCCTCACTAAAG) 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: ATGGTGAACGTTCCTAAGACC (a 5'

sense oligo) and the 21mer L41.5'Δα: GGTCTTAGGAACGTTCACCAT (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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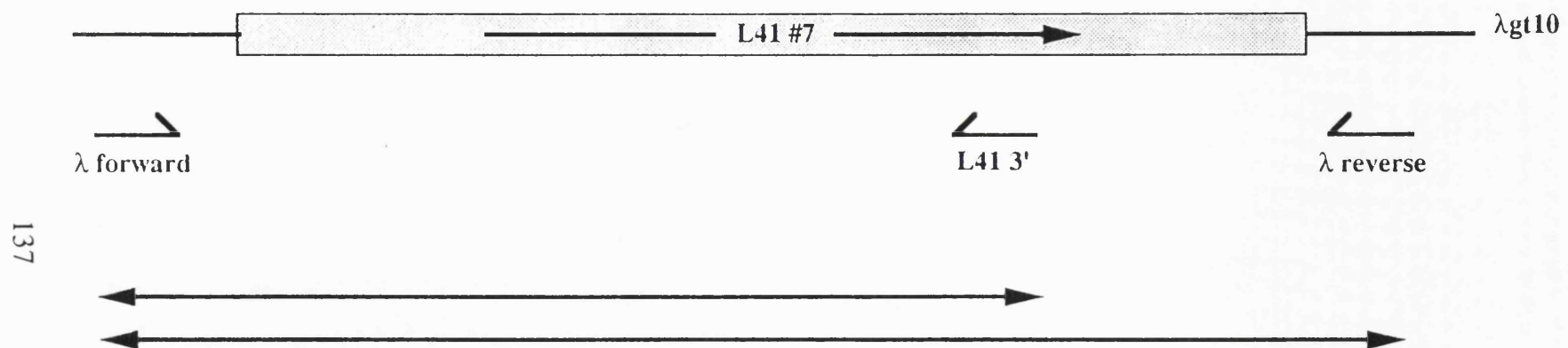
Figure 5. 1 Orientation of L41#7 in λgt10. PCR reactions using combinations of; λgt10 forward, λgt10 reverse and L413'primers (single head arrows) gave products (double headed arrows) only between λgt10forward/λgt10 reverse and between λgt10 forward/L413' pairs of primers, indicating the orientation of the L41#7 cDNA insert to be as indicated.
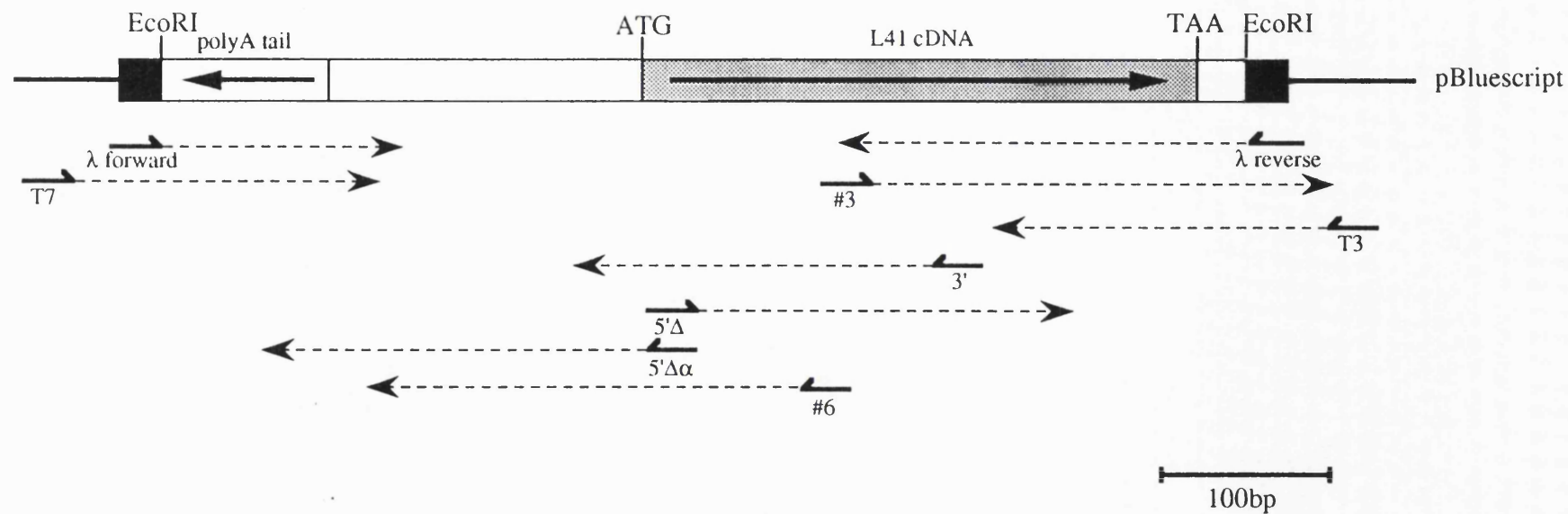
Figure 5.2 Sequencing strategy for pKSL41#7. Primers used are indicated by solid arrows and the sequence raised from them indicated by broken arrows. Scale is indicated by the size bar (100bp).

The nucleotide sequence of *RPL*41 coding region and the predicted amino acid sequence of the L41 ribosomal protein is presented as figure 5.3. The *RPL*41 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 *RPL*41 (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 *RPL*41 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 *RPL*41, 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

```
AGCCAGGAAAA ATG GTG AAC GTT CCT AAG ACC AAG CGG GCG TTC TGC AAG GGG TGC AAG AAG   62
            M   V   N   V   P   K   T   K   R   A   F   C   K   G   C   K   K

CAC ATG ATG ATG AAG GTC ACC CAG TAC AAG ACT GGC AAG GCC TCC CTC TAC GCG CAG GGC  122
H   M   M   M   K   V   T   Q   Y   K   T   G   K   A   S   L   Y   A   Q   G

AAG CGC CGC TAC GAC CGC AAG CAG TCG GGT TAC GGT GGT CAG ACC AAG CCC GTC TTC CAC  182
K   R   R   Y   D   R   K   Q   S   G   Y   G   G   Q   T   K   P   V   F   H

AAG AAG GCC AAG ACC ACC AAG AAG ATC GTG CTG CGC ATG CAG TGC CAA GAG TGC AAG CAG  242
K   K   A   K   T   T   K   K   I   V   L   R   M   Q   C   Q   E   C   K   Q

ACC TGC ATG AAG GGC CTG AAG CGC TGC AAG CAC TTC GAG ATC GGT GGT GAC AAG AAG AAG  302
T   C   M   K   G   L   K   R   C   K   H   F   E   I   G   G   D   K   K   K

GGC AAC TAA ACGTGGCGCC GTACCGGCGG TTAGG   336
G   N   *
```

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.

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	A		C		G		U	
AA	Lys	0	Asn	2	Lys	23	Asn	0
AC	Thr	0	Thr	6	Thr	0	Thr	1
AG	Arg	0	Ser	0	Arg	0	Ser	0
AU	Ile	0	Ile	2	Met	6	Ile	0
CA	Gln	1	His	3	Gln	6	His	0
CC	Pro	0	Pro	1	Pro	0	Pro	1
CG	Arg	0	Arg	5	Arg	1	Arg	0
CU	Leu	0	Leu	1	Leu	2	Leu	0
GA	Glu	0	Asp	2	Glu	2	Asp	0
GC	Ala	0	Ala	2	Ala	2	Ala	0
GG	Gly	0	Gly	4	Gly	1	Gly	5
GU	Val	0	Val	2	Val	2	Val	1
UA	STOP	1	Tyr	4	STOP	0	tyr	0
UC	Ser	0	Ser	1	Ser	1	Ser	0
UG	STOP	0	Cys	6	Trp	0	Cys	0
UU	Leu	0	Phe	3	Leu	0	Phe	0

Table 5.1 Codon usage in the *Chlamydomonas reinhardtii RPL*41 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 pH10.3 and will therefore have a net positive charge at physiological pH (+25.2 at pH7.0).

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 *Pst*I digested DNA reveals two, low molecular weight bands.

```
                      1                                                     55

C. reinhardtii   MVNVPKTKRAFCKG..CKKHMMMKVTQYKTGKASLYAQGKRRYDRKQSGYGGQTK
       human     MVNVPKTRRTFCKK..CGKHQPHKVTQYKKGKDSLYAQGRRRYDRKQSGYGGQTK
         rat     MVNVPKTRRTFCKK..CGKHQPHKVTQYKKGKDSLYAQGKRRYDRKQSGYGGQTK
   T. brucei     MVNYPKKKKMHCPDERCNAHKSFKVVQYKAGKARLYARGKRRYDRKQSGYGGQTK
S. cerevisiae    MVNVPKTRKTYCKGKTCRKHTQHKVTQYKAGKASLFAQGKRRYDRKQSGFGGQTK
P. gilliermondii MVNVPKTRRTYCKGKDCRKHTQHKVTQYKAGKASLFAQGKRRYDRKQSGFGGQTK
   C. maltosa     MVNIPKTRNTYCKGKGCRKHTIHKVTQYKSGRASLFAQGKRRYDRKQSGYGGQTK
  C. tropicalis   MVNIPKTRNTYCKGKGCRKHTIHKVTQYKAGRASLFAQGKRRYDRKQSGYGGQTK
   K. fragilis    MVNVPKTRKTYCKGKACRKHSQHKVTQYKAGKASLYAQGKRRYDRKQSGFGGQTK
    K. lactis     MVNVPKTRKTYCKGKECRKHAQHKVTQYKAGKASLYAQGKRRYDRKQSGFGGQTK
Sw. occidentalis MVNVPKTRKTYCKGKECRKHTQHKVTQYKAGKASLFAQGKRRYDRKQRGYGGQTK


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C. reinhardtii   PVFHKKAKTTKKIVLRMQCQE..CKQTCMKGLKRCKHFEIGGDKKKGN.....
       human     PIFRKKAKTTKKIVLRLECVEPNCRSKRMLAIKRCKHFELGGDKKRKGQVIQF
         rat     PIFRKKAKTTKKIVLRLECVEPNCRSKRMLAIKRCKHFELGGDKKRKGQVIQF
   T. brucei     PIFHKKAKTTKKIVLKLQC..SNCKSIIQNVLKRTKHFELNDKKKTGNKDPTW
S. cerevisiae    PVFHKKAKTTKKVVLRLECV..KCKTRAQLTLKRCKHFELGGEKKQKGQALQF
P. gilliermondii PVFHKKAKTTKKVVLRLECV..VCKTKAQLSLKRCKHFELGGDKKQKGQA...
   C. maltosa     QVFHKKAKTTKKIVLKLECT..VCKTKKQLPLKRCKHIELGGEKKQKGQALQF
  C. tropicalis   QVFHKKAKTTKKIVLKLECT..VCKTKKQLPLKRCKHIELGGEKKQKGQALQF
   K. fragilis    QIFHKKAKTTKKVVLRLECM..SCKTKTQLALKRCKHFELGGEKKQKGQALQF
    K. lactis     QIFHKKAKTTKKVVLRLECM..SCKTKTQLALKRCKHFELGGEKKQKGQALQF
Sw. occidentalis QIFHKKAKTTKKVVLKLECV..VCKTKAQLSLKRCKHFELGGDKKQKGQALQF
                      *
```

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.
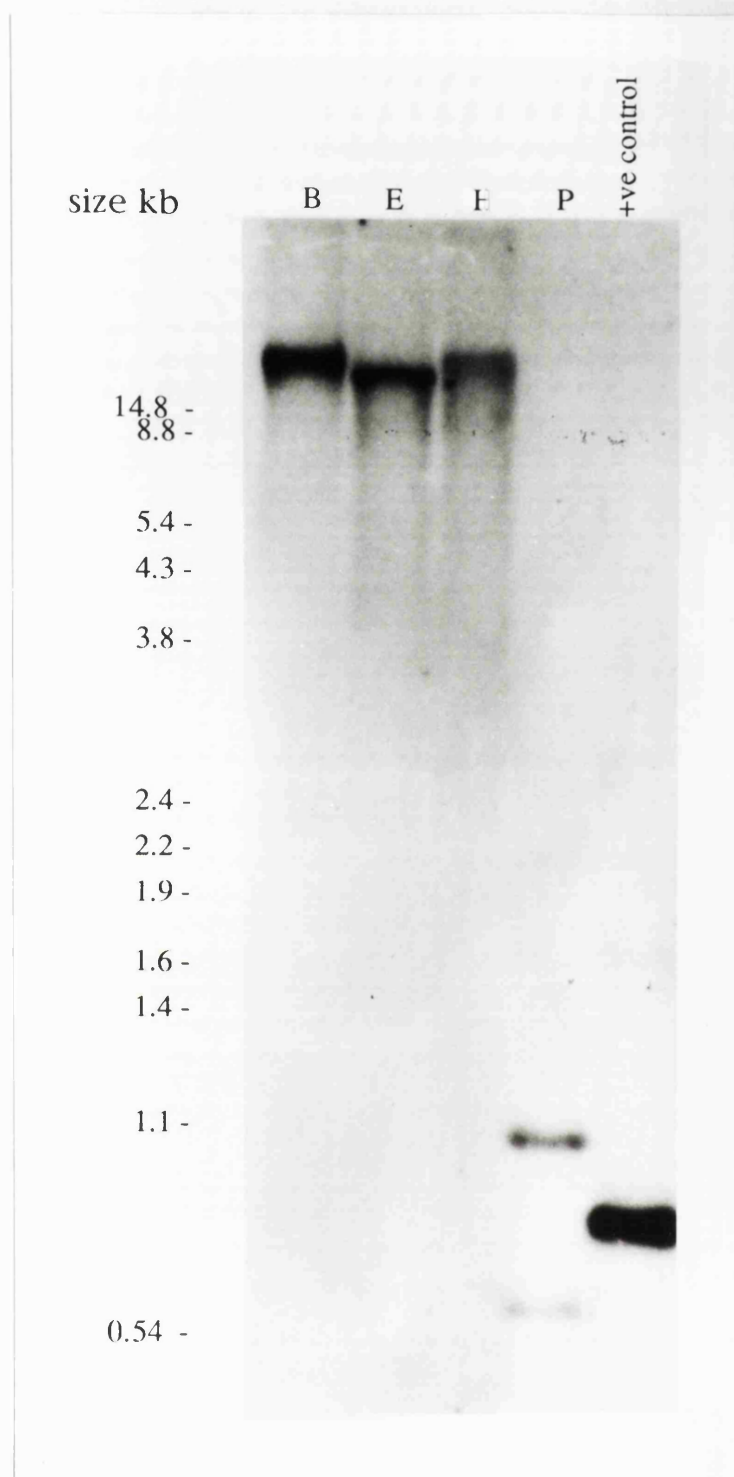
Figure 5.5 Southern analysis of the *RPL*41 gene from wild type *Chlamydomonas reinhardtii* cells. DNA was extracted from wild type cells and digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H) and *Pst*I (P) and hybridised with a probe specific for *RPL*41 synthesised from a PCR product generated using the L41#3 and λgt10 reverse primers. The positive (+ve) control is the *Eco*RI insert from λgt10#7 clone and the sizes of markers are in kilobase pairs.

Nucleotide sequence analysis of the region covered by the probe reveals that no *Pst*I 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 Cyh^R 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* Cyh^R 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 Cyh^R phenotypes attempts were made to clone this region of *RPL*41 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
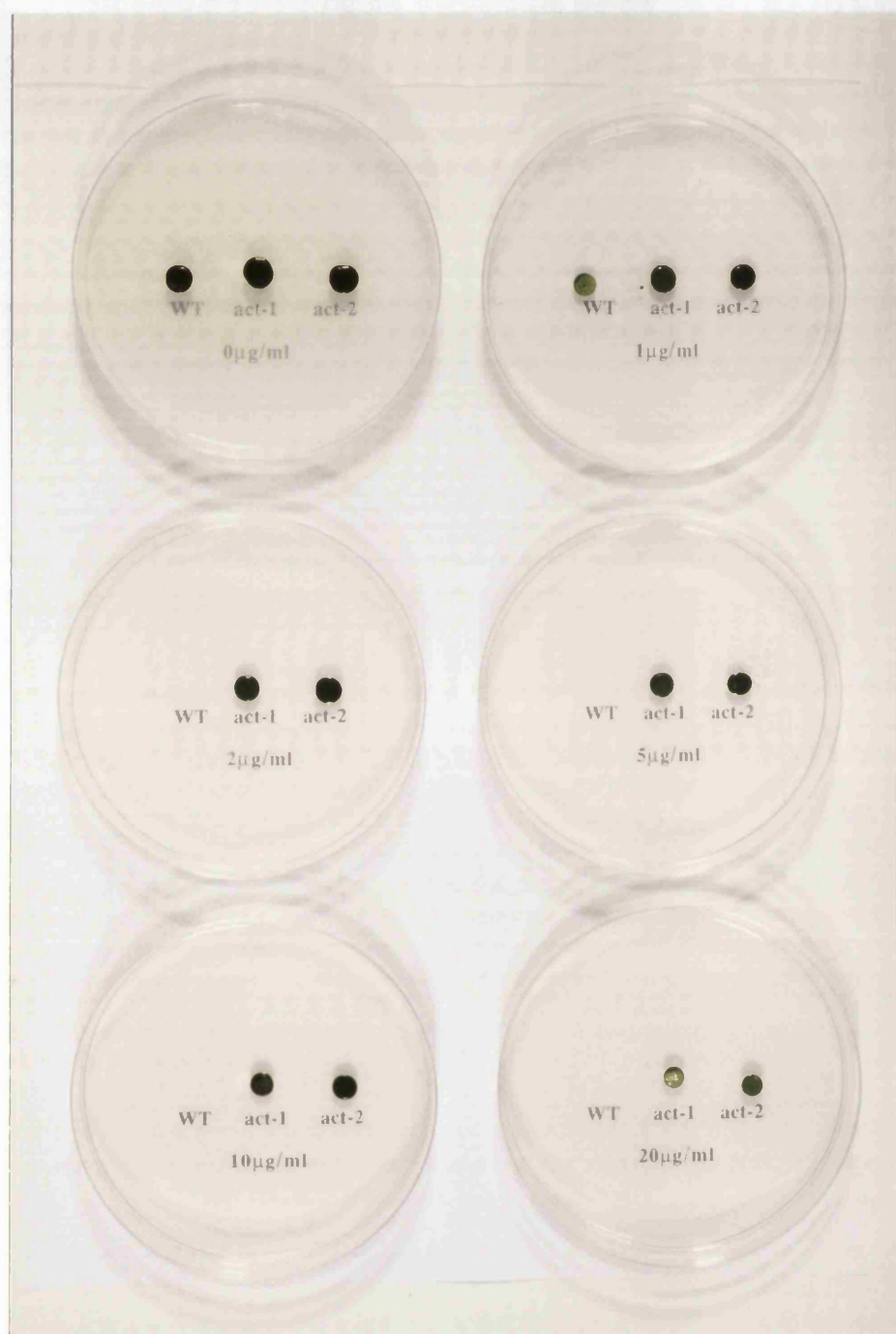
Figure 5.6 Sensitivity of wild type (WT), *act*-1 and *act*-2 strains of *Chlamydomonas reinhardtii* to cycloheximide. Cultures of each strain were spotted to TAP-agar plates supplemented with cycloheximide at the concentrations indicated and allowed to grow at 25^{0}C in the light for seven days.

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.

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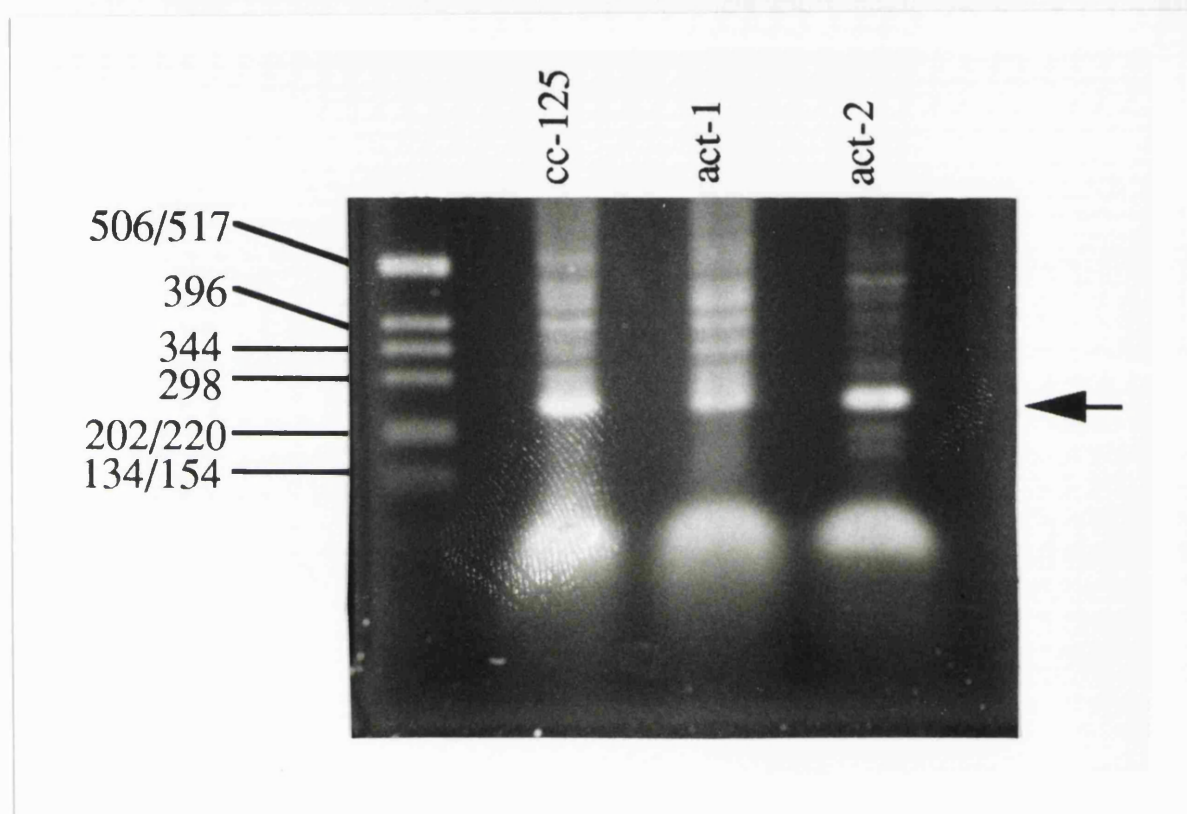
Figure 5.7 Amplification of a fragment of the *RPL*41 gene from wild type (CC-125), *act*-1 and *act*-2 DNA by PCR using the L415'Δ and L413' primers. The PCR products were separated on a 2% agarose gel. The major product is indicated with an arrow and the sizes of markers are given in base pairs.

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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 *RPL*41 sequence. Given that it has been demonstrated that by using this method it is possible to amplify the region of interest of *RPL*41 from genomic DNA, it should now be possible to clone and sequence this fragment from both *act*-1 and *act*-2.
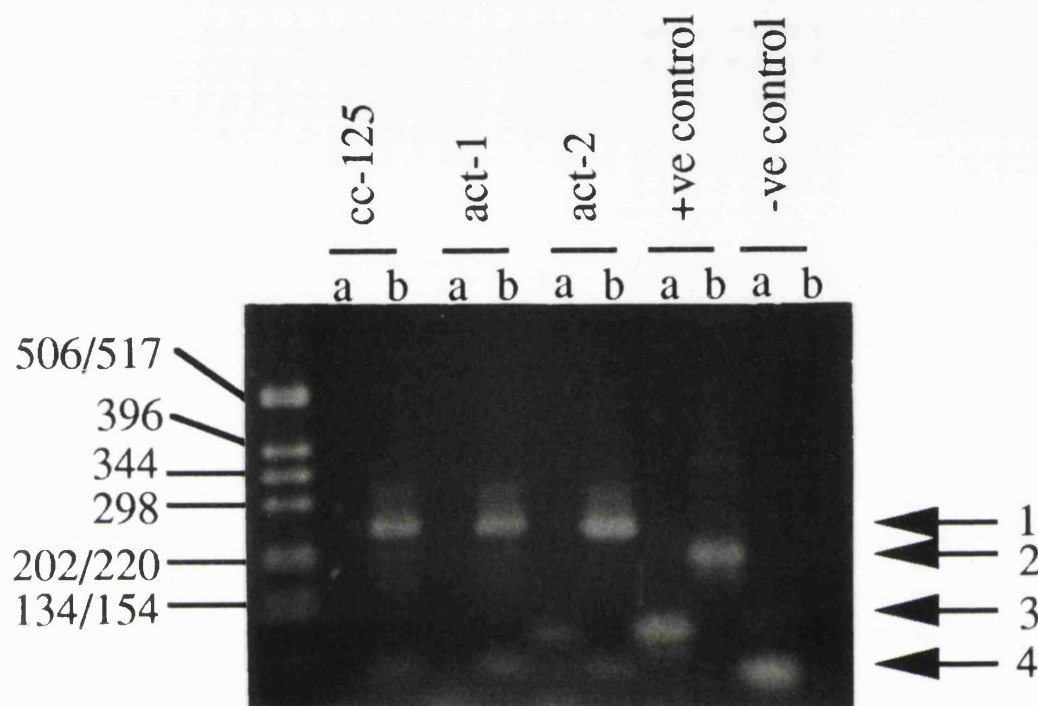
Figure 5.8 Diagnostic PCR screening of the *RPL*41 genomic PCR products from figure 5.7. PCR reactions (a) were performed using L415'Δ and L413' primers. 'Nested' PCR reactions (b) were performed using L41#3 and L413' primers. In addition; positive (+ve) control (pKSL41#7) and negative (-ve) control (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 *Eco*RI 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.

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The sequence of the *RPL*41 coding region is however typical of *C. reinhardtii* nuclear genes in so much as it has a high GC content (57.5%) - *C. reinhardtii* nuclear genome GC content (61.6%) (Jarvis *et al.* 1992). This high GC content is reflected in a codon usage that is typical of *C. reinhardtii* nuclear genes (Sharpe & Day, 1993).

The predicted amino acid sequence of the *C. reinhardtii* L41 ribosomal protein gene (figure 5.3) shows a high level of similarity to other L41 proteins (*e.g.*72% amino acid identity with the L41 subunit of| *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 *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 *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^S phenotype of WT *C. reinhardtii* cells (this residue is present as a proline in Cyh^S organisms but as a glutamine in Cyh^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^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 *Pst*I 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 *Pst*I 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 *RPL*41 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 Cyh^R phenotype of either of these strains.

The work presented in this chapter represents the initial stages of the development of a marker based on Cyh^R for the nuclear transformation of *C. reinhardtii*. To construct a marker based on an altered copy of the L41 ribosomal protein a copy of *RPL*41 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

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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 Pm[R]

transformants. Analysis of PmS and PmR 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 PmR and PmS 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 *RBCS2* promoter elements contained within the pSP108 construct are insufficient in themselves to drive transcription of the *ble* gene and that those copies of the *ble* gene that are expressed have fortuitously integrated close to an enhancer or other *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 *RBCS2* promoter has been used to efficiently express endogenous *Chlamydomonas* genes reintroduced in transformation (*e.g.* Kozminski, 1993) would suggest that all *cis* acting elements necessary for expression are present. To investigate the possibility that too little of the cloned and available RBCS2 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 *ble* gene an element identified as a scaffold attachment region from pea (Pwee & Gray, 1993) was included on the *RBCS2/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 *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 *OEE*1 gene (Mayfield *et al.*, 1989) linked to *RBCS2* controlling elements fails to rescue an *oee*1- 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

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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 *OEE*1 gene. *OEE*1 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 *oee*1- strain to photosynthetic growth (Mayfield & Kindle, 1990). Using established molecular biology techniques the coding region of the *OEE*1 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 *oee*1- 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 *nit*1- cells had a marked beneficial effect. Purton & Rochaix (1995) however found that including 5% PEG in transformations with *ARG*7 did not alter transformation rates. Work presented here demonstrates that whilst transformation efficiency when using cw_d

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 RBCS2/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 ARG7 (Debuchy et al., 1989) and NIT1 (Kindle et al., 1989) and, unlike CRY1-1 (Nelson et al., 1994), the marker is truly dominant (not co-dominant). In contrast to resistance to emetine and cryptopleurine, spontaneous mutants to Pm^R have not been observed and, furthermore, the protocol for transformation with ble is somewhat simpler than that employed with CRY1-1. Given that the use of autolysin renders walled cells susceptible to transformation by the glass bead method (Kindle, 1990) the RBCS2/ble construct should be suitable for transforming any C. reinhardtii strain.

The ble marker also benefits from its small size, being markedly smaller than those which are presently used: 9 kb for NIT1 (Nelson et al., 1994); 7.6 kb for ARG7 (Purton & Rochaix, 1995) and 4.5 kb for RBCS2/CRY1-1 (Nelson et al., 1994). The RBCS2/ble construct is portable as a 1.4 kb EcoRI cassette or as a slightly larger 1.7 kb 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 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 *Eco*RI 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[S] strain of *S. cerivisiae* with a resistant copy of the gene resulted in the generation of Cyh[R] transformants (Kawai *et al.*, 1992). The sequence of *RPL*41 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[S] phenotype of wild type *C. reinhardtii* cells. There are however Cyh[R] 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 basisof 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 α-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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