The Development of Transgenic Aequorin as an Indicator for Cytosolic Free Calcium in *Neurospora crassa*

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Doctor of Philosophy
University of Edinburgh
1996

This thesis has been composed by myself. The research of which it is a record, was carried out by me. All sources of information have been acknowledged by means of reference.

I dedicate this thesis to my parents and grandparents.
I could not have achieved it without them.

Acknowledgements

I am especially grateful to Prof. J.R.S. Fincham F.R.S., third supervisor from 1993 onwards, for his guidance and support, and reading of this thesis.

Thanks to:

Dr. A. Hudson and members of his research group, for the use of equipment, and advice.

Tony Rednall, for friendship and helping to keep the 'wolf from the door.'

Dr. N. Read and Prof. A.J. Trewavas for giving me the opportunity to pursue this course of research.

Other 'aequorin workers' for their helpful advice.

Tony Collins, for fashion advice and help with computing.

Matt Turmaine, for love and support.

My friends in Edinburgh: Barbara, Marianne and Nikki; also Matt and Nikki for appearing unexpectedly!

'Wee Newty' for support.

For accompanying me through the long, dark hours in the luminometer room, and beyond: especially Kate Bush and The Shamen; also Avebury, Delirium, Galliano, Massive Attack, Megadog, Moby, Michael Nyman, Paul Oakenfold, Pulp, Pure, Orbital, Ozric Tentacles, Portishead, J.R.R. Tolkien and The Tor.

Finally in the words of Dave Clarke: "If your not on this list you're either meant to be or not meant to be (your conscience will tell you...)."

Abstract

The aim of this project was to develop techniques for the measurement of cytosolic free calcium ($[Ca^{2+}]_c$) in *Neurospora crassa*, using the Ca^{2+}-sensitive photoprotein aequorin. Aequorin is a tightly bound complex of apoaequorin (apoprotein), coelenterazine (luminophore) and oxygen. On binding Ca^{2+} aequorin is discharged emitting light (λ_{max}=465) which is proportional to the concentration of free Ca^{2+}.

Two expression vectors were constructed, in which apoaequorin was under the control of a promoter from either the native malate synthase (pNCAEQ1) or glucose-repressible gene (pNCAEQ3). The plasmids were introduced into the wild type and *Band* mutant of *N. crassa* using either chemical or electro-transformation techniques. Stable transformants were selected on the basis of their resistance to either hygromycin B (pNCAEQ1) or phosphinothricin (pNCAEQ3).

An *in vitro* assay of aequorin luminescence was developed for the screen of apoaequorin yield in primary transformants. The assay confirmed that the apoprotein produced by *N. crassa* was fully functional. The yield of apoaequorin from both transformant series' was low, at best 13 *fg*/µg total soluble protein. Isolation of homokaryotic derivatives from primary transformants improved this level by up to 11-fold, though this was still 32-times lower than that obtained in apoaequorin transformed *Nicotiana plumbaginifolia*.

Possible reasons for poor transgene expression were explored. Southern analysis showed that virtually all transformants contained at least one intact apoaequorin expression cassette. Northern analysis revealed an apoaequorin mRNA which corresponded to the expected

transcript size. Apoaequorin mRNA abundance in *N. crassa* primary transformants was at best approximately 2-fold less than in *N. plumbaginifolia,* this was not reflected in the protein yield which was almost 400-fold lower. Northern analysis confirmed that the block acting on apoaequorin yield must be at translation or beyond. The codon requirements of apoaequorin and codon usage in a number of apoaequorin transformed species was examined. In general, species which showed a much more compatible codon usage produced more apoaequorin. Apoaequorin was shown to be somewhat unstable in *N. crassa* with a half life of approximately 45 minutes, which may be a contributory factor to the low yield.

Methods were developed whereby the transformed strains were able to report changes in their $[Ca^{2+}]_c$. Both apoaequorin and coelenterazine were shown to be non-toxic, and aequorin was regenerated in cells simply by incubation with coelenterazine. Background sources of luminescence were identified and methods for the lysis of *N. crassa* cells were developed. Lysis is a prerequisite for the calibration of Ca^{2+} concentration from aequorin light emission.

In spite of the very low levels of aequorin present in *N. crassa,* a clear increase in $[Ca^{2+}]_c$ was detected by light emission from aequorin-containing cells on treatment with exogenous Ca^{2+}.

Abbreviations

Standard SI units (International System of Units) were used throughout this thesis. Non-SI units and abbreviations are listed below:

ADP	adenosine diphosphate
approx.	approximately
APS	ammonium persulphate
AR	aequorin constitution (reconstitution)
ATP	adenosine triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
$[Ca^{2+}]_c$	cytosolic free calcium
$[Ca^{2+}]_e$	extracellular free calcium
CaM	calmodulin
CCD	charged coupled device
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate
CHX	cycloheximide
CIAP	calf intestinal alkaline phosphatase
CICR	calcium induced calcium release
CsCl	caesium chloride
cys	cysteine
d.H_2O	distilled water
DEAE	diethylaminoethyl
DG	diacylglycerol
DMSO	dimethyl sulphoxide
d.NTP	deoxynucleotide triphosphate

DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis-(ß-aminoethylether)N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
EtBr	ethidium bromide
EtOH	ethanol
FDA	fluorescein diacetate
G-protein	guanosine triphosphate binding protein
GDH	glutamate dehydrogenase
GFP	green fluorescent protein
h	hour or hours
HEWL	hen egg white lysozyme
his	histidine
HPLC H_2O	high power liquid chromatography purifed water
HRP	horse radish peroxidase
hyg	hygromycin B
IP_3	inositol-1,4,5-trisphosphate
IP_4	inositol-1,3,4,5-tetraphosphate
IPTG	isopropyl ß-D-thiogalactoside
kb	kilo-base pairs
kcal	kilo-calorie
kD	kilo-Daltons
LB	luria-bertani
mcs	multiple cloning site
MeOH	methanol
min	minute or minutes
MOPS	3-[N-morpholino]propanesulfonic acid
mol	mole
mRNA	messenger ribonucleic acid

msec	millisecond or milliseconds
m.wt	molecular weight
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced
NMR	nuclear magnetic resonance
NP40	octylphenoxy polyethoxy ethanol
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	propidium iodide
PIP2	phosphatidylinositol-4,5-bisphosphate
PK	protein kinase
PLC	phospholipase C
Ponceau S	3-hydroxy-4-[2-sulfo-4(sulfo-phenylazo)phenylazo]-2,7 naphthalenedisulfonic acid
PPT	phosphinothricin
RE	restriction endonuclease
RNA	ribonucleic acid
RNA-ase	ribonuclease
RIP	repeat induced point mutation
rpm	revolution per minute
sec	second or seconds
s.d.H_2O	sterile distilled water
SDS-PAGE	sodium dodecyl sulphate - polyacylamide gel electrophoresis
TEMED	N,N,N',N-tetramethylethylenediamine
tRNA	transfer ribonucleic acid
tsp	total soluble protein
TFP	trifluoperazine
trp	tryptophan
Tween 20	polyoxyethylene sorbitan monolaurate
UV	ultra-violet

VS	1 x Vogels medium N + 2% sucrose
VSH	1 x Vogels medium N + 2% sucrose + 200 µg/ ml hygromycin B
v/v	volume per volume
W5	N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide
W7	N-(4-aminohexyl)-5-chloro-1-naphthalenesulfonamide
W13	N-(4-aminobutyl)-5-chloro-2-naphthalene-sulfonamide
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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Chapter One

1. Review of the Literature

1.1 The role of Ca^{2+}as a second messenger

It is now generally accepted that cytosolic free calcium ($[Ca^{2+}]_c$) forms a critical link in the stimulus-response coupling of numerous physiological processes in eukaryotic systems. Changes in $[Ca^{2+}]_c$ transduce information from primary signals received at the plasma membrane into specific intracellular responses (Campbell, 1983). Three second messenger pathways have been identified in eukaryotic systems: the cyclic-adenosine monophosphate (c-AMP) pathway, the cyclic-guanosine monophosphate (c-GMP) pathway and the Ca^{2+}-pathway. In the Ca^{2+}-pathway $[Ca^{2+}]_c$ is central but at least two other second messengers are involved. Evidence for all of these second messengers has been found in filamentous fungi (Gadd, 1994).

The properties of the calcium ion which predisposed it to evolution as a universal second messenger are cytotoxicity, abundance and flexible atomic structure (Campbell, 1983; Hepler and Wayne, 1985). Due to the reaction of Ca^{2+} with phosphate, $[Ca^{2+}]_c$ must be maintained at a low concentration (i.e. around 100 nM), and this leads to a high concentration gradient across the plasma membrane which shows a low permeability for it. The atomic structure of Ca^{2+} allows flexibility in the formation of co-ordinate bonds of different lengths, thus permitting the design of ligands with a much higher affinity for Ca^{2+}, as opposed to its antagonist Mg^{2+}.

The Ca^{2+} pathway has been studied intensively, predominantly in mammalian systems. Studies in filamentous fungi are few in comparison, but both Ca^{2+}-regulated responses (table 1.1) and a number of components from the Ca^{2+} pathway (table 1.2) have been identified.

Resting levels of $[Ca^{2+}]_c$ are generally in the range of 100-200 nM. On stimulation this may rise to greater than 300-400 nM, and in specific cases to supra micromolar levels (Schroeder and Thuleau, 1991). Due to the toxicity of Ca^{2+}, the cytosol can only tolerate these levels for brief periods so transient increases are co-ordinated by tightly regulated mechanisms of influx (i.e. Ca^{2+} channels), efflux (i.e. Ca^{2+}- ATP-ase, Ca^{2+}-Na^+ antiport, Ca^{2+}-H^+-antiport) and binding to intracellular elements. It is the Ca^{2+} signal itself which modulates cell activity by direct enzyme activation or by binding to effector proteins (e.g. calmodulin). However, the form of this signal will be very much dependent on the other intracellular messengers of the Ca^{2+}pathway: inositol-1,4,5-trisphosphate (IP_3), inositol 1,3,4,5 tetraphosphate (IP_4), and diacylglycerol (DG), as well as $[Ca^{2+}]_c$ itself (Berridge, 1987). The generation of these messengers and their regulative properties is described.

The Ca^{2+}pathway (Fig. 1.1) is initiated when an agonist binds to a plasma membrane spanning GTP-binding protein (G-protein)-linked or tyrosine kinase-linked receptor. A conformational change following agonist binding to a G-protein-linked receptor is responsible for activating the G-protein which dissociates into the subunits G_α and $G_{\beta\gamma}$, both of which activate isozymes of the enzyme phospholipase C (PLC). PLC catalyses the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) yielding IP_3 and DG. Activated tyrosine kinase linked-receptors, interact directly with another isozyme of PLC. This type of receptor has yet to be identified in filamentous fungi.

Table 1.1 : Responses shown to be regulated by Ca^{2+} or Ca^{2+} /calmodulin (CaM) in fungi. The abbreviations used in column three are explained in the notes at table end.

Organism	Response	Evidence for Ca^{2+} or Ca^{2+} /calmodulin	Reference
OOMYCOTA			
Achyla ambisexualis	Asexual spore release	Inhibited by trifluoroperazine (TFP)	Suryanarayana and Thomas, 1986
Dictyuchus monosporus	Asexual spore release	Inhibited by TFP	Suryanarayana and Thomas, 1986
Phytophthora cactorum	Sporangium and oospore production	Inhibited by TFP	Elliot, 1986
P. cinnamoni	Cytokinesis in zoosporangia	Blocked by BAPTA	Jackson and Hardham, 1996
P. infestans	Germ tube growth	Low conc. A23187 stimulatory, high inhibitory	Temperli *et al.*, 1991
P. palmivora	Sporangium production	Inhibited by TFP and EDTA	Elliot, 1986
	Zoospore differentiation	Requirement for external Ca^{2+}	Griffith et al., 1988
Pythium sp	Zoospore adhesion and germination	Suppressed by a range of Ca^{2+} modulators and CaM inhibitors	Deacon and Donaldson, 1993
	Zoospore motility	Swimming pattern modified by a range of Ca^{2+} modulators and CaM inhibitors	Donaldson and Deacon, 1993
Saprolegnia ferax	Hyphal tip growth	Requirement for external Ca^{2+}	Jackson and Heath, 1989; Garrill *et al.*, 1993
	Asexual spore release	Inhibited by Gd^{3+}	Suryanarayana and Thomas 1986
	Establishment of growth axis	Inhibited by TFP	Hyde and Heath, 1995
ZYGOMYYCOTA			
Basidiobolus magus	Polarised cytoplasmic movement	Requirement for external Ca^{2+}	McKerracher and Heath, 1986
Phycomyces blakesleanus	Chitin synthase activation	Inhibited by TFP and EDTA	Martinez-Cadena and Ruiz-Herrera, 1987
Phycomyces sp.	Bending of sporangiophores	$LaCl_3$ and EGTA exert an inhibitory effect	Sineshchekov and Lipson, 1992
Zoophthora radicans	Appressorium formation	Inhibited by Nd^{3+}, nifedipine, TFP, W7, W5	Magalhaes *et al.*, 1991
BASIDIOMYCOTA			
Rhodosporidium toruloides	Pheromone induced differentiation	Efficiency of reception dependent on external Ca^{2+}	Miyakama *et al.*, 1985
Coprinus cinereus	Transduction of gravitropic impulse	Perception effected various Ca^{2+} modulators	Frazer and Moore, 1993

Table 1.1 continued:

Organism	Response	Evidence for Ca^{2+} or Ca^{2+}/calmodulin	Reference
ASCOMYCOTA			
Aspergillus nidulans	Cell cycle	Ca^{2+} /CaM required	Lu *et al.*, 1992, 1993
Ceratocystis ulmi	Fungal dimorphism	Ca^{2+} /CaM interaction required for yeast to mycelium switch	Muthukumar and Nickerson , 1984
Neurospora crassa	Apical growth	Decrease in $[Ca^{2+}]_c$ loss of apical dominance	Schmid and Harold, 1988
N.crassa	Circadian conidiation rhythm	Phase shifted by Ca^{2+} A23187, TFP, W7, W13, chlorpromazine,	Nakashima, 1984 and 1986
Saccharomyces cerevisiae	Cell Cycle	Inhibited by TFP, events dependent on time of TFP addition	Eilam and Chernichovsky, 1988
DEUTEROMYCETES			
Candida albicans	Germ tube growth	Inhibited by A23187. Protein phosphorylation induced by Ca^{2+}	Paranjape *et al.*, 1990
Fusarium graminearum	Hyphal extension	Low external Ca^{2+} induces increased braching and irregular morphology	Robson *et al.*, 1991
Metarhizium anisopliae	Conidial germination	A23187 caused loss of germ tube apical dominance, increasing branching	St Leger *et al.*, 1990
Penicillium species	Induction of conidiation	Requirement for external Ca^{2+}	Pitt and Ugalde, 1984
P. notatum		Calmidazolium blocked sporulation when added before Ca^{2+}	Pitt and Barnes, 1993
Sporothrix schenckii	Conidial germination	Inhibited by A23187	Rivera-Rodriguez and Rodrigues-del Valle, 1992

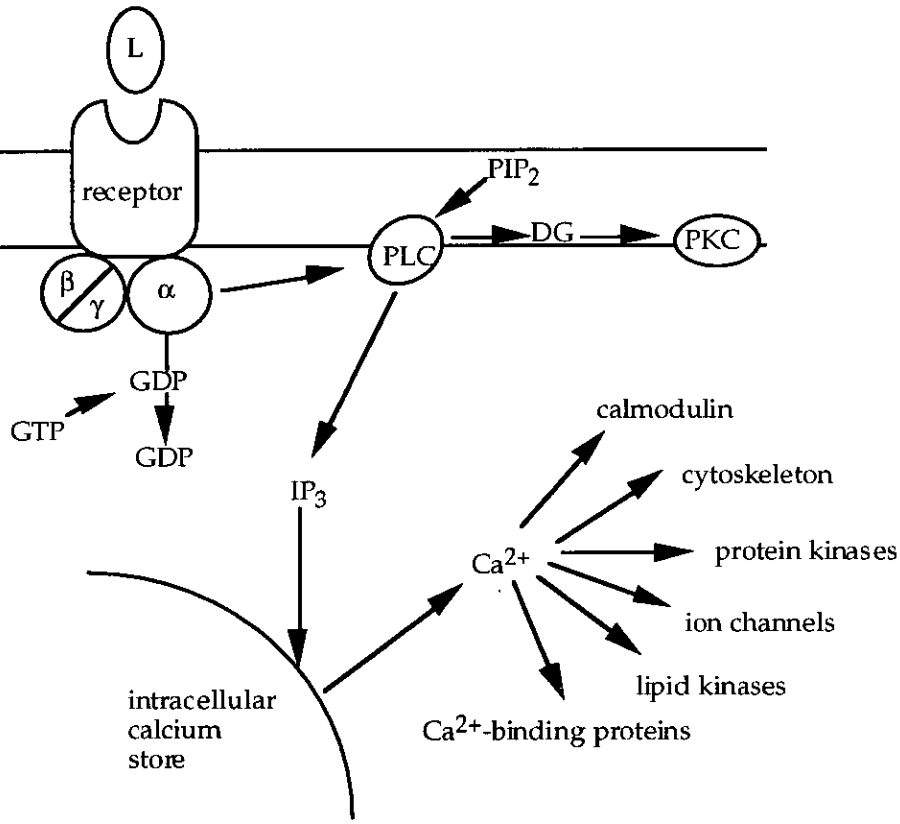
Notes:
TFP (trifluoroperazine), calmidazolium, W5, W7, W13 are all calmodulin antagonists
BAPTA, EGTA and EDTA are all Ca^{2+} chelators
A23187 is a Ca^{2+} ionophore
Gd^{3+} (gadolinium) blocks stretch activated ion channels
$LaCl^{3+}$ (lanthanium chloride), Nd^{3+} (neodynium), and nifedipine all block Ca^{2+} channels

Table 1.2: Components of the Ca^{2+} signal transduction pathway identified in the filamentous fungus *Neurospora crassa*.

Component	Evidence	Reference
Plasma membrane receptor	66 kD insulin-binding protein	Kole *et al.*, 1991
Low resting $[Ca^{2+}]_c$	Mean $[Ca^{2+}]_c$ = 92 +/-15 nM	Miller *et al.*, 1990
Phosphatidylinositol-4-5-bisphosphate(PIP_2)	Lithium-induced decrease correlated with abnormal morphology	Hanson, 1991
Inositol-1,4,5-trisphosphate (IP_3)	Lithium-induced decrease correlated with abnormal morphology	Hanson, 1991
IP_3 -regulated Ca^{2+} channels	In vacuole membrane, Ca^{2+} efflux K_m=5.3 μM. Channel inhibited by dantrolene. IP_3 released approximately 37% of Ca^{2+} from the vacuole	Cornelius *et al.*, 1989
H^+ / Ca^{2+} antiport	Preliminary evidence from uncoupling studies.	Miller *et al.*, 1990
Calmodulin	Isolation 149 amino acids, 85% homology to human CaM Single copy gene	Cox, 1982 Melnick *et al.*, 1993 Capelli *et al.*, 1993
Protein kinase C	Partial purification	Favre and Turian, 1993
Other protein kinases	3 PK peaks resolved by DEAE cellulose column chromatography PKII activity stimulated by Ca^{2+} and CaM	Ulloa *et al.*, 1991
Protein phosphatases	CaM dependent, 75 % homology with mammalian calcineurin Activity of 3 serine/ threonine protein phosphatases characterised	Higuchi *et al.*, 1991 Zapella *et al.*, 1996
Ca^{2+} /CaM activated proteins	3 microtubule-associated proteins which bind CaM	Perez *et al.*, 1994
Further downstream	Central regulator of blue light responses is a zinc finger protein	Ballario *et al.*, 1996

Notes:
Lithium affects the phosphatidylinositol cycle
Dantrolene is an intracellular Ca^{2+} channel blocker
PK denotes protein kinase; CaM denotes calmodulin

Figure 1.1: Simplified diagram of the Ca^{2+} pathway, in which Ca^{2+} is released from intracellular stores (based on Gadd, 1994). L, ligand; G-protein composed of G_α and $G_{\beta\gamma}$ subunits, G_α exchanges bound GDP for GTP before interacting with PLC; PLC, phospholipase C; PIP_2, phoshatidylinositol-4,5-bisphosphate; DG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C.



IP_3 is water soluble and passes into the cytosol where it initiates the release of Ca^{2+} from stores sensitive to it. DG remains in the plasma membrane where it may undergo hydrolysis to phosphatidic acid by DG kinase or cleavage by DG ligase releasing arachidonic acid (another intracellular messenger). Alternatively, DG may bind to the serine/ threonine phosphorylating enzyme protein kinase C (PKC), thereby activating the PKC branch of the Ca^{2+} pathway. The two branches of the Ca^{2+} pathway directed by IP_3 and DG can act independently but also exhibit

a high degree of co-operative regulation, both being required for the full expression of a response (Berridge, 1984, 1987).

In the cytosol, IP_3 triggers the rapid release of Ca^{2+} from those Ca^{2+} stores which are sensitive to it. This is facilitated by Ca^{2+} channels present in certain endomembranes which are gated by IP_3. In animals these are located in the endoplasmic reticulum (ER) membrane; in plants and fungi they are in the tonoplast (Alexandre *et al*, 1990; Johannes *et al*, 1991; Cornelius *et al.*, 1989). The IP_3 receptor of the ER is also the Ca^{2+} channel and Ca^{2+} acts as a co-agonist. A family of IP_3-gated Ca^{2+} channels have now been characterised and channels show variation in the number of molecules required for activation (one to four)(Berridge, 1993). In general, IP_3 receptors possess a large N-terminal domain which lies free in the cytoplasm and, has a C-terminal TXCFICG motif which is characteristic of Ca^{2+} channels. The IP_3 receptor does not become desensitised to IP_3 but switches to a low affinity state for its co-agonist Ca^{2+} at concentrations above approximately 300 nM (Berridge, 1993). Iino and Endo (1992) showed that by releasing caged IP_3 or caged Ca^{2+} into the cytoplasm Ca^{2+} exerted first positive and then negative feedback during the rising phase of a Ca^{2+} spike, thereby generating a temporally abrupt Ca^{2+} transient. Transients in $[Ca^{2+}]_c$ were observed in *S. cerevisiae* in response to the addition of glucose to starved cells (Nakajima-Shimada *et al.*, 1991b). Refilling of the IP_3 sensitive pool is thought to occur by a capacitive mechanism whereby the Ca^{2+} content of the ER pool determines Ca^{2+} influx across the plasma membrane (Putney, 1986; Berridge, 1995). This hypothesis requires that information is transmitted across the gap separating the ER from the plasma membrane. Suggested means of relaying this information have included G-proteins, an unidentified Ca^{2+} influx factor and models based on protein-protein interactions such as protein phosphorylation and conformational coupling (Berridge, 1995).

While all the mechanisms may contribute to the refilling of the ER, recent evidence has suggested that conformational coupling is central. It has been proposed that the large N-terminal head of the IP_3 receptor transmits information directly or via the cytoskeleton to appropriate Ca^{2+} channels present in the plasma membrane (Berridge, 1995). This model requires that the ER and plasma membrane are closely associated, cell fractionation (Rossier *et al.*, 1991) and electron microscopy (Chadwick *et al.*, 1992) studies have revealed that this is the case.

An interesting property of IP_3-induced release of Ca^{2+} is its all or nothing response. Releasing low levels of IP_3 into the cytoplasm of *Xenopus* oocytes from its caged precursor resulted in localised small puffs of Ca^{2+} as measured by fluo-3 (a Ca^{2+}-indicator). At higher IP_3 concentrations Ca^{2+} release from local sites formed a focus for the initiation of a propagating Ca^{2+} wave (Parker and Yao, 1991) . A Ca^{2+} wave across a defined area of the cell, which results from crossing a localised threshold for IP_3 may be generated by the two types of Ca^{2+} channel identified, the IP_3 receptor or the receptor for the plant alkaloid ryanodine which has been observed to release Ca^{2+} on the addition of cyclic ADP-ribose (Berridge, 1993). In both cases Ca^{2+} discharge from the stores is mediated by the cytosolic Ca^{2+} concentration and so the wave generating process is termed Ca^{2+}-induced Ca^{2+} release (CICR). Ca^{2+} waves have been visualised in a wide range of cells and variation has been witnessed in their velocity, amplitude and frequencies (Jaffe, 1993).

Oscillations in $[Ca^{2+}]_c$, i.e. repetitive pulses of high Ca^{2+}, have been observed in both animal cell cultures and plants (Berridge, 1990; Johnson *et al*, 1995; McAinsh *et al.*, 1995). They vary in temporal characteristics, requirements for external Ca^{2+} and dependency on agonist concentration. They are mainly derived from intracellular stores as shown by the

persistence of spikes for a few cycles on transfer to media without Ca^{2+} (Tsien and Tsien, 1990). Critical to the sustained generation of oscillations is the regenerative process of CICR coupled with the influx of external Ca^{2+} to replenish the stores. One hypothesis on the maintenance of oscillations is that there is a positive feedback effect of Ca^{2+} on phospholipase C generating pulses of IP_3; this could be coupled with simultaneous refilling of stores by the capacitative mechanism (Berridge, 1993).

It has been observed using both Ca^{2+}-sensitive fluorescent dyes and the Ca^{2+}sensitive photoprotein aequorin, that Ca^{2+} waves are not restricted to individual cells but can be transmitted across entire tissues and organs in a diverse range of organisms (Osipchuk and Cahalan, 1992, Knight *et al*, 1993; Cubitt *et al.*, 1995). In clusters of leukaemia cells, such intercellular Ca^{2+} waves were shown to be generated by ATP secreted into the intercellular space (Osipchuk and Cahalan, 1992). In the multicellular stage of *Dictyostelium*, c-AMP was the suggested transmission signal (Cubitt *et al.*, 1995). In cells which are cytoplasmically joined via plasmodesmata it is possible that $[Ca^{2+}]_c$ or IP_3 could transmit the signal.

Recently it was proposed in mammalian cells that extracellular Ca^{2+} ($[Ca^{2+}]_e$) could act as a primary signal and the cloning of an extracellular Ca^{2+}-sensing receptor supports such an informational role for $[Ca^{2+}]_e$ (Brown *et al.*, 1993; Brown *et al.*, 1995).

As stated previously, elevations in $[Ca^{2+}]_c$ are transient due in part to the binding of Ca^{2+} to intracellular elements. Ca^{2+} may activate specific enzymes, e.g. the Ca^{2+}/phospholipid dependent lipid and protein kinase of *N. crassa* (Turian and Favre, 1990), ion channels and cytoskeletal elements directly, but frequently the Ca^{2+} signal is transduced into the intracellular

response by interaction with a Ca^{2+}-modulated protein. Ca^{2+}-modulated proteins (e.g. calmodulin, troponin-C, parvalbumin) undergo specific conformational changes on binding Ca^{2+} which renders them able to interact with and thereby activate other proteins. Certain members of this family may be involved in Ca^{2+} buffering, translocation and storage (e.g. pavalbumin, calbindin, calreticulin and calsequestrin).

Ca^{2+}-modulated proteins are an evolutionarily ancient group which all bind Ca^{2+} in a similar way. One member, calmodulin (CaM), has been shown to be ubiquitous to all tissues studied. Calmodulin was first detected as an activator of bovine brain cyclic 3',5' nucleotide phosphodiesterase (PDE)(Cheung, 1970). Calmodulin from bovine brain is well characterised: it is composed of 148 amino acids (m.wt=16.7 kD), acidic (pI= 4.3) due to the high content of aspartate and glutamate (>30%), thermally stable, and has a binding affinity for Ca^{2+} in the μM range (Cheung, 1980). The absence of cysteine, hydroxyproline, or tryptophan provides CaM with a highly flexible tertiary structure necessary to interact with its multiple receptor proteins (Cheung, 1980). The amino acid sequence of CaM is highly conserved in eukaryotes with plant CaM's generally showing > 90% homology with bovine brain CaM; fungal CaMs exhibit 60-80% similarity. Homology reflects the essential role of CaM in modulating basic cellular functions. This has been verified experimentally by producing *S. cerevisiae* and *A. nidulans* strains which were conditional for CaM expression (Ohya and Anraku, 1989, Ping Lu *et al.*, 1992). Most Ca^{2+}-modulating proteins possess the characteristic Ca^{2+}-binding domain of 11-12 amino acids in a helix-loop-helix configuration (the EF hand). Four EF hands have been identified in all CaMs with the exception of yeast CaM which possesses three complete hands (Davis *et al.*, 1986). Aspartate and glutamate at positions 1 and 12 in the EF hand are essential for Ca^{2+}-binding and along with four other residues provide the

negatively charged carbonyl groups which are the oxygen donor ligands for Ca^{2+} (Williams, 1986). The two EF hands at the C terminus bind Ca^{2+} tightly (KD = 10^{-6} M) the two N-terminal EF hands show a lower affinity for Ca^{2+} (KD = 10^{-5} M) (Linse *et al.*, 1991). Occupancy of the four Ca^{2+} binding sites is not essential for the activation of certain Ca^{2+}-CaM regulated proteins, indicating that a stepwise conformational change must occur as Ca^{2+} is bound. On binding Ca^{2+}, CaM shows an increase in α-helical content and two hydrophobic patches are exposed which bind specifically to CaM binding domains on a variety of proteins (James *et al.*, 1995). CaM binding domains from structurally and functionally diverse proteins show significant homology (James *et al.*, 1995).

Inactive CaM can bind to various Ca^{2+}-CaM activated proteins and in doing so increases the affinity of CaM for Ca^{2+} (Allen and Hepler, 1989). Ca^{2+}-CaM activated proteins require different parts of the CaM molecule for activation, in general they can be divided into three classes based upon this (Klee *et al*, 1987). The physiological significance of theses observations will be the sensitising of CaM for Ca^{2+}, thereby facilitating protein activation during small $[Ca^{2+}]_c$ transients, and specifying the activation of proteins at different Ca^{2+} concentrations. The ability of a mutant CaM to function without binding Ca^{2+} suggests that CaM may have additional roles in the cell which are Ca^{2+} independent (Geiser *et al*, 1991).

The main target proteins of Ca^{2+}-CaM activation fall broadly into five groups, involved respectively in Ca^{2+} transport, cyclic nucleotide metabolism, the cytoskeleton and, phosphorylation/dephosphorylation. Thus, a decoded Ca^{2+} signal will bring about Ca^{2+} signal termination, influence other second messenger systems and trigger structural and genetic changes in the cell.

All the principal second messenger systems in eukaryotic cells mediate the action of the primary stimulus by modulating the activity of protein kinases and protein phosphatases (Cohen, 1992). Phosphorylation of serine threonine and tyrosine residues by the evolutionary related serine/threonine and tyrosine kinases causes conformational changes in proteins which will alter activity. The relative levels of resistance of these phosphate groups to the action of protein phosphatases will affect the duration of cellular change initiated by protein kinases. The harnessing of protein kinases in sequential cascades of phosphorylation events, allows tremendous amplification of the initial physiological stimulus, thus increasing the sensitivity of the cell to the primary stimulus. These cascades ultimately converge in the nucleus to phosphorylate and regulate the activity of transcription factors (Herschman, 1991).

In summary, the Ca^{2+} pathway possesses a central role in eukaryotic signal transduction. The evolutionary conservation of components coupled with the diversity apparent in individual species suggests that the pathway emerged early on in evolution and was then modified to meet the unique signal transduction requirements of different cell types. Table 1.2 summarises the evidence for the Ca^{2+} pathway presented above, in the filamentous fungus, *Neurospora crassa*. To date, changes in $[Ca^{2+}]_c$ during stimulus response coupling have not been demonstrated in this or any other filamentous fungus. However, the identification of key pathway components (Table 1.2) combined with the proposed role for Ca^{2+} in various responses (Table 1.1) provides good circumstantial evidence for the Ca^{2+} pathway in filamentous fungi.

1.2 Monitoring $[Ca^{2+}]_c$: aequorin as an intracellular Ca^{2+} indicator

The majority of evidence which has accumulated for the role for $[Ca^{2+}]_c$ as a second messenger filamentous fungi has been obtained indirectly using Ca^{2+}-channel blockers, calmodulin antagonists, Ca^{2+} ionophores and Ca^{2+} chelators (see Table 1.1). Information on the specificity of channel blockers and antagonists is limited, whilst data available for Ca^{2+} ionophores and Ca^{2+} chelators shows some worrying unspecific effects (Youatt, 1993). To show definitively that $[Ca^{2+}]_c$ is acting as a second messenger, intracellular indicators which permit the measurement of free Ca^{2+} in living cells are essential. An ideal indicator of $[Ca^{2+}]_c$ should meet the following criteria (Campbell, 1988):

(a) Sensitive to the entire physiological range of $[Ca^{2+}]_c$, pCa 5-8 (pCa $=\log_{10}[Ca^{2+}]$).
(b) High specificity for Ca^{2+}.
(c) Detect $[Ca^{2+}]_c$ transients, which vary in amplitude and duration
(d) Incorporation without significant disturbance to the cell or pertubation of the Ca^{2+} balance.
(e) Not so much diffusion as to affect the signal from discrete $[Ca^{2+}]_c$ transients.
(f) Localise distribution of free Ca^{2+} within the cell.

Only when these requirements are fulfilled can the site of Ca^{2+} activity and compartmentation be defined, and changes in $[Ca^{2+}]_c$ in space and time identified.

There are three classes of $[Ca^{2+}]_c$ indicators: Ca^{2+}-sensitive fluorescent dyes, Ca^{2+}-sensitive microelectrodes and Ca^{2+}-sensitive photoproteins. The Ca^{2+}-dyes are structurally based on the Ca^{2+} chelator BAPTA. On binding Ca^{2+}, dye fluorescence is shifted in wavelength due to the withdrawal of

nitrogen lone pair electrons from the aromatic rings. A combination of the Ca^{2+}-dyes with microscope technologies such as fluorescence ratio imaging or confocal scanning laser microscopy allows subcellular localisation and quantification of $[Ca^{2+}]_c$. The dyes are commonly incorporated into cells by microinjection, electroporation, or as lipophilic acetomethyl esters (Read *et al.*, 1992; Tsien, 1981, Gilroy *et al.*, 1989). The available loading techniques are primarily applicable to individual cells or cell populations, and not to tissues, organs and multicellular organisms. In addition, there are a number of problems which have restricted the use of these dyes, including cytotoxicity, dye photobleaching during prolonged illumination of dye loaded cells, and dye compartmentalisation. In filamentous fungi sequestration of the Ca^{2+}-dyes into organelles has severely limited their application. Sequestration results in a greatly reduced concentration of the dye in the cytosol to levels which prevent accurate $[Ca^{2+}]_c$ measurement. Within the organelles, the Ca^{2+}-dye is saturated due to the high concentration of Ca^{2+} giving maximal fluorescence which confuses $[Ca^{2+}]_c$ measurement (Knight *et al.*, 1993). Preliminary evidence obtained recently in *N.crassa* whereby a 10 kD dextran-conjugated Ca^{2+}-dye was introduced by pressure microinjection and did not show sequestration is promising (S. Fischer, pers. comm.).

A Ca^{2+}-sensitive microelectrode is composed of a thin glass tube drawn to a fine tip (< 1 µm dia) which is filled with a lipophilic cocktail including an ion sensor (e.g. the neutral ligand ETH 1001). The cocktail forms a membrane between the electrode filling and the test solution. Neutral ligands strip the ion of its hydration shell binding it tightly and selectively and thus it is effectively solubilized in this membrane. If the movement of the ion-ligand complex is adequate, a Nernstian potential will develop rapidly across the membrane in accordance with the activity ratio of the ion (Felle, 1989).

Ca^{2+} microelectrodes have been used to measure the resting level of $[Ca^{2+}]_c$ in *N. crassa* (Miller *et al.*, 1990). They have also provided evidence for IP_3 gated receptors on the vacuolar membrane of *N. crassa* (Cornelius *et al.*, 1989) and IP_3-dependent and IP_3-independent Ca^{2+} channels in *C. albicans* (Calvert and Saunders, 1995). The application of Ca^{2+} microelectrodes is limited by the lack of spatial resolution and restriction to single cells studies. In addition, a response time to changes in calcium concentration of several seconds may prevent the detection of rapid $[Ca^{2+}]_c$ transients.

The first application of a Ca^{2+}-sensitive photoprotein to a filamentous fungal system is presented in this thesis. Recent advances in photoprotein biochemistry and molecular biology, and low light level imaging technology have dramatically improved the scope of photoprotein application and the quality of data generated. Ca^{2+}-sensitive photoproteins now provide the best overall fulfilment of the criteria required of an ideal $[Ca^{2+}]_c$ indicator.

Photoprotein is the term used to define a bioluminescent substrate-enzyme system in which the components are tightly or covalently bound together and can be isolated as a whole complex (Shimomura *et al.*, 1962). All bioluminescent systems use chemiluminescence which differs from fluorescence (i.e. Ca^{2+}-dyes) in that the actual light emitter is chemically different from the initial chromophore, and as a consequence of this it can only produce a photon once. The bioluminescent substrate-enzyme system is composed of a luciferin (chemiluminescent substrate), a luciferase (catalyst), and molecular oxygen. In addition, up to three other factors may be required to facilitate the bioluminescent reaction: an energy

yielding metabolite (eg NADH, ATP), a cation (Cu^{2+}, Ca^{2+}, Mg^{2+}) and an energy transfer acceptor (Campbell and Sala-Newby, 1994).

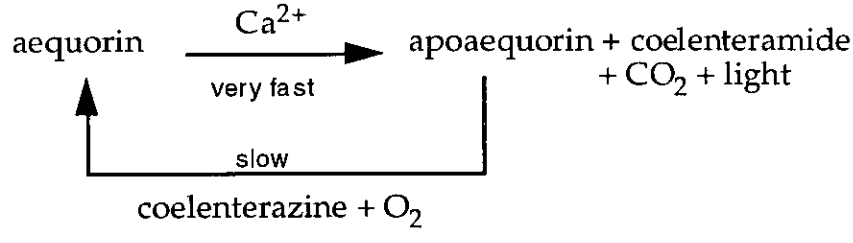
Bioluminescent systems occur in evolutionarily diverse organisms (e.g. fungi, worms, jellyfish and fish) but are most common in marine organisms. In the coelenterates at least five photoproteins have been identified in which the luminesent reaction is triggered by the addition of Ca^{2+}. Aequorin (λ_{max}=465) was the first Ca^{2+}-sensitive photoprotein to be isolated, from the jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962). In *A. victoria* aequorin is distributed in specialised cells (photocytes) present in the circumoral ring of the umbrella. Luminescence is produced from the photocytes on mechanical stimulation and yields a bluish-green light (λ_{max}=508) due to the close association of aequorin with the green fluorescent protein (GFP). GFP is thought to be activated by radiation-less energy transfer from the excited state of the aequorin light emitter (Ward and Cormier, 1979).

A thorough understanding of the components of aequorin has been obtained over the last 30 years. The Ca^{2+}-sensitivity of aequorin led to its immediate recognition as a potential indicator for Ca^{2+} in biological systems (Shimomura *et al.*, 1963). Since its first application in barnacle muscle cells (Ridgeway and Ashley, 1967), aequorin has been used to detect changes in Ca^{2+} in a wide range of systems including mammals (Button and Brownstein, 1993), plants (Williamson and Ashley, 1982; Knight *et al.*, 1991a and b) and yeast (Nakajima-Shimada *et al.*, 1991b).

Aequorin is composed of apoaequorin (the luciferase), coelenterazine (the luciferin) and bound oxygen. Coelenterazine (m.wt 423) is a substituted dihydropyrazinimidazolone ring system, a common substrate in the bioluminescent reactions of marine organisms (Campbell and

Herring, 1990). On binding Ca^{2+} aequorin is converted into the blue fluorescent protein, a readily dissociable complex which contains the light emitter coelenteramide, the product of coelenterazine oxidation (Shimomura and Johnson, 1969; 1973). NMR studies have shown that a singlet oxygen is attached to the C-2 of coelenterazine (Musicki *et al.*, 1986) and the other O is presumed to be joined to an amino acid residue via a peroxide bridge. The energy released as luminescence (60 kcal/mol) obtained by the oxidation of coelenterazine is thought to be generated by the formation of a cyclic oxygen intermediate (dioxetanone). The chemical synthesis of potential intermediates of coelenterazine has recently afforded the first evidence of this (Usami and Isobe, 1995). The observation that active aequorin could be regenerated from spent aequorin by the addition of coelenterazine and molecular oxygen showed that in fact the photoprotein is a very stable intermediate in the bioluminescence reaction (Shimomura and Johnson, 1975). Figure 1.2 summarises the Ca^{2+} regulated discharge of aequorin and its constitution. The extent to which regeneration occurs in *A. victoria* is unclear. Synthesis of coelenterazine occurs in oceanic decapods (Thomson *et al.*, 1995) and it may also be acquired in the diet.

Figure 1.2: Scheme showing the discharge of aequorin by Ca^{2+} and constitution of active aequorin from apoaequorin, coelenterazine and oxygen.



The low level of light emitted by aequorin requires a highly sensitive detector (i.e. which can detect down to one photon per second with low

noise) with a wide sensitivity range (over at least six orders of magnitude). The development of detectors with these characterisitcs has permitted aequorin detection down to as low as 1×10^{-18} g.

Aequorin luminescence can be detected by using either a chemiluminometer (Campbell, 1988) or a photon imaging detector (Campbell, 1988; Miller *et al.*, 1994), the latter has spatial resolution. The basis of a chemiluminometer is; a light tight sample housing in which a sample cuvette can be brought adjacent to the light receiver (photocathode) of a photomultiplier tube. Due to the photoelectric effect, when the photocathode receives a photon from the sample it emits an electron which is focused and electrostatically accelerated to hit the first in a series of dynodes. The first dynode emits several electrons which then hit a second dynode, thus in a series of 13 dynodes there is massive amplification of the original photon before the electrons are collected as a pulse of charge at the anode. This process is fast, usually <100 ns (Campbell, 1988). The pulse is then delivered via a preamplifyer to a discriminator set to select charge over a certain energy level. The acccumulated photon counts can then be displayed using a scaler, chart recorder or computer interface. The blue sensitivity, amplification properties and speed of response makes the chemiluminometer an excellent detector for aequorin which emits in the blue region (λ_{max}=465) with a half rise time of reaction of 8 msec.

Most systems for imaging aequorin (i.e. giving spatial resolution) are based around the charged-coupled devise (CCD). A CCD is a solid state two dimensional array of semiconductors, each of which acts as an individual light dectector. Photons which hit the individual well insulated semiconducters build up a charge, quantification of the charge allows a pattern of light detection to be built up which can be

reconstructed into an image using a computer. Knight *et al.* (1993) used an intensified CCD camera to image changes in $[Ca^{2+}]_c$, by detecting aequorin light emission from transgenic *Nicotiana plumbaginifolia.*

Ca^{2+} shows a strong affinity for aequorin (KD = 0.14 μm). However, a range of other ions can also trigger (Sr^{2+}, Pb^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+} and lanthanides), competitively inhibit (Na^+, K^+, Mg^{2+}) or non-competitively inhibit (Ag^{2+}, Hg^{2+}) luminescence to differing extents. The majority of these ions will not occur in sufficient amounts to effect accurate Ca^{2+} detection in biological systems, with the exception of Mg^{2+} and to a lesser extent K^+. These ions will desensitise aequorin to small changes in Ca^{2+}.

Binding of Ca^{2+} ions occurs at three sites (EF hands) present in the apoprotein. These show homology in both amino acid sequence and position to three of the Ca^{2+} binding sites of calmodulin and troponin C, suggesting a common evolutionary origin (Charbonneau *et al.*, 1985). The validity of these putative Ca^{2+} binding sites has been confirmed by synthesising peptides of these regions and examining Ca^{2+} binding by measuring the removal of free Ca^{2+} using a Ca^{2+}-microelectrode (Oishi *et al.*, 1992). From the dissociation constants obtained (KD's= 10^{-3} to 10^{-4}) it could be seen that other apoprotein characteristics contribute to the high Ca^{2+} affinity (Oishi *et al.*, 1992). An interesting discrepancy in Ca^{2+} binding to aequorin has commonly been noted in log-log plots of luminescence intensity versus Ca^{2+} concentration. The data gave slopes from 2-3 depending on the conditions used, indicating that either 2 or 3 Ca^{2+} ions triggered luminescence. Recently, to clarify the ambiguity in the stoichometery, Ca^{2+} titrations of aequorin measuring free Ca^{2+} and light emission were carried out, after it was shown that the dissociation of Ca^{2+} from spent aequorin did not effect results (Shimomura 1995a; Shimomura and Inouye, 1996). Using the homogenous recombinant aequorin, results

showed clearly that two Ca^{2+} ions were required to trigger luminescence and that the affinity of aequorin for the initial two Ca^{2+} ions is roughly 22 times stronger that that for the third (Shimomura and Inouye, 1996). Site directed mutagenesis of single calcium binding sites in which the highly conserved 6th position glycine was changed to arginine (neutral to positive charge) have supported this observation showing that the third calcium binding site is non-essential for luminescence (Tsuji *et al.*, 1986).

Site-directed mutagenesis has been used to examine the role of specific amino acids in aequorin, to determine the sites of coelenterazine and oxygen binding. The amino acids selected for mutagenesis (e.g. trp, his, cys) were those which are uncommon in Ca^{2+} binding proteins, but occur quite frequently in apoaequorin. Two studies indicated through complete loss in activity that a histidine (either his 58 or his 169) was probably the amino acid oxygen binding site (Tsuji *et al.*, 1986; Ohmiya and Tsuji, 1993). Changing tryptophan residues to phenylalanine (both possess large aromatic side chains) resulted in small changes in secondary structure as determined by circular dichronism and reductions in luminescence (Ohmiya *et al.*, 1992). However, the modification of trp 86 resulted in an aequorin with a dual emission spectrum, indicating that this residue may be important in the production of the excited state of coelenteramide (Ohmiya *et al.*, 1992). Replacement of the three cysteine residues with serine to obtain information on disulphide bond formation was inconclusive (Kurose *et al.*, 1989). Analysis of tryptic digests before and after reduction with dithiothreitol (DTT) by mass spectrometry showed that a disulphide bond was formed between cys 145 and cys 152 (Ohmiya *et al.*, 1993). The essential requirement for ß-mercaptoethanol or DTT in the *in vitro* regeneration of aequorin (Shimomura and Johnson, 1975) and spontaneous weak luminescence seen on damaging the disulphide bond (Kemple *et al.*, 1984) suggest that the thiol groups play an important role

in the incorporation of coelenterazine into the apoprotein. The C-terminal proline also seems to be important for the long term stability of bound coelenterazine (Watkins and Campbell, 1993).

The cloning of two apoaequorin genes from *A. victoria* (*aeq1*, Prasher *et al.*, 1985, 1986; *AQ440* Inouye *et al.*, 1986) and expression in *E. coli* (Cormier *et al.*, 1989) has produced high yields of readily purified homogenous apoprotein which has not exhibited any of the toxic side effects sometimes seen with native aequorin (preparations of native aequorin contain up to 10 isoaequorins)(Shimomura, 1991). cDNA sequencing indicated that the polypeptide was composed of 196 amino acids and this was confirmed by the rapid isolation of aequorin from *A. victoria* (Prasher *et al.*, 1987). It had been proposed that the mature apoprotein started with the seventh amino acid valine, but the loss of this peptide is now known to be the result of the isolation procedure (Prasher *et al.*, 1987).

Luminescence of recombinant aequorin, native aequorin and the isoforms of native aequorin have been calibrated to Ca^{2+} concentrations under conditions which mimic the physiological situation and can all be used to accurately measure Ca^{2+} over a wide dynamic range pCa 5.2-6.7 (Shimomura, 1991).

At very low Ca^{2+} concentrations aequorin exhibits Ca^{2+}-independent luminescence. Allen *et al.* (1977) under a specific set of conditions showed that a log-log plot of luminescence versus Ca^{2+} concentration was essentially flat at Ca^{2+} concentrations $< 10^{-8}$ M. Properties of $\cdot Ca^{2+}$-independent luminescence include; pH dependency, temperature dependency, enhancement by Ag^{2+}, and influence by [KCl] (Allen *et al.*, 1977). Mg^{2+} protects aequorin from Ca^{2+}-independent discharge (Ray *et al*,

Figure 1.3: DNA and amino acid sequence of the apoaequorin A cDNA, *aeq1* (Prasher *et al.*, 1985).

met	thr	ser	glu	gln	tyr	ser	val	lys	leu
ATG	ACC	AGC	GAA	CAA	TAC	TCA	GTC	AAG	CTT
thr	pro	asp	phe	asp	asn	pro	lys	trp	ile
ACA	CCA	GAC	TTC	GAC	AAC	CCA	AAA	TGG	ATT
gly	arg	his	lys	his	met	phe	asn	phe	leu
GGA	CGA	CAC	AAG	CAC	ATG	TTT	AAT	TTT	CTT
asp	val	asn	his	asn	gly	arg	ile	ser	leu
GAT	GTC	AAC	CAC	AAT	GGA	AGG	ATC	TCT	CTT
asp	glu	met	val	tyr	lys	ala	ser	asp	ile
GAC	GAG	ATG	GTC	TAC	AAG	GCG	TCC	GAT	ATT
val	ile	asn	asn	leu	gly	ala	thr	pro	glu
GTT	ATA	AAC	AAT	CTT	GGA	GCA	ACA	CCT	GAA
gln	ala	lys	arg	his	lys	asp	ala	val	glu
CAA	GCC	AAA	CGT	CAC	AAA	GAT	GCT	GTA	GAA
ala	phe	phe	gly	gly	ala	gly	met	lys	tyr
GCC	TTC	TTC	GGA	GGA	GCT	GGA	ATG	AAA	TAT
gly	val	glu	thr	glu	trp	pro	glu	tyr	ile
GGT	GTA	GAA	ACT	GAA	TGG	CCT	GAA	TAC	ATC
glu	gly	trp	lys	arg	leu	ala	ser	glu	glu
GAA	GGA	TGG	AAA	AGA	CTG	GCT	TCC	GAG	GAG
leu	lys	arg	tyr	ser	lys	asn	gln	ile	thr
TTG	AAA	AGG	TAT	TCA	AAA	AAC	CAA	ATC	ACA
leu	ile	arg	leu	trp	gly	asp	ala	leu	phe
CTT	ATT	CGT	TTA	TGG	GGT	GAT	GCA	TTG	TTC
asp	ile	ile	asp	lys	asp	gln	asn	gly	ala
GAT	ATC	ATT	GAC	AAA	GAC	CAA	AAT	GGA	GCT
ile	ser	leu	asp	glu	trp	lys	ala	tyr	thr
ATT	TCA	CTG	GAT	GAA	TGG	AAA	GCA	TAC	ACC
lys	ser	ala	gly	ile	ile	gln	ser	ser	glu
AAA	TCT	GAT	GGC	ATC	ATC	CAA	TCG	TCA	GAA
asp	cys	glu	glu	thr	phe	arg	val	cys	asp
GAT	TGC	GAG	GAA	ACA	TTC	AGA	GTG	TGC	GAT
ile	asp	glu	ser	gly	gln	leu	asp	val	asp
ATT	GAT	GAA	AGT	GGA	CAG	CTC	GAT	GTT	GAT
glu	met	thr	arg	gln	his	leu	gly	phe	trp
GAG	ATG	ACA	AGA	CAA	CAT	TTA	GGA	TTT	TGG
tyr	thr	met	asp	pro	ala	cys	glu	lys	leu
TAC	ACC	ATG	GAT	CCT	GCT	TGC	GAA	AAG	CTC
tyr	gly	gly	ala	val	pro				
TAC	GGT	GAA	GCT	GTC	CCC				

1985). The influence of different factors on Ca^{2+}-independent discharge of aequorin illustrates the need to monitor these factors in *in vitro* and where possible in *in vivo* calibrations.

The range over which Ca^{2+} can be measured using aequorin has been extended by the development of synthetic analogues of coelenterazine and the inclusion of these into the photoprotein to yield recombinant or native semi-synthetic aequorins (Shimomura *et al.*, 1988, 1989; Shimomura *et al*, 1990). Table 1.3 summarises the types and pCa ($\log_{10}[Ca^{2+}]$) ranges of semi-synthetic aequorins, the λ_{max} range is between 440 and 475. Certain semi-synthetic aequorins show fast rise times (half rise time of normal aequorin = 8 msec) which are useful for studying rapid changes in Ca^{2+}, and bimodel spectra in which the ratio of the two peaks is dependent on the Ca^{2+} concentration alone. The formation of a wide range of active semi-synthetic aequorins indicates a high degree of conformational flexibility at the coelenterazine binding site. Most of the spectral variations in luminescence can be explained on the basis of hydrophobic and ionic interaction between coelenteramide and apoaequorin (Shimomura, 1995b).

The information reviewed in this section illustrates a number of advantages of using aequorin as an indicator for $[Ca^{2+}]_c$. To summarise, these include a wide range of concentrations over which Ca^{2+} can be measured, contrast enhancement as luminescence is proportional to 2nd to 3rd power of Ca^{2+}, and a lack of Ca^{2+} buffering and toxicity within the cell. In addition, detecting luminescent light is advantageous as, unlike autofluorescence, autoluminescence is practically absent from most natural systems and aequorin is very stable within the cytosol (Miller *et al.*, 1994). The only real limitation with using aequorin has been ensuring that enough light is emitted for detection; the system being studied may be

too small, the free Ca^{2+} levels too low, the spatiotemporal resolution required too high, or the methods of introduction too inefficient. In systems other than the filamentous fungi fluorescent dyes may complement the use of aequorin in these circumstances.

Table 1.3: The types of semi-synthetic aequorins and the range of pCa ($log_{10}[Ca^{2+}]$) measurable. The coelenterazine analogue incorporated is indicated by a lower case prefix e.g. *e*-aequorin. Table based on Shimomura, 1991.

Aequorin type	Example aequorin shown by prefix	pCa range*
Slightly sensitized form	*h, f, cp*	5.7-7.2
Moderately sensitized form	*ip, hch, fch*	6.0-7.5
Highly sensitized form	*hcp, fcp, fip, efcp*	6.5-8.0
Low sensitivity form	*i, n*	4.4-6.2
Fast response aequorin	*ch, e, ech*	5.2-6.7
	cp, eh	5.7-7.2
	fch, efcp	6.0-7.5
	hcp, efcp	6.5-8.0
e-type aequorin	*e, ech*	5.2-6.7
	eh, ehch	5.7-7.2
	efch, ehcp	6.0-7.5
	efcp	6.5-8.0

Notes:
*Approximate optimum range under physiological conditions (ionic strength 0.1-0.15; 1-2 mM Mg^{2+}. In the absence of Mg^{2+} pCa values should be increased by 0.5.

1.3 Transgenic aequorin and general problems of transgene expression

Aequorin can be introduced into the cell by any one of five methods: micro-injection, transient cell permeablization, vesicle-cell fusion, internal release from phagocytosed vesicles and finally by the introduction of genetic material (Campbell and Sala-Newby, 1994). Microinjection and transformation are generally an order of magnitude more efficient at

delivering aequorin into the cell than the other methods (Miller *et al.*, 1994). Genetic transformation has the over-riding advantage of introducing aequorin into all the cells of any genetically amenable species.

Transformation has been used to introduced apoaequorin into plants (Knight *et al.*, 1991b; Haley *et al.*, 1995), yeast (Nakajima-Shimada *et al.*, 1991a,b), *Dictyostelium* (Saran *et al.*, 1994; Cubitt *et al.*, 1995), *E. coli* (Knight *et al.*, 1991a) and mammalian cell lines (Button and Brownstein, 1993; Sheu *et al.*, 1993). In all cases $[Ca^{2+}]_c$ could be detected in the organisms following a period of incubation with coelenterazine, which readily permeates cell membranes, yielding active aequorin. Using this technology, aequorin has also been used to report free Ca^{2+} within subcellular compartments or specific subcellular locations, by fusing apoaequorin to organelle targeting signals (Rizzuto *et al.*, 1992) or to the coding sequence of a normally resident protein (Rizzuto *et al.*, 1994). Aequorin has now been used to detect changes in free Ca^{2+} in the nucleus (Brini *et al.*, 1993; Badminton *et al.*, 1995), endoplasmic reticulum (Kendall *et al.*, 1992, 1994; Button and Eidsath, 1996), mitochondria (Rizzuto *et al.*, 1992, 1994), vacuole (M. Knight, pers. comm.), locations adjacent to the plasma membrane (H. Knight, pers. comm.), and chloroplasts (Johnson *et al.*, 1995). By pursuing these studies in a model organism e.g. the plant *Nicotiana plumbaginifolia*, major advances have been made in our understanding of $[Ca^{2+}]_c$ fluxes i.e. types of transients, spatiotemporal patterns, Ca^{2+} mobilizable pools, and intercellular Ca^{2+} waves. In some cases luminescence has been detected originating from single cells (N. Wood, pers. comm.).

Few studies have estimated the concentration of aequorin in transformed cells, as the levels obtained usually permit $[Ca^{2+}]_c$ measurement. Those studies in which an estimate has been made

include; in the yeast cytosol 1 μM during the exponential cell growth phase (Nakajima-Shimada *et al.*, 1991b), 0.2-10 ng/mg protein in mammalian cell nuclei, (Brini *et al.*, 1993), and a few pg protein per mg fresh *N. plumbaginifolia* tissue (Knight and Knight, 1995).

There is only a single study in which the *in vivo* stability of apoaequorin has been assessed, aequorin is known to be stable over several days *in vivo*. Badminton *et al.* (1995) estimated the half life of apoaequorin in the cytosol of mammalian cells to be approximately 20 min. There was a two to three fold increase in aequorin levels when the growing cells were incubated with coelenterazine i.e. apoaequorin was stabilized as aequorin (Badminton *et al.*,1995). Apoaequorin which was directed to subcellular organelles was found to be more stable. This may be due to a combination of the protection afforded by additional sequences (luciferase-apoaequorin shows greater stability) and conditions within the organelles (Badminton *et al.*, 1995).

Transformation of a filamentous fungus with apoaequorin and subsequent formation of aequorin in transformed cells may allow; the detection, measurement and imaging of $[Ca^{2+}]_c$ in individual cells, cell populations and across the fungal colony. Introduction of apoaequorin by transformation is feasible as suitable fungal expression cassettes and transformation systems are available. In addition, a number of systematic studies on foreign transgene expression in filamentous fungi have provided some insights into techniques for optimising protein yield.

For the expression of any gene in a transformed host, expression signals which are functional in that organism must flank the protein coding sequence. To maximise the potential for high expression of a transformed gene the expression signals (i.e. the promoter and

terminator) should be native to the host and from a highly expressed gene. The range of promoters available for vector construction depends upon the genes which have been previously cloned and characterised, or the development of techniques for selecting sequences with promoter activity (Turgeon *et al.*, 1987). The relative strength of promoters can then be assessed by making fusion vectors with reporter genes (Vanwert and Yoder, 1994). It is not essential that the expression signals are native to the host as the transcription control and translation initiation regions contained in a promoter from one fungal species may function adequately in a wide range of related species (van den Hondel *et al.*, 1991). Even a plant virus promoter was recognised by the transcriptional machinery of *Uromyces appendiculatus* (Li *et al.*, 1993). The role of the terminator in an expression cassette is not clearly defined. The terminator possesses sequences for transcription termination and the polyadenylation signal; it is assumed that these are required for optimal mRNA production and stability.

DNA-mediated transformation requires that an organism can take up and express exogenous DNA that can confer a selective advantage on those cells which receive it. There are three criteria which must be meet to achieve successful transformation (Rambosek and Leach, 1987): the preparation of competent cells, the induction of those cells to take up DNA and the application of selective pressure to identify those cells which contain the foreign DNA.

The preparation of competent cells in filamentous fungi usually involves removal of the cell wall and treatment with polyethylene glycol and calcium chloride. However, certain methods by-pass these steps by permeabilizing intact cells and introducing the DNA directly (e.g. by electroporation). Factors within the cell which govern transformation are

not defined but they are known to be a property of the nucleus as opposed to the plasma membrane (Pandit and Russo, 1992; Grotelueschen and Metzenberg, 1995).

Following transformation, the identification of those cells which have incorporated the foreign DNA can be achieved by selecting for the expression of a marker gene included in the transforming DNA. Generally these fall into two types: normal wild-type genes which complement a pre-existing (usually auxotrophic) mutation and lead to prototrophy; and foreign genes encoding a new function, usually antibiotic resistance.

Drug resistance markers have an advantage over auxotrophic markers in that they facilitate transformation of wild-type strains of species for which auxotrophic mutants will be difficult to obtain (e.g. obligate pathogenic and polyploid species, and genetically uncharacterised species). However, their use tends to generate high levels of unstable background colonies, termed 'abortives.' Abortive transformants show strong growth initially but are unable to maintain it on vegetative transfer. It is thought that they arise from the ability of the introduced DNA to replicate outside of the chromosome, and the subsequent loss of this property during growth (Fincham, 1989).

Many of the published reports on heterologous gene expression provide only qualitative data regarding the production of the foreign protein, making it difficult to compare the efficiency of different expression systems. The systematic studies conducted have concentrated on *Aspergillus sp.* and *Trichoderma reesei*, species' favoured because of the industrial applications of the former and the prodigious secretion

capacity of the latter. The relitively few examples of heterologous gene expression in *N. crassa* are less numerous and are summarised in table 1.4.

Table 1.4: Examples of heterologous gene expression in *N. crassa*. For each part of the expression cassette, the origin, gene symbol and protein product is given.

Promoter	Coding Sequence	Terminator	Reference
A.nidulans *trp C* anthranilate synthetase	bacterial *hph* hygromycin B phosphotransferase	*A. nidulans* *trp C* anthranilate synthetase	Staben *et al.*, 1989
N. crassa *am* glutamate dehydrogenase	bacterial transposon T5 *ble* bleomycin resistance gene	*N. crassa* *am* glutamate dehydrogenase	Austin *et al.*, 1990
N. crassa *acu5* acetyl coenzyme A synthase	bacterial *cat* chloramphenicol acetyltransferase	unspecified	Zoysa and Connerton, 1994
N. crassa *Bml* ß-tubulin	bovine preprochymosin	*N. crassa* *Bml* ß-tubulin	Nakano *et al.*, 1993
N. crassa *grg-1* glucose repressible gene	bovine preprochymosin	*N. crassa* *grg-1* glucose repressible gene	Nakano *et al.*, 1993
N. crassa *mtr* methyl tryptophan resistance	bacterial *bar* phosphinothricin acetyltransferase	*A. nidulans* *trpC* anthranilate synthetase	Pall *et al.*, 1993
N. crassa *arg-2* carbamyl phosphate synthetase A - small subunit	bacterial *lac Z* β-galactosidase	unspecified	Sachs and Ebbole, 1990
N. crassa *con-10* conidiation specific gene 10	bacterial *lac Z* β-galactosidase	*A. nidulans* *trpC* anthranilate synthetase	Sachs and Ebbole, 1990

Transformation of spheroplasts originating from multinucleate conidia (e.g. *N. crassa*: Vollmer and Yanofsky, 1986) or mycelial compartments (e.g. *N. crassa*: Buxton and Radford, 1984) is expected to give rise to heterokaryons which can be purified by repeated conidial isolation or by outcrossing. The homokaryotic progeny may differ in protein yield as some may be affected by the poorly understood phenomenon of gene silencing (see below). Homokaryons which are unaffected by gene silencing will show improved protein yield as the number of transformed nuclei in the mycelium has been increased.

Transforming DNA in filamentous fungi is sometimes homologously integrated (i.e. by addition or replacement) but ectopic integration is more common and sometimes occurs in multiple copies present in tandem arrays. This may arise from homologous recombination between plasmids prior to integration or a single integration may occur followed by successive integration of further plasmid copies by homology with the first one. Evidence obtained in *S. cerevisiae* provides strong evidence for the latter (Orr-Weaver and Szostak, 1983). Transformants which possess multiple copies of the transgene may reflect the high competence of a specific nucleus to assimilate DNA into the genome. The factors which determine a competent nucleus are unknown, but it has been suggested that privileged chromosomal sites may exist (Grotelueschen and Metzenberg, 1995).

In *N. crassa*, outcrossing ectopic transformants can lead to gene silencing by RIP (methylation and GC to AT mutations). Tandemly duplicated gene copies rarely escape RIP. Unlinked gene duplications are altered at frequencies of 10% to 70% (Singer and Selker, 1995).

Heavy methylation of multicopy transgenes correlated with low expression has been observed in *N. crassa* (Bull and Wootton, 1984). This suggests that tandem arrays attracted *de novo* methylation. Growth in the presence of 5-azocytidine prevented methylation but did not increase gene expression (Bull and Wootton, 1984). Pandit and Russo (1992) showed that duplication by transgenes could trigger reversible inactivation which was associated with heavy methylation; they called this transgene-induced gene silencing *quelling*. Recently it was shown that quelling can occur in a methylation deficient strain and that a transgene-derived sense RNA was present in quelled but not in revertant strains suggesting that transgene transcription was somehow required for silencing (Cogoni *et al.*, 1996). Quelling might seem to pose a general threat to transgene expression but, in fact, it seems to be the exception, not the rule.

Yield of transgene protein product increases with extra gene copies but is not strictly proportional to copy number as some ectopic sites of integration give more transcription that others (Baron *et al.*, 1992). In addition, when excessive copy numbers are introduced, expression of the transgene and the native gene from which the promoter originates may decrease, due to the titration of trans-acting regulatory proteins (Verdoes *et al.*, 1994).

To increase the transgene product, different or improved promoters, multiple copies, or where available, optimised strains may be used. Punt *et al.*(1992) included a short sequence from the *gpdA* promoter in the *amdS* promoter and showed that it yielded a 30-fold increase of ß-galactosidase (*lacZ*) in *A. nidulans* transformants.

The inclusion of native protein sequences making a hybrid protein sometimes improves yield. Such translational fusions have been shown

to increase secreted protein yield in *Trichoderma reesei* (Harkki *et al.*, 1989; Nyyssönen *et al.*, 1993), *Aspergillus niger* (Broekhuijsen *et al.*, 1993; Jeenes *et al.*, 1994) and *Tolypocladium geodes* (Baron *et al.*, 1992). In *Trichoderma reesei* yield was increased from 1 mg/l to 150 mg/l (Nyyssönen *et al.*, 1993) and in *A. niger* from 50 mg/l to 1 g/l (Jeenes *et al.*, 1993). These protein hybrids not only facilitate better secretion but have also been shown to increase mRNA stability (Jeenes *et al.*, 1994; Nyyssönen and Keranen, 1995).

A common observation in transgene studies is that the expression of the optimised transgene is very low compared to the amount of native protein produced from a natural gene using the same promoter in the same strain. Nyyssönen *et al.* (1993) estimated the yield of a cellobiohydrolase I (*cbhI*) - heavy Fd chain translational fusion (with the *cbhI* promoter) to be 150 mg/l as compared to 1-10 g/l of cellobiohydrolase I. Such a drastic reduction could be due either to a low level of transcription or to post-transcriptional factors. Nyyssönen and Keranen (1995) observed very low levels of mRNA when the expression cassette did not contain *cbh1* sequences beyond that encoding the signal sequence for secretion, in ectopically integrated transgenes; much higher levels of mRNA were observed in gene fusions which included part of the *cbh1* coding sequence. A similar situation was found in *A. niger* transformants secreting hen egg white lysozyme (HEWL) where the transgene alone exhibited a ten-fold higher mRNA : protein ratio as compared to a glucoamylase- HEWL gene fusion (Jeenes *et al.*, 1994).

It is probable that codon usage differences between the transgene and the host organism will influence the level of protein production. mRNA stability will also influence protein yield, though the factors which govern this are poorly understood.

Differences in codon usage are possible whereever there are two or more synonymous codons for the same amino acid. It was first shown in *E. coli* that the choice of codon was strongly correlated with the abundance of the corresponding tRNA (Ikemura, 1981). The bias was greatest in highly-expressed genes and reduced or absent in low-expressing genes. Ikemura (1985) found a similar situation in *S. cerevisiae*. Bias in codon usage has now been shown in a wide range of species including filamentous fungi (Lloyd and Sharp, 1991; Edelmann and Staben; 1994). In both *A. nidulans* and *N. crassa* pyrimidines (especially cytosine) are strongly favoured over purines in the third positions of codons and, where a purine is obligatory, guanine is strongly favored over adenine.

The importance of optimal codon usage has been shown experimentally in a number of species. In *N. crassa* the introduction of three rare codons with third position adenine into the *am* gene caused a 70% loss in protein production without any appreciable reduction in mRNA levels (Kinnaird *et al.*, 1991). In *E. coli* insertion of rare arginine codons (AGG and AGA) in the template and their relative position had a significant effect on the level of gene expression. Translation was particularly disadvantaged when two of these codons were adjacent to each other producing a significant amount of polypeptides of incomplete length (Gurski *et al.*, 1992). It has been suggested that the presence of rare codons in the mRNA slows down polypeptide production as the ribosome will stall at a rare codon until the corresponding tRNA becomes available. If the rare codons occur in a cluster the ribosomes will jam and translation will be greatly reduced. As each species has an individual pattern of codon usage, the expression of a heterologous gene may be influenced by the acceptability of its codons in the new host and the arrangement of any now rare codons in the gene. To eliminate this possible rate-limiting step

in foreign protein production a number of researchers have synthesised genes which reflect the codon bias of the host in which the protein is to be expressed. These studies have yielded mixed results. A re-engineered GFP gene sequence which favored codons of highly expressed humam proteins gave 20-fold higher GFP expression in *Zea mays* cells than the original jellyfish gene (Chiu *et al.*, 1996). In *S. cerevisiae* no change in expression of the murine interleukin-2 gene was observed when twelve consecutive codons were replaced by those optimal for the host (Demolder *et al.*, 1992). In *Streptomyces lividans* replacing certain rare codons in the mouse tumour necrosis factor α gene did not improve yield, and when five of these were adjacent a drastic reduction in protein was observed due to a marked decrease in mRNA stability (Lammertyn *et al.*, 1996). By expressing codon-adjusted synthetic α and β-globin genes in *E. coli*, a high protein yield was obtained but only when the two genes were expressed as a fusion protein (Hernan *et al.*, 1992). Codon usage as a possible limitation on transgene expression remains a matter of concern, but the extent of its influence is still quite problematical.

The protein yield from a transgene will depend strongly on the half life of the protein in its new environment. The intracellular stability of a foreign protein will be governed by (a) its liability to the action of various selective proteases present in the cytosol and (b) its export into the lysosome or vacuole where those proteases of broader specificity are located. It is well known that cells recognise proteins of altered conformations (e.g. due to mutation, denaturation or premature chain termination) and rapidly degrade them. Rapid degradation also acts on unassembled components of multicomponent complexes. Features which may increase the turnover rate of mature proteins include sequence specific motifs (e.g. the pro-glu-ser-thr sequence) and global properties such as size, negative charge, thermal instability and

hydrophobicity (Rechsteiner *et al.*, 1987). Unfortunately at present there is not enough information avaliable to predict how well a specific heterologous protein will survive in a novel cytosolic environment.

1.4 Outline of the thesis

The initial aim of this study was to apply the transgenic aequorin approach for investigating $[Ca^{2+}]_c$ to the filamentous fungi. The species selected for the study was *Neurospora crassa*. Its advantages include: the avaliability of suitable expression cassettes, tried and tested transformation protocols, considerable evidence for the Ca^{2+} pathway (see Table 1.2) and mutants in proposed Ca^{2+}-regulated responses.

The objectives of the work recorded in this thesis were: (1) to obtain *N. crassa* strains stably transformed with the apoaequorin gene, (2) to develop methods for measuring apoaequorin production by these transformants, (3) to maximize apoaequorin yield, (4) to characterise apoaequorin production, (5) to use the transformed strains for the detection of changes in $[Ca^{2+}]_c$ in response to various stimuli.

Chapter Two

2. Materials and Methods

2.1 Chemicals

Unless otherwise specified media reagents were purchased from BDH Ltd (UK) and biochemicals from Sigma (UK). Restriction endonucleases and modification enzymes were from Boehringer Mannheim (UK).

2.2 Organisms

Manipulation of organisms was carried out using established sterile technique. Genetically modified *N. crassa* and *E. coli* were both containment level 1 organisms and the correct procedures for handling and disposal were followed (Genetic Manipulation and Biological Safety Committee, University of Edinburgh).

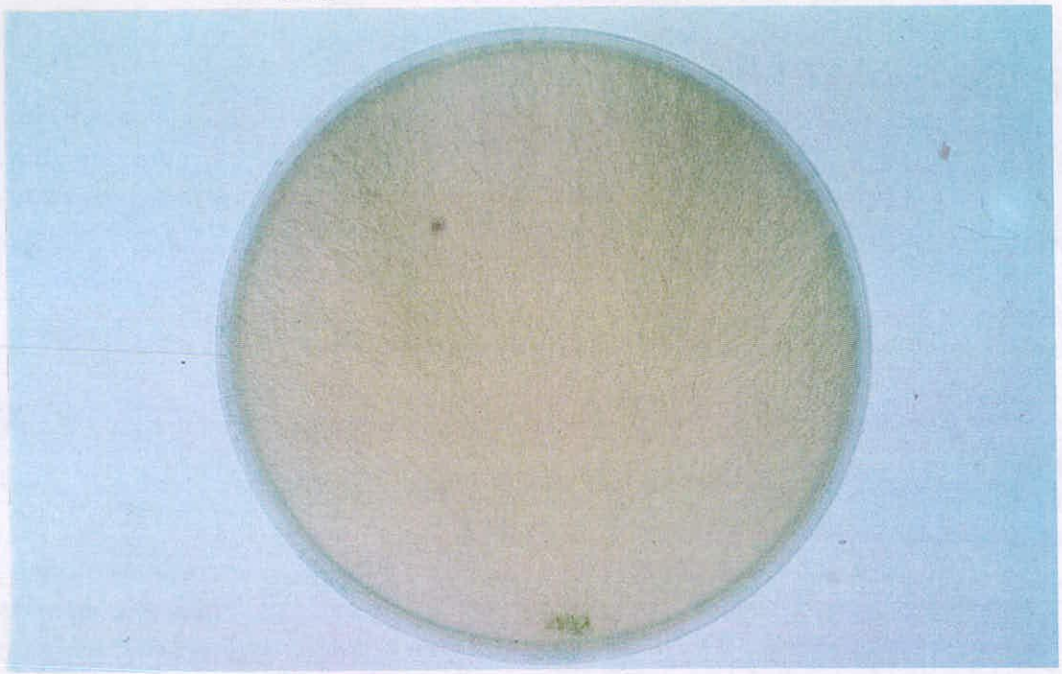
2.2.1 Neurospora crassa

2.2.1.1 Strains

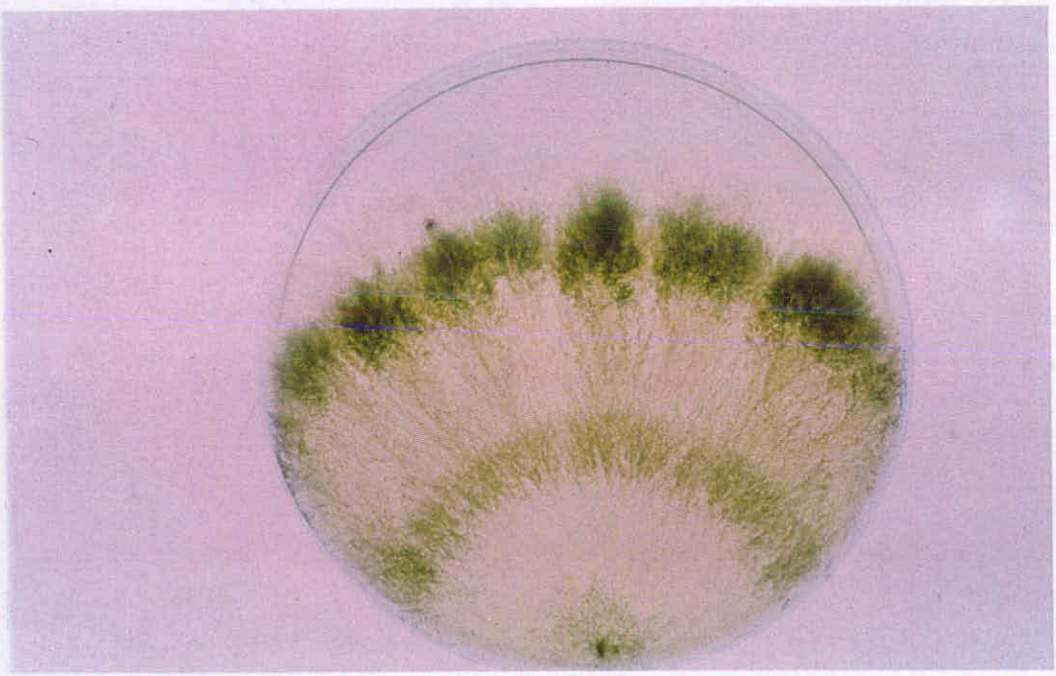
The wild type strain St Lawrence (St) 74 mating type *A* (*74A*) (Fungal Genetics Stock Centre strain 262) and the *Band* (*Bd*) mutant mating type *A* (*BdA*) (FGSC strain 1858) were transformed with the apoaequorin gene. The *Bd* mutant shows a pronounced circadian rhythm of conidiation when grown on solid medium in closed culture (Sargent *et al.*, 1956). The morphology of the two strains is shown in Fig. 2.1.

Figure 2.1: *N. crassa* strains grown on 1x Vogel's medium N, 2% maltose and 2% agar.

St Lawrence 74 mating type *A*



Band* mutant mating type *A



2.2.1.2 Culture media

The strains of *N. crassa* were grown on 1x Vogel's medium N (Vogel's salts)(Vogel, 1956) using either sucrose, glucose, glucose and fructose, or maltose as the carbon source. For solid media 2% Oxoid Number 3 agar (Oxoid, UK) was added. All media were sterilised prior to use at 121 °C for 16 min at 1 bar and if glucose was present at 121 °C for 11 min at 1 bar. Percentage solutions are weight : volume (w:v) unless stated otherwise.

Vogel's medium N x 50 stock solution:

Na_3 citrate.$2H_2O$	126.7 g
KH_2PO_4	250 g
NH_4NO_3	100 g
$MgSO_4.7H_2O$	10 g
$CaCl_2.2H_2O$	5 g
D-Biotin solution[a]	5 ml
Trace element solution[b]	5 ml
Distilled water (d.H_2O)to 1 l	

Chloroform (3 ml) was added as a preservative and the solution stored at room temperature.

[a] 5 mg of D-biotin (Sigma, UK) was dissolved in 100 ml of 50% volume : volume (v/v) ethanol (EtOH). The solution was stored at 4 °C.

[b] Trace elements solution

citric acid.$1H_2O$	5.0 g
$ZnSO_4.7H_2O$	5.0 g
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1.0 g
$CuSO_4.5H_2O$	0.25 g
$MnSO_4.1H_2O$	0.05 g
H_3BO_3	0.05 g
$Na_2MoO_4.2H_2O$	0.05 g
d.H_2O	to 100 ml

Chloroform (1 ml) was added as a preservative and the solution was stored at room temperature.

For sexual crosses, synthetic crossing medium (omitting sucrose) was used; the low nitrogen content promotes development of protoperithecia and perithecia (see Davis and deSerres, 1973, for Westergaard and Mitchells synthetic crossing medium).

x 1 synthetic crossing medium (minus sucrose)

KNO_3	1.0 g
KH_2PO_4	0.7 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2$	0.5 g
NaCl	0.1 g
Biotin solution[a]	0.1 ml
Trace element solution[b]	0.1 ml
d.H_2O	to 1 l

[a] and [b]: as for Vogel's medium N.

For solid medium 2% agar (Oxoid number 3) was added.

2.2.1.3 Culturing *N. crassa*

2.2.1.3.1 Inoculation procedure

Unless otherwise specified, cultures were inoculated using a sterile platinum wire loop. A meniscus of sterile d.H_2O (s.d.H_2O) in the loop allowed conidia to be taken into suspension during the transfer and thereby minimise the risk of dispersal.

2.2.1.3.2 Types of culture

2.2.1.3.2.1 Slants

Stock cultures were maintained on 1 x Vogel's medium N, 2% sucrose, 2% agar (VS agar). Ten ml of unsolidified medium was poured into a sterile test tube (150 mm x 15 mm dia) and allowed to set at an angle of approximately (approx.) 30°. Inoculated cultures were incubated at 27 °C in continuous illumination for 7 days. Mature cultures could be stored at 4 °C for up to 3 months.

2.2.1.3.2.2 Swirl flasks

To obtain high concentrations of conidia (>1 x 10^9), inoculation was made onto 50 ml of VS agar in a 250 ml conical flask. The medium had previously been solidified by rotating at an angle under a stream of cold water. This resulted in the adhesion of a thin layer of agar to the sides of the flask with a thick layer over the base. Inoculated cultures were incubated at 27 °C in continuous illumination for 6-7 days.

2.2.1.3.2.3 Shaking liquid culture

For bulk conidial germination as required for transformation experiments, conidia were harvested from a swirl flask in 50 ml s.d.H_2O. Conidia were concentrated, washed and finally resuspended in 5 ml s.d.H_2O. The suspension was then added to 150 ml 1x Vogel's medium N,

1.5% sucrose, in a 500 ml conical flask. This was incubated overnight at 15-18 °C, shaking at 200 rpm.

2.2.1.3.2.4 Still liquid culture

For DNA, RNA, and protein extractions from mycelium, a conidial inoculation (quantified when necessary) was made into 50 ml 1x Vogel's medium N, 2% sucrose (VS medium) contained in a 250 ml conical flask. Cultures were incubated standing in darkness at 25 °C for the required time.

2.2.1.3.2.5 Race tubes

Growth rate of strains was assessed by inoculating 5 x 10^6 conidia at one end of a horizontal race tube (350 mm x 12 mm dia) containing a level VS agar (15 ml) strip along the tube base. Race tubes were incubated at 25 °C in darkness. Growth was measured at regular intervals.

2.2.1.3.2.6 Crossing slants

The strain Sta 73*a* (FGSC strain 3834) was grown at 25 °C for 6 days on synthetic crossing medium (minus sucrose), 2% agar, with a strip of Whatman 3MM filter paper providing a minimal carbon source.

2.2.2 *Escherichia coli*

Two *E. coli* strains were used in the subcloning of pBluescript (Stratagene) and pUC (Gibco BRL, UK) based plasmids.

2.2.2.1 Strains

JM101: *supE, thi,* Δ(*lac-pro*AB) F'[*tra*D36, *pro*AB, *lac*Iq *lac*ZΔM15]

XL1-blue: *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac* -,
F' [*pro*AB, *lac*Iq, *lac*ZΔM15, Tn10 (Tet^r)]

JM101 and XL-1 blue can be used for the colour-based selection of plasmid insertions. Both strains carry a mutant *lac Z* which codes for the carboxy terminal end of ß-galactosidase. The plasmid pBluescript possesses the regulatory and first 146 amino acid sequence of this gene adjacent to the multiple cloning site. Following pBluescript transformation the two inactive fragments can combine to produce enzymatically active ß-galactosidase. In the presence of isopropyl ß-D-thiogalactoside (IPTG) as inducer and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) these colonies appear blue. Those colonies carrying pBluescript with an insertion at the multiple cloning site are incapable of producing potentially active protein and are white.

2.2.2.2 *E.coli* culture media

All media was sterilised by autoclaving for 20 min at 121 °C at 1 bar.

Luria-Bertani (LB) broth:
1% bacto tryptone
0.5% w/v bacto yeast extract
1% NaCl, pH 7.0

For Luria-Bertani agar (LB agar) 1.5% bacto agar (Difco, UK) was added to LB broth.

2.2.2.3 Antibiotics

For the selection of XL-1 blue LB broth and agar was supplemented with 10 µg/ml tetracycline. For the selection of pBluescript and pUC based plasmids carrying an ampicillin resistance gene, 100 µg/ml ampicillin was added to the media.

2.2.2.4 Colour selection

For colour-selection of pBluescript based plasmids agar was supplemented with 238 mg/ml IPTG and 20 mg/ml X-Gal.

2.2.2.5 *E.coli* cell culture

2.2.2.5.1 Solid medium

LB agar was sterilised and cooled to approx 50 °C before any supplements were added. The agar was dispensed into sterile 9 cm Petri dishes and allowed to set. To dry condensation from the agar surface and dish lids, plates were inverted with the lid removed in a drying oven for a brief period. Plates were stored at 4 °C and used within 2 weeks.

Bacterial suspensions were applied to the agar surface using a sterile glass spreader, the plates were incubated overnight at 37 °C by which time bacterial colonies (each the progeny of a single cell) were visible.

2.2.2.5.2 Liquid medium

2.2.2.5.2.1 Mini-cultures

For the isolation of small amounts of plasmid DNA a single bacterial colony was inoculated into 5 ml of LB broth with the appropriate antibiotic, and incubated overnight at 37 °C shaking at approx. 300 rpm.

2.2.2.5.2.2 Maxi-cultures

For the isolation of large quantities of plasmid DNA a miniculture was added to 500 ml LB broth supplemented with the appropriate antibiotics and incubated overnight shaking at approx. 300 rpm.

2.3 Extraction and purification of plasmid DNA from *E.coli*

2.3.1 Small scale preparation

This method was adapted from the protocol of Holmes and Quigley (1981). Minipreparations (mini-preps) of plasmid DNA were made from a 1.5 ml aliquot of a 5 ml mini-culture. Cells were harvested in a

microcentrifuge (MSE, UK) at 13,500 rpm for 5 min, the supernatant was removed and the cell pellet resuspended in 250 µl STET buffer (8% sucrose, 5% Triton, 50 mM EDTA, 50 mM Tris.Cl, pH 8.0). Ten µl of 10 mg/ml lysozyme was then added to the suspension and mixed (vortexed) using a whirlmixer (Hook and Tucker Instruments, UK). The tube was placed in a 100 °C dry heating block (Tecam®, UK) for 40 sec and immediately transferred on to ice and 270 µl of 5 M lithium chloride was added. After 30 min incubation the tube was centrifuged at 13,500 rpm for 5 min at 4 °C, following which the pellet was removed using a sterile toothpick. Ethanol precipitation of the supernatant (section 2.5.1) for 30 min at -80 °C was used to concentrate the DNA following which it was dissolved in 50 µl TE, pH 8.0 (10 mM Tris.Cl, 10 mM EDTA pH8.0) or s.d.H_2O. If the pellet was difficult to dissolve the tube was heated for 5 min at 65 °C to aid solution. Miniprep DNA was stored at -20 °C.

2.3.2 Large scale preparation

Maxipreparations (maxi-preps) of closed circular plasmid DNA were obtained by equilibrium centrifugation of cell lysates in caesium chloride (CsCl)/ethidium bromide (EtBr) gradients. The topological constraints of closed circular plasmid limits EtBr binding and its differential density allows it to be resolved as a separate band following ultracentrifugation. Maxi-preps were made from maxicultures of *E. coli* (OD_{600} = 1). Cells were harvested by centrifugation at 7000 rpm, for 10 min, at 4 °C in a Sorvall® GSA rotor using a RCB5 centrifuge (DuPont, USA). The supernatant was decanted and the tubes inverted to remove any remaining culture fluid. The cells were resuspended in 3 ml of sucrose mix (25% sucrose, 40 mM EDTA pH 8.0, 50 mM Tris.Cl pH 8.0); this was followed by the addition of 500 µl of 10 mg/ml lysozyme and 500 µl of 500 mM EDTA, pH 8.1. The contents were mixed gently by swirling and placed on ice for 5 min. After this, 6.5 ml of Triton mix (1% v/v Triton x100, 62.5 mM EDTA pH 8.0, 50

mM Tris.Cl pH 8.0) was added and the tube was left on ice for a further 10 min, swirling occasionally to mix and thereby aid cell lysis. The cell lysate was aliquoted into tubes and centrifuged for 45 min at 18,000 rpm in a Sorvall® SS34 rotar (Du Pont, USA). The supernatant was decanted into a measuring cylinder and the volume adjusted with TE, pH 8.0 so that the final volume was a multiple of 8 ml. CsCl (0.95 g per ml of lysate) was added and dissolved by mixing gently, followed by the addition of EtBr (0.1 ml of a 5 mg/ml EtBr stock solution per ml lysate). The solution was aliquoted into Beckman quick seal ultracentrifuge tubes (Beckman, USA) using a Pasteur pipette, balanced and sealed. The tubes were centrifuged at 38,000 rpm in an I1270 rotor in a OTD 50B centrifuge (Dupont, USA) for 2-3 days at 20 °C. The band of closed circular plasmid DNA was removed from the gradient using a hypodermic needle under ultra violet (UV) illumination into glass corex tubes. The EtBr was removed by extraction with NaCl-saturated isopropanol. An equal volume was added to the DNA solution and mixed well prior to centrifugation at 2000 rpm for 3 min in an HB-4 rotor. This step was repeated until the pink coloration (i.e. EtBr) was removed and then the CsCl was discarded by EtOH precipitation of the DNA at -20 °C (section 2.5.1).

2.4 *E. coli* transformation

2.4.1 Transformation using $CaCl_2$ competent cells

This method was based on the protocol developed by Cohen *et al.* (1972). A 1.5 ml aliquot of an overnight mini-culture of JM101 or XL-1 blue was added to 48.5 ml of LB broth in a 250 ml conical flask. Cells were grown at 37 °C, shaking at approx. 300 rpm until the OD_{600} was approx. 0.2 (for JM101) and 0.3 (for XL-1 blue). The culture was transferred to a chilled 50 ml sterile polypropylene tube (Greiner, UK) and centrifuged for 5 min at 4 °C at 3000 rpm. The culture medium was decanted and the tube inverted for 1 min to allow drainage of the cell pellet. The pellet was

resuspended in 25 ml of 100 mM $CaCl_2$ and placed on ice for at least 30 min. The cell suspension was centrifuged as before, the solution decanted and the cells gently resuspended in 0.5 ml of $CaCl_2$. The cell suspension was then placed on ice for at least 4 hours. 100 µl aliquots of the competent cells were used for transformation or stored at -70 °C until use. If cells had been stored they were defrosted slowly on ice prior to transformation.

For transformation, 2-10 µl of a DNA solution was added to the competent cell suspension, mixed gently by tapping the tube, and incubated on ice for 15 min. The cells were heat shocked in a 42 °C water bath (Grant, UK) for exactly 3 min and 1 ml of LB broth was added to the tube prior to a 1 hour outgrowth period at 37 °C. Serial dilutions were made using LB broth, and 200 µl of each dilution was plated onto LB agar plates containing the appropriate antibiotics. Plates were incubated upright at 37 °C for 5 min and then inverted. Bacterial colonies were visible on the plates following overnight incubation at 37 °C.

2.4.2 Transformation by electroporation

Electrotransformation has become an established technique for the introduction of plasmid DNA into *E. coli*. Dower *et al.* (1988) estimated it to be 10-20 times more efficient than that obtained with maximally competent cells prepared by chemical methods. Optimisation of electroporation conditions for a wide range of bacterial strains has simplified its application. The protocol followed for the preparation and subsequent electroporation of *E. coli* was supplied with the Bio-Rad Gene Pulser system (Bio-Rad, UK).

A maxi-prep of JM101 was grown until the OD_{600} was 0.5-0.7. The cell culture was chilled on ice for 30 min and the cells were harvested by

centrifugation at 4 °C at 3600 rpm. The medium was removed and the cell pellet was resuspended in 500 ml 10% glycerol. Following identical centrifugation steps, the cells were resuspended in 25 ml of 10% glycerol, and finally 3 ml of 10% glycerol. The cell suspension was divided into 40 µl aliquots in Eppendorf tubes used fresh, or snap frozen in liquid nitrogen and stored at -70 °C until use.

Stored competent cells were defrosted slowly on ice and electroporation cuvettes (0.1 cm width) were also chilled on ice. A 2 µl aliquot of a DNA solution was added to the cell suspension, mixed gently and then transferred to a cuvette using a Pasteur pipette. The cuvette was placed in the safety chamber of the pulse controller (Bio-Rad, UK) ensuring contact with the electrodes, and a single 2.5 kV square electrical pulse was administered from the adjoining gene pulser (Bio-Rad, UK). The characteristics (resistance 200 Ω, capacitance 25 µF, time constant 4-5 msec, field strength 25 kV/cm) of this pulse had previously been shown to be optimal for *E. coli*. The cuvette was immediately removed from the apparatus and 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added. The cells were gently resuspended in this using a Pasteur pipette, transferred to a 15 ml polypropylene tube and incubated at 37 °C for 1 hour shaking at 225 rpm. Individual aliquots of 200 µl of the cell suspension were applied to LB plates supplemented with the appropriate antibiotics and incubated overnight at 37 °C after which time bacterial colonies were visible.

2.5 Purifying and concentrating nucleic acids

The methods in this section followed those of Sambrook *et al.* (1989) with the exception of sub-sections 2.5.2.1 and 2.5.2.3.

2.5.1 Concentrating DNA by EtOH precipitation

Nucleic acids can be concentrated by precipitation with EtOH in the presence of moderate concentrations of monovalent cations. To the DNA solution, 0.1 volumes of 3 M sodium acetate (NaAc) pH 5.5, was added followed by 2 volumes of cold 100% EtOH. Precipitation occurred immediately though in some cases it was promoted by incubating samples at -80 °C for 30 min. The DNA was concentrated by centrifugation at 13,000 rpm for 5 min and the pellet was washed in 70% v/v EtOH. Centrifugation was repeated and after removing the supernatant the pellet was dried under a vacuum of -1 bar (Giro vap, Howe). The dried pellet was dissolved in the required volume of TE, pH 8.0 (10 mM Tris.Cl pH 8.0, 1mM EDTA pH 8.0).

2.5.2 Purification of nucleic acids

For the removal of contaminating proteins and the isolation of DNA fragments from agarose gels, DNA was purified either by extraction with phenol:chloroform or by using the Gene Clean II® Kit (Bio 101 inc, USA).

2.5.2.1 Elution of DNA from agarose gels prior to phenol:chloroform extraction

The required DNA fragment was cut out of the agarose gel using a sterile scalpel and placed on a 100 µl bed of glass beads in a 750 µl Eppendorf tube. This tube had a pin hole in the base and was placed within a larger 1500 µl tube. The two tubes were spun at 13,000 rpm for 3 min. The agar remained in the smaller tube and a liquid fraction

containing the DNA was collected in the larger. The liquid was then extracted using the phenol:chloroform protocol.

2.5.2.2 Extraction with phenol : chloroform

Water-saturated phenol (Rathburns, UK) was equilibrated using 100 mM Tris.Cl, pH 7.4 and a 1:1 mixture of equilibrated phenol:chloroform made up. An equal volume of this mixture was added to the nucleic acid sample, and mixed to form an emulsion. The two phases were resolved by centrifuged at 13,000 rpm for 5 min and the aqueous phase removed to a clean tube. This step was repeated and to the isolated aqueous phase of the second extraction an equal volume of chloroform was added to remove any contaminating phenol. Following centrifugation the aqueous phase was removed to a clean tube and precipitated with EtOH (see section 2.5.1).

2.5.2.3 Gene Clean II® Kit (Bio 101 inc, USA)

The protocol used followed the manufacturers recommendations. Three volumes of 6 M sodium iodide was added to the nucleic acid sample. If the sample was in agarose gel then this mixture was heated at 55 °C for 5 min before proceeding to the next step. To the solution 5 µl Glassmilk® (a suspension of silica matrix which binds DNA) was added, and the tube incubated on ice for 5 min. The Glassmilk® DNA complex was then pelleted by a 5 sec centrifugation at 13,000 rpm and the supernatant decanted. The pellet was then washed 3 times in "New Wash". Each wash involved resuspending the pellet in x 50 volume of New Wash followed by centrifugation as above to recover the washed pellet. Following this, the pellet was resuspended in TE and incubated at 55 °C for 3 min to elute the DNA from the silica matrix. The DNA was recovered in the supernatant of a final 30 sec, 13,000 rpm spin.

2.5.3 Estimating nucleic acid purity and concentration.

To determine the concentration and purity of DNA and RNA preparations, 1-10 µl of the solution was diluted to 600 µl in d.H_2O, and placed in a quartz cuvette with a light path of 1 cm. If the DNA was suspended in TE 1-10 µl TE in 600 µl d.H_2O was used as the blank. The absorption of the solution was measured over a UV wavelength scan of 220 nM to 320 nM using a Beckman DU®-64 spectrophotometer (Beckman, USA). The purity of the nucleic acid preparation was estimated by the ratio OD_{260}/OD_{280} (pure DNA=1.8, pure RNA=2.0). The concentration of the sample can be calculated from the observation that an $OD_{260} = 1$ represents approx 50 µg/ ml for double stranded DNA and 40 µg/ ml single stranded DNA or RNA.

2.6 Agarose gel electrophoresis

Agarose is a linear polymer composed of D-galactose and 3,6-anhydro L-galactose units. Agarose forms a gel in which the pore size and therefore the separation properties are determined by its concentration. In this study 0.7% agarose gels were used (effective separation 0.8 kb to 10 kb).

2.6.1 Making agarose gels

Agarose gels were made up as follows. The required amount of agarose (Severn Biotechnology Ltd, UK) was added to the final volume of 1 x TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) and heated in a microwave oven until the solution was clear and transparent. The agarose solution was allowed to cool to approx. 50 °C and the volume corrected for loss through evaporation with d.H_2O. EtBr (10 mg/ml stock solution) was then added to a final concentration of 1 µg/ml and the gel was cast into a gel tray with sample combs in position and allowed to set on a levelling table. After setting, the sample comb was removed from the gel along

with any tape for sealing the gel tray edges. The cast gel in the tray was then placed in a gel tank which had been filled with 1 x TAE to a level approx. 1 cm over the gel surface. All DNA samples prior to electrophoresis had sample buffer added at 1/6 final volume (6 x sample buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15 % Ficoll, in d.H_2O). The wells flanking the sample tracks were always loaded with standards to form a kb DNA "ladder" (0.1 µg/µl working concentration) so that the sizes of the sample DNA fragments could be estimated.

A range of different agarose gels were cast for diagnostic use and for obtaining fragments of DNA for use in subcloning. A summary of the types follows.

2.6.2 Meniscus gels

This small gel was ideal for isolating DNA for subcloning with minimal agarose contamination. To 50 ml of 1 x TAE was added 0.35 g of agarose, and processed as described in section 2.6.1. Twenty-five ml of the agarose/EtBr solution was then poured onto a glass plate producing a gel of 15 cm x 6 cm x 0.3 cm. This gel was used for analysing up to 27 samples of 10 µl or less and run at 8-15 V/cm.

2.6.3 Mini-gels

Agarose (0.35 g) was added to 50 ml of 1 x TAE and treated as in section 2.6.1. The entire 50 ml was then cast into a gel tray (10 cm x 7 cm x 1.5 cm) with an 8 well sample comb in place. The maximal well loading was 25 µl. Minigels were electrophoresed at 7-10 V/cm.

2.6.4 Midi-gels

Midigels allowed the analysis of up to 40 samples of 25 µl or less in one gel and were particularly useful for the analysis of mini-prep DNA. To 150 ml of 1 x TAE was added 1.15 g agarose and processed as described in section 2.6.1. The agarose/ EtBr solution was cast into a gel tray (10 cm x 16 cm x 0.6 cm) and allowed to set. The gel was electrophoresed at 6-10 V/cm.

2.6.5 Analysis of agarose gels

Following electrophoresis DNA bands were visualised by placing the gel on a UV transilluminater (Hybaid) and the image recorded using a high performance CCD camera (Cohu) linked to a video copy processor (Mitsubishi). DNA fragments to be used in subcloning were removed into Eppendorf tubes using a sterile scalpel and purified using one of the methods described in section 2.5.2.

2.7 Manipulating DNA using restriction endonucleases and modifying enzymes.

2.7.1 Restriction endonuclease digests

Restriction endonucleases (restriction enzymes) cleave double stranded DNA at specific sites within or adjacent to a particular sequence. They have a central role in sub-cloning, first in the generation of DNA fragments for incorporation in ligation reactions and second as a diagnostic tool for analysing the products of these ligations.

Typically reaction digests included up to 1 µg of plasmid DNA and 0.2-0.3 units per µl (U/µl) of the appropriate restriction enzyme (1 unit is defined as the amount of enzyme required to digest 1 µg of substrate DNA completely in 60 min under the specified conditions for that enzyme). Restriction enzyme buffer (supplied with the enzyme) was present at 1x

strength and s.d.H_2O added for a final volume of 20 µl. Digest reactions were vortexed then centrifuged briefly and incubated for 2-3 hours at the required temperature (37 °C or 25 °C). The reaction products were analysed by gel electrophoresis (section 2.6).

2.7.2 Filling recessed 5′ and 3′ termini

Recessed 5' and 3' termini created by digestion with certain restriction enzymes, or 3' protruding overhangs generated by the PCR can be filled in or flushed using the Klenow fragment of *E. coli* DNA polymerase I or bacteriophage T4 DNA polymerase (T4 DNA pol), supplemented with the appropriate deoxynucleotide triphosphates (d.NTPs). For 3' to 5' exonuclease activity T4 DNA pol is preferred because of its greater 3'-to-5' activity.

2.7.2.1 Klenow fragment of DNA polymerase I

After completion of the RE digest, d.NTPs were added to the reaction at a final concentration of 1 mM. One µl of Klenow fragment (5 U/µl) was added, the tube contents mixed and incubated at room temperature for 15 min. Following incubation, the Klenow and restriction enzymes were inactivated by heating at 75 °C for 10 min and the DNA was then extracted with phenol:chloroform (section 2.5.2.2).

2.7.2.2 Bacteriophage T4 DNA polymerase

The PCR product (40 µl) was mixed with d.NTP's to a final concentration of 100 µM and 1.5 µl of T4 DNA pol (1 U/µl) and mixed well. The reaction was incubated at 12 °C for 15 min and the modified DNA recovered by phenol:chloroform extraction (section 2.5.2.2).

2.7.3 Calf intestinal alkaline phosphatase (CIAP)

CIAP catalyses the hydrolysis of phosphate monoesters from a variety of substrates. CIAP was used to remove the 5' phosphates from linearised plasmids prior to their incorporation in ligation reactions to prevent their self-ligation. Up to 1 µg of linearised DNA was treated with 1 µl (1 U/µl) CIAP, 2 µl of x10 CIAP buffer (supplied with CIAP) in a final volume of 20 µl with s.d.H_2O. The tube was incubated at 37 °C for 30 min and following this CIAP was inactivated by heating at 75 °C for 10 min or removed by phenol:chloroform extraction (section 2.5.2.2).

2.8 Ligation reactions

The joining of linearised DNA (i.e. plasmid vector and gene or cDNA insert) used bacteriophage T4 DNA ligase. The enzyme catalysed the joining of cohesive or blunted termini of double stranded DNA molecules, by ligating a 5' terminus carrying a phosphate group to a 3' terminus hydroxyl group.

To promote the formation of useful recombinant molecules the concentration of the two DNA fragments in the ligation reaction was adjusted to give an insert:vector molar ratio of 3:1 for cohesive end ligation and 10:1 for blunt end ligations.

A typical ligation experiment using phosphatased vector DNA included four separate reactions:

Control 1: To determine the frequency of self-ligation
unphosphatased vector, - DNA ligase

Control 2: To test efficiency of DNA ligase
unphosphatased vector, + DNA ligase

Control 3: To test the efficiency of CIAP activity
phosphatased vector, + DNA ligase
Ligation reaction: phosphatased vector, + insert + DNA ligase

The final volume of each reaction was typically 20 µl; this included no more than 500 ng total DNA, 1.5 µl of 1 U/µl T4 DNA ligase and 1x strength ligase buffer; the volume was made up with s.d.H_2O. Ligation reactions were incubated at 15 °C overnight. A 2-10 µl aliquot of each reaction was then transformed into *E.coli* (section 2.4).

2.9 Polymerase Chain Reaction (PCR)

The PCR was used to simultaneously amplify the apoaequorin cDNA and incorporate a *Nde*I site at each end. This was achieved by using specifically designed oligodeoxynucleotide primers which incorporate the restriction enzyme site as a 5' add-on (section 3.2.1.2).

The PCR was carried out in a volume of 50 µl, it contained 50 pg target DNA , 0.92 µg forward primer (721), 1.05 µg reverse primer (722), 2.5 U of Taq polymerase, 1x strength TAQ polymerase buffer (supplied with enzyme), 200 µM d.NTPs and s.d.H_2O to the final volume. Mineral oil (50 µl) (Sigma, UK) was overlaid on to each reaction to prevent evaporation. Two controls were included in the PCR experiment; one omitted the target DNA, the other used another primer which had previously been used to amplify the target. Thermocycling was accomplished using a Hybaid Omnigene Thermal Cycler (Hybaid, UK), and the following programme which had been tested previously with this target DNA (M.Knight, pers. comm.) was used.

Cycle 1:	95 °C, 5 min; 40 °C, 1 min; 70 °C, 2 min
Cycle 2:	95 °C, 3 min; 40 °C, 1 min; 70 °C, 2 min

Cycle 3:	95 °C, 1 min; 40 °C, 1 min; 70 °C, 2 min
Cycles 4-30:	95 °C, 25 sec; 40 °C, 1 min; 70 °C, 2 min
Cycle 31:	95 °C, 30 sec; 40 °C, 1 min; 70 °C, 5 min

Following thermal cycling the reaction products were examined by gel electrophoresis (section 2.6).

2.10 Transformation of *N. crassa*

Plasmid DNA was transformed into *N. crassa* using two methods; chemical transformation using polyethylene glycol with $CaCl_2$ and by electroporation. Both procedures involved the formation of spheroplasts prior to transformation.

2.10.1 Spheroplast formation

Spheroplasts formation (see Fig. 2.2) was based on the method of Vollmer and Yanofsky (1986) using germinated macroconidia (referred to as conidia). Conidia were isolated from a swirl flask (section 2.2.1.3.2.2) by adding 30 ml of s.d.H_2O to the culture swirling gently and then pouring the conidial suspension through a double layer of sterile muslin into a 50 ml screw cap polypropylene tube. Conidia were washed twice in s.d.H_2O by centrifugation at 2500 rpm for 3 min (MSE Mistral 1000, UK) and finally resuspended in 5 ml s.d.H_2O. The conidia were germinated in a shaking liquid culture (section 2.2.1.3.2.3). The germinated conidia were washed twice in s.d.H_2O and then resuspended in 10 ml of 1 M sorbitol (osmotic protectant) and placed in a sterile 250 ml flask. Five ml of 5 mg/ml filter sterilised Novozyme 234 (Novo Nordisk, Denmark) in 1 M sorbitol was added to the spheroplast suspension and this was incubated at 30 °C for 1 hour shaking at 100 rpm. The spheroplast mix was decanted into a 50 ml polypropylene tube and the volume made up to 50 ml with 1 M sorbitol and centrifuged at 2500 rpm for 3 min to concentrate the cells. After removing the supernatant the pellet was resuspended in 50 ml of 1 M

sorbitol and centrifuged. The pellet was then resuspended in 50 ml STC mix (1 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris.Cl, pH 8.0) for the PEG + $CaCl_2$ transformation or 1 M sorbitol for electroporation.

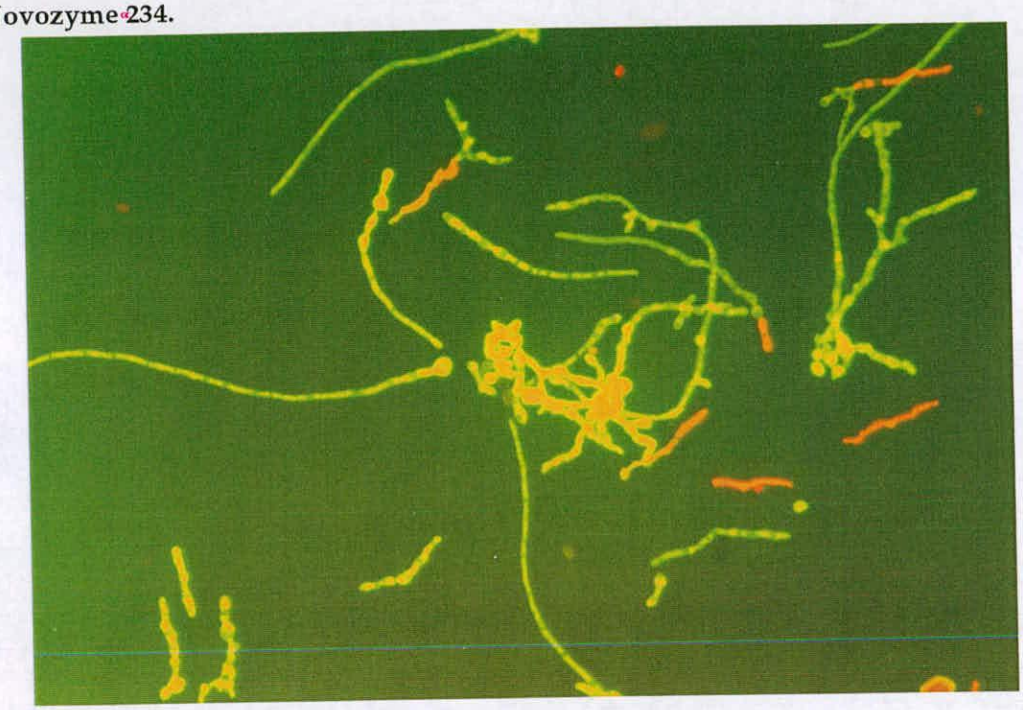
2.10.2 PEG + $CaCl_2$ transformation and transformant regeneration

The method was based on a modified method of Vollmer and Yanofsky (1986) (Dr. J. Bond, pers. comm.). Isolated spheroplasts were washed once in 50 ml STC mix then resuspended in STC mix. One quarter of that volume of PTC mix (40% w/v PEG 3350, 50 mM $CaCl_2$, 50 mM Tris.Cl, pH 8.0) was then added together with dimethyl sulphoxide (DMSO) to a final concentration of 1%. The conidial density was determined by a haemocytometer count. In an Eppendorf tube 1 µl (2 µg) of plasmid DNA was mixed with 2 µl of 50 mM spermidine.3HCl and 5 µl of 5 mg/ml heparin (in 1 M sorbitol) and placed on ice. An aliqout of 100 µl (approx. $1x10^7$) of the spheroplast suspension was added to the Eppendorf tube and incubated on ice for 30 min. One ml of PTC mix was added and the suspension incubated at room temperature for 20 min.

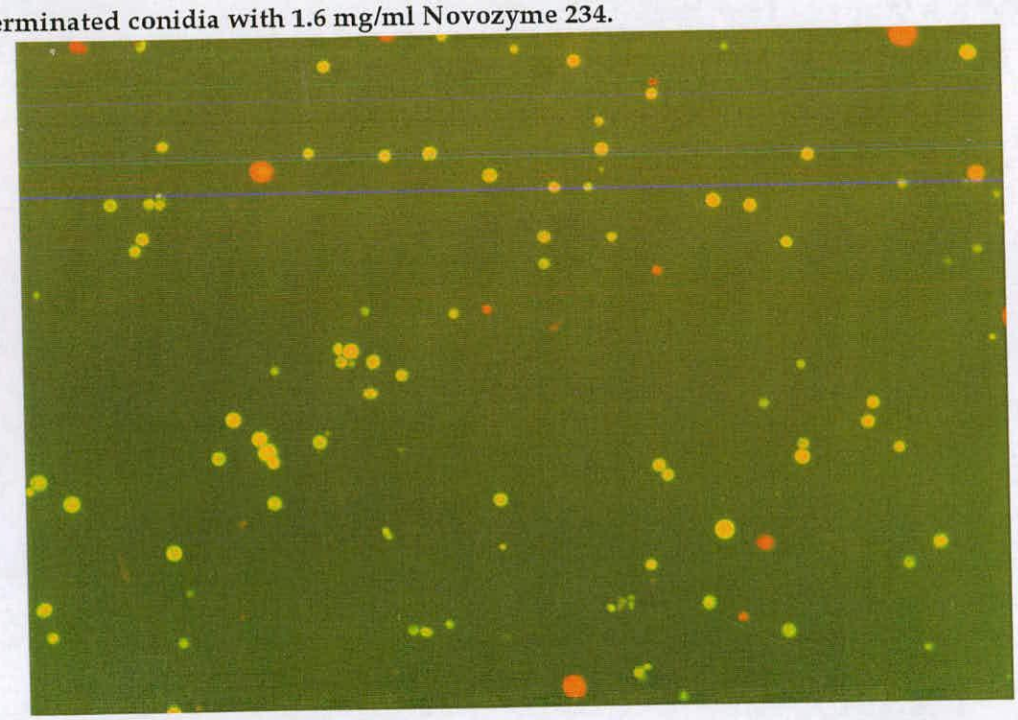
For the regeneration of transformants a dilution series of the transformation mix was made in 1 ml lots of 1 M sorbitol. The 1 ml dilutions were each added to 15 ml of molten agar (approx. 55 °C) containing 1x Vogel's medium N, 0.5% glucose, 0.5% fructose, 2% sorbose and 1 M sorbitol and mixed by inversion. This overlay was then poured onto a 1x Vogel's medium N, 0.5% glucose, 0.5% fructose, 2% sorbose agar plate supplemented with 200 µg/ml hygromycin B (Boehringer Mannhein). The plates were incubated at 25 °C in darkness and transformant growth was visible after 2-3 days. Transformants that showed strong growth were removed from the plate and inoculated onto a VS agar slant supplemented with 200 µg/ml hygromycin B (VSH slant).

Figure 2.2: Spheroplast formation in *N. crassa*. 74*A*.

A: alive/dead staining of germinated conidia (in d.H_2O) prior to treatment with Novozyme 234.



B: alive/dead staining of spheroplasts (in 1 M sorbitol) formed from the treatment of germinated conidia with 1.6 mg/ml Novozyme 234.



Transformants were designated as stable if they showed strong growth and conidiation on slants.

2.10.3 Transformation by electroporation of spheroplasts

Efficient electrotransformation of *N. crassa* spheroplasts was developed in this research programme, and is presented in section 3.3.

2.11 Analysis of apoaequorin by immunodetection

2.11.1 Protein extraction

Transgenic and untransformed *N. crassa* was inoculated into standing liquid culture (section 2.2.1.3.2.4) and incubated for 41 hours at 25 °C. A number of different extraction protocols were tested, and the following yielded consistently good extracts. The mycelium was separated from the culture medium by filtering through muslin and then rinsed in d.H_2O. Excess water was removed by pressing the mycelium between sheets of Whatman 3 MM paper and the damp mycelium was weighed, and ground with sand in 1x Laemelli sample buffer excluding bromophenol blue (1.25% SDS, 10% glycerol, 100 mM dithiothreitol, 60 mM Tris.Cl, pH 6.8) at a 3:7 tissue to buffer ratio. The slurry was transferred to an Eppendorf tube, vortexed for 30 sec and centrifuged at 13000 rpm for 5 min. The supernatant was removed to a new tube, snap frozen in liquid nitrogen and stored at -70 °C until use. The protein concentration of extracts was measured using the Bradfords protein assay (Bio-Rad, UK)(section 2.11.2)

2.11.2 Quantification of total soluble protein in extracts.

Quantification of total protein in the mycelial extracts used the Bio-Rad Bradford's Protein Assay (Bio-Rad, UK)(Bradford, 1976). The assay was based on the observation that the absorbance maximum for an acidic

solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein and that it shows differential colour change in response to differing concentrations of protein. The assay was performed using bovine serum albumin (BSA) as the protein standard. Dilution of samples (1:10 or 1:20) was made in d.H_2O. The appropriate buffer at the same dilution was used as the sample blank. BSA standards were made from a 1.4 mg/ml stock in the range 70 µg/ml and 630 µg/ml. One ml of dye reagent (diluted 1 part dye to 4 parts d.H_2O) was then added to each tube, mixed gently, incubated for 5-20 min and measured at OD_{595} versus the reagent blank (d.H_2O plus dye). Plotting the OD_{595} against the known concentration of the standards allowed unknown sample concentrations to be read from this standard curve (standard range 14 µg-126 µg protein). Actual protein concentration was obtained by adjusting for the dilution factor.

2.11.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

2.11.3.1 Large SDS-PAGE gels

Large SDS-PAGE gels were cast into the space between two glass plates clipped together and separated along three edges by three 3 mm deep spacers (gel dimensions 22.5 cm x 17.5 cm x 0.3 cm). Where two spacers came into contact, the joint was sealed with silicon grease. Leakage from this casting system was checked by filling with d.H_2O. A Teflon well comb supported by the top of two spacers was positioned between the gel plates and a line 3 cm below the comb teeth was marked on the glass plate.

A 50 ml 10% (linear separation range 16-68 kD) resolving gel was made by mixing together 20 ml d.H_2O, 16.6 ml 30% w/v acrylamide / *bis*acrylamide (29:1) (Anachem, UK), 12.5 ml 1.5 M Tris base (pH 8.8) and 0.5 ml of 10% SDS. This was followed by 0.5 ml of 10% ammonium persulphate and 20 µl of N,N,N',N'-tetramethylethylenediamine (TEMED) the initiator of polymerisation. The solution was mixed

following TEMED addition and immediately poured into the gel space up to the marked line. Water-saturated butanol was overlaid onto the gel solution to remove any air bubbles and the gel was allowed to set for 1 hour. After setting, the water-saturated butanol was removed and a 5% stacking gel solution was poured on top of the setting gel. The stacking gel was prepared in the same way as the separating gel with 6.8 ml $d.H_2O$, 1.7 ml 30% w/v acrylamide/*bis*acrylamide (29:1), 1.25 ml of 1.0 M Tris (pH 6.8), 100 µl of 10% SDS, 100 µl of 10% ammonium persulphate and 10 µl of TEMED (total volume 10 ml). The comb was immediately inserted, a thin layer of water-saturated butanol applied to the exposed gel surface, and the gel was allowed to set (30 min). After polymerization, the comb and bottom spacer were removed and the wells rinsed with $d.H_2O$.

2.11.3.2 SDS-PAGE minigels

SDS-PAGE mini-gels replaced the large gels in later experiments as up to five could be made at one time in a casting system (Biotechnology Instruments Ltd, UK) with minimal leakage, smaller volumes of the gel solutions were required and electrophoresis was quicker. Mini-gels (7.5 cm x 9.5 cm x 0.5 cm) were used as either 10% (as above) or 15% (linear separation range 12-43 kD) gels. The composition of a 50 ml solution of a 15% gel was 11.4 ml $d.H_2O$, 25 ml 30% w/v acrylamide/*bis*acrylamide (29:1), 12.5 ml 1.5 M Tris base (pH 8.8), 0.5 ml of 10% SDS, 0.5 ml of 10% ammonium persulphate and 20 µl of TEMED. The stacking gel was identical to that used in the large gels. Gel solutions were made up in the same way as for large gels but included an additional step of filter-sterilising the solution prior to the addition of TEMED.

2.11.4 Gel electrophoresis of proteins

Both gel types were treated in an identical way in relevant vertical electrophoresis apparatus. The gel was clipped to the electrophoresis tank

with the wells upright and then the upper and lower buffer reservoirs of the electrophoresis equipment were filled with 1x Laemmli running buffer (25 mM Tris base pH 8.3, 250 mM glycine, 0.1 % SDS). The wells were cleaned of any excess gel matrix by washing with running buffer through a 10 ml syringe, and any air bubbles trapped at the lower end of the gel were removed using a 10 ml syringe and bent needle. Samples and protein markers were combined with 5% v:v bromophenol blue solution. They were then heated at 100 °C for 5 min, pulse-centrifuged to reincorporate any condensation into the solution, and then loaded on to the gel using a Hamilton syringe. The quantity of protein samples loaded into each well varied between gels but was standard for each gel. Molecular weight markers either Rainbow Markers (Amersham, UK) or SDS-7 markers (Amersham, UK) were used at 4 µl and 6 µl respectively. Large gels were run at 2.6 V/cm until the dye line had left the stacking gel and then increased to 4.4 V/cm through the separating gel. Minigels were run at 5.3 V/cm and 13 V/cm respectively. Both gels were run until the dye line was within 0.5 cm of the bottom of the gel.

2.11.5 Transfer of proteins to a solid support

Transfer of electrophoresed proteins to a solid support was achieved using wet electrophoretic elution (Towbin *et al.*, 1979). One sheet of Hybond-ECL™ nitrocellulose membrane (Amersham, UK) and four sheets of Whatman 3 MM paper were cut so that they were slightly larger than the gel. The nitrocellulose was allowed to wet in d.H_2O by capillary action and then submerged for 2 min. The membrane was then soaked for 5 min in transfer buffer (25 mM Tris.Cl, 380 mM Glycine, 0.1% SDS, 20% methanol). The gel, filter paper, and four support pads were also soaked thoroughly in transfer buffer. With care to remove any air bubbles, the Western transfer sandwich was constructed as follows. Two support pads were placed on a plastic frame and two sheets of filter paper

were laid on top, the gel was positioned on the paper and the nitrocellulose overlaid on to the gel. Two sheets of filter paper were placed on the nitrocellulose and two support pads on top of this. The frame was clipped shut to ensure the components were held tightly together and this transfer assembly was soaked in transfer buffer for 5 min. The transfer assembly was placed in the transfer tank which had been filled with transfer buffer with the membrane closest to the anode. Large gel transfer was carried out at 50 V overnight at 4 °C, mini-gels were transferred for 1 hour at 120 V.

Following transfer, the gel was stained with Coomassie Blue (section 2.11.7) and the filter stained with Ponceau S (section 2.11.6) to verify transfer.

2.11.6 Staining immobilised proteins with Ponceau S

Concentrated Ponceau S (3-hydroxy-4-[2-sulfo-4(sulfo-phenylazo)phenylazo]-2,7-naphthalene disulfonic acid) solution (2% Ponceau S, 30% trichloroacetic acid, 30% sulfosalicylic acid) was diluted 1 part in 10 with d.H_2O. The nitrocellulose filter was washed once and then incubated for 10 min in this working solution. Following several washes in Tris buffered saline (20 mM Tris base, 137 mM NaCl, pH 7.6) the protein tracks were discernible and the position of the molecular weight standards marked in pencil. The stain did not need to be removed prior to the immunodetection protocol.

2.11.7 Staining gels

Following Western transfer, the polypeptides remaining on the gel were simultaneously fixed and stained in filtered Coomassie Brilliant Blue solution (0.25 g Coomassie Brilliant Blue R250, 10 ml glacial acetic

acid, 90 ml 1:1 d.H_2O : methanol), by immersion for 2-3 hours on a slowly rotating platform. After staining, the gel was removed to a clean container rinsed in 100% EtOH and 100 ml of destain (10% glacial acetic acid, 90% 1:1 H_2O : methanol) was added. After 30 min incubation the destain was removed and 100 ml fresh destain applied. Small pieces of sponge were included in the second destain incubation of over 8 hours, the sponge absorbed the dye as it was leached from the gel. After destaining the gel was dried.

2.11.8 Drying gels

To obtain a permanent record of each protein gel following staining, it was dried onto a solid support. Large gels were laid out on a piece of wet Whatman 3 MM paper which was slightly larger than the gel. This was then covered in Saran™ Wrap (Dow Chemical Company, USA) and dried under vacuum in a gel dryer (Bio-Rad, UK).

Small gels were dried between two layers of cellophane (Biorad, UK). The membrane was soaked in d.H_2O, and laid over a sheet of Perspex half the membrane size. The gel was positioned on this and the membrane was folded back over the gel, care being taken to remove any air bubbles. A perspex frame was placed over this and the assembly clipped together. After several days the assembly had dried and the protected gel could be removed.

2.11.9 Immunological detection of immobilised proteins using ECL™ Western Blotting (Amersham, UK)

For the detection of apoaequorin immobilised on nitrocellulose filters the ECL™ Western Blotting (Amersham, UK) kit was used. The principle of this detection system was to directly conjugate the antigen via a primary

antibody (mouse antiaequorin antisera) with a horseradish peroxidase-labelled secondary antibody (sheep antimouse antisera). In the presence of hydrogen peroxide, horseradish peroxidase catalyses the oxidation of luminol, the subsequent decay from its excited to its ground state yields light (λmax = 528). The chemiluminescence generated by this reaction was used to detect the presence of the antigen using blue light sensitive autoradiography.

Application of the manufacturer's protocol to the detection of apoaequorin required some optimisation. The method described below is the optimised protocol which yielded an apoaequorin signal.

To prevent non-specific adsorption of immunological reagents to the nitrocellulose membrane, it was blocked overnight with a solution of 5% low fat dried milk in TBS-T (TBS + 0.2% Tween™ 20) at 4 °C. All further incubations were carried out at room temperature. After blocking the filter was rinsed twice in TBS-T, and washed 1 x 15 min, 2 x 5 min in TBS-T. The primary antibody was diluted to 1:1000 in TBS-T in a final volume of 40 ml and the filter was incubated in this for 1 h. Following primary antibody binding the filter was rinsed twice in TBS-T and washed 1 x 1 h and 2 x 10 min in TBS-T. The secondary antibody was diluted in 40 ml TBS-T to 1 : 5000 and the filter incubated in this for 1 h. Washing steps were identical to those used after primary antibody incubation. For detection of the labelled antigen equal amounts of the two detection reagents were mixed together to cover the filter with 0.125 ml/cm^2. The filter was removed to a fresh container and the detection reagent mix applied to the surface carrying the protein and incubated for 60 sec. The detection reagent was drained off and the filter sealed between two layers of Saran™ wrap (Dow Chemical Company, USA) . Autoradiography was performed using the blue light sensitive Hyperfilm-ECL™ (Amersham, UK). Exposure times were determined empirically.

2.11.10 Stripping filters for reprobing

Primary and secondary antibodies were completely removed from the membrane by submerging it in stripping buffer (100 mM ß-mercaptoethanol, 2% SDS, 62.5 mM Tris.Cl, pH 6.7) and incubating for 30 min at 60 °C. Following this treatment the membrane was washed for 2 x 10 min in 200 ml of TBS, and the blocking and immunodetection protocols could then be repeated.

2.12 *In vitro* constitution and luminometry of aequorin from protein extracts

2.12.1 Luminometer

Luminometry was initially performed using a digital chemiluminometer equipped with a 9757AM 1" diameter photomultiplier tube (PM tube) at 1150 V with a discriminator, and linked to an 8 digit scaler. Later this was replaced with an integrated system (Thorn EMI, UK) containing a 2" diameter photomultiplier tube (type 9829A) which was maintained at -20 °C to minimise thermal noise in a FACT 50 air cooled housing. The PM tube was linked via an amplifier discriminator (AD2) to a counter timer board in an IBM-based computer. The optimal operating voltage of the PM tube was determined by using computer software designed specifically for this purpose (Thorn, EMI, UK). The optimal (knee) working voltage was 1550 V, the greatest signal to noise ratio was obtained at 1300 V. The optimal working voltage was used throughout this work.

2.12.2 Coelenterazine

Coelenterazine and synthetic coelenterazines were purchased from Cambridge Bioscience (UK). It was essential that any manipulation of coelenterazine was performed in near-darkness. Large quantities were separated into smaller aliquots by dissolving in cold 100% MeOH dispensing the solution into Eppendorf tubes using a Hamilton syringe and drying down under vacuum. Tubes were wrapped in aluminium foil and stored at -70 °C. Before use coelenterazine was dissolved in 100% MeOH prior to the addition of AR buffer for *in vitro* experiments, or for *in vivo* experiments 1M sorbitol, Tris buffered sorbitol (1 M sorbitol, 10 mM Tris.Cl pH 7.6) or 1 x Vogel's medium N.

2.12.3 Protein extraction and *in vitro* constitution and luminometry of aequorin from different sources

2.12.3.1 *N. crassa*

Mycelia from *N. crassa* were grown for the required period in still liquid culture (section 2.2.1.3.2.4). The mycelium was extracted with either acid-washed sand (section 2.11.1) using an optimised w:v ratio (see section 4.XX) in aequorin constitution (AC) buffer (10 mM EGTA, 50 mM Tris. Cl, pH 7.4, 500 mM NaCl, 10 mM β-mercaptoethanol, 0.1% BSA, in HPLC H_2O) or in liquid nitrogen. HPLC purifed H_2O contained less contaminating Ca^{2+} than normal d.H_2O. Liquid nitrogen extraction was used for time course studies. Cultures were strained, rinsed, blotted dry, and weighed. They were then placed in separate aluminium foil wallets, frozen in liquid nitrogen and stored at -70 °C. Protein extraction was performed by adding the frozen mycelium to a pre-cooled pestle and mortar containing liquid nitrogen and grinding it to a fine powder. The powder was transferred to a 15 ml polypropylene tube containing AC buffer, vortexed for 30 sec then aliquoted into Eppendorf tubes and

centrifuged at 13,000 rpm for 5 min. The supernatants for individual samples were recombined in a clean Eppendorf tube and stored in 100 to 200 µl aliquots at -70 °C. Protein quantification used the Bradfords Bio-Rad Protein Assay (section 2.11.2).

Optimisation of *N. crassa* aequorin *in vitro* constitution and luminometry is presented in section 4.4. A preliminary assay used the method for *N. plumbaginifolia* aequorin.

2.12.3.2 *N. plumbaginifolia*

In vitro constitution and luminometry was performed on apoaequorin-transformed tobacco extracts following the method of Knight *et al.* (1991b). Protein was extracted from *N. plumbaginifolia* leaf in a 10% w/v ratio in AC buffer. Quantified *N. plumbaginifolia* extract was added to AC buffer and 10 µl of 25 µM coelenterazine solution, in a final volume of 100 µl. Reactions were incubated for 3 hours at 25 °C in darkness. A dilution (1:10) was made in TE pH 7.0 (200 mM Tris.Cl, 0.5 mM EDTA, pH 7.0) and light emission measured on the addition of 100 mM $CaCl_2$ in the luminometer.

2.12.3.3 Recombinant aequorin

Recombinant aequorin was purchased from Molecular Probes. Aequorin was stored in 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 40 mM EDTA at -70 °C, however over prolonged storage breakdown occurred necessitating its constitution with fresh coelenterazine. This was achieved by incubating aliquots in 1.25 µM coelenterazine for 4 hours.

2.13 Formation of homokaryons

Macroconidia are mainly multinucleate (average = 2-3 nuclei), and therefore it was assumed that most of the primary transformants would be heterokaryotic i.e only one nucleus out of several would be transformed. As *N. crassa* is haploid a transformed genome can be purified by extracting a homokaryon state. Two methods were employed, the isolation of hygromycin-resistant progeny from sexual crosses and the serial isolation of hygromycin-resistant macroconidia.

2.13.1 Sexual crossing of *N. crassa* primary transformants

Crossing slants were produced as in section 2.2.1.3.2.6. Individual primary transformants were all *A* mating type. A conidial inoculum was diluted in 5 ml s.d.H_2O and a loop of this suspension was spread over the 73a cultures. Slants were incubated for 18 days after which time ripe ascospores had been ejected from the asci onto the side of the test tube. Ascospores were isolated by adding 2 ml of s.d.H_2O to the tube and using a platinum wire to dislodge them. The suspension was decanted into a clean tube and centrifuged briefly to sediment the ascospores free of any conidia. The ascospores, resuspended in s.d.H_2O were heat shocked at 60 °C for 1 hour and then plated onto 1x Vogel's medium N with 0.05% glucose, 0.05% fructose, 2% sorbose and 200 µg/ml hygromycin B and incubated at 25 °C. Single colonies were isolated using a dissecting microscope and transferred onto VSH slants.

2.13.2 Serial transfer of macroconidia

Conidia from a primary transformant were inoculated into 5 ml of s.d.H_2O, the suspension was mixed by vortexing and a 200 µl aliquot of the suspension was spread on to a 1x Vogel's medium N, 0.05% glucose, 0.05% fructose, 2% sorbose, 200 µg/ml hygromycin B agar plate. Plates were

incubated at 25 °C in darkness for 3 days. Isolated colonies from these plates were inoculated onto VSH slants and incubated in continuous illumination at 27 °C for 7 days to obtain sufficient conidia for the second round of single colony isolation. The plating and colony isolation procedure was repeated 3 times for individual primary transformants. After 3 serial isolations the probability of the strain remaining heterokaryotic is likely to be 10% or less.

2.14 Southern analysis of genomic DNA from *N. crassa*

2.14.1 DNA extraction from *N. crassa*

Still liquid cultures of individual transformants were grown up (section 2.2.1.3.2.4). Culture contents were poured onto two layers of sterile muslin supported over a 1 l plastic beaker to separate the mycelium from the growth medium. The mycelium was washed in 300 ml of s.d.H_2O and excess water was removed by squeezing the mycelium against the cheesecloth with a pair of sterile forceps. The mycelium was then added to a mortar which had been pre-cooled and filled with liquid nitrogen. The mycelium was ground with liquid nitrogen and transferred to a 50 ml polypropylene tube containing 15 ml of DNA extraction buffer (10 mM EDTA, 100 mM NaCl, 0.5% SDS, 10 mM ß-mercaptoethanol, 40 mM Tris.Cl, pH 8.0). The tube was vortexed for 30 sec, snap-frozen in liquid nitrogen and stored at -70 °C. Samples were thawed rapidly in a 65 °C water bath to minimise exposure to nucleases and immediately 1 volume of water saturated phenol (equilibrated to pH 7.4 with 100 mM Tris.Cl, pH 7.4) was added. The tube was shaken well and centrifuged at 3600 rpm for 3 min, the aqueous phase was removed to a clean tube and a second phenol extraction performed. This was followed by a single chloroform extraction to remove contaminating phenol. The DNA was precipitated with 10% v:v 3 M NaAc, pH 5.5, and two volumes 100% EtOH. The DNA was recovered by pelleting it by centrifugation at 3600

rpm for 3 min. After desiccation the DNA pellet was redissolved in 500 µl TE (10 mM Tris.Cl, pH 7.6, 1 mM EDTA pH 8.0). A 250 µl aliquot of the sample was then incubated with 2 µl of RNA-ase (10 U/ µl) at 37 °C for 30 min; this was followed by the phenol:chloroform extraction procedure described in section 2.5.2.2 to remove contaminating protein. An UV wavelength scan was performed (section 2.5.3) to estimate the concentration and purity of the genomic DNA and gel electrophoresis (section 2.6) used to examine its integrity.

2.14.2 Restriction enzyme digests of genomic DNA

Genomic DNA (10 µg) was digested with 15 U of the appropriate restriction enzyme and 1x buffer in a final volume of 20 µl in d.H_2O. After 3 hours an additional 15 U of enzyme was added and the reaction incubated for a further 2-3 hours. A 2 µl aliquot of the reaction was examined by gel electrophoreis for complete DNA digestion and concentration estimation.

2.14.3 Gel electrophoresis of genomic DNA

Samples for Southern analysis were resolved in large (20 cm x 20 cm x 0.6 cm) gels of 0.7% agarose in 1 x TAE. Gels were poured on a level table to ensure equal distribution of the gel matrix across the tray. The gels were electrophoresed at 2-3 V per cm overnight.

2.14.4 Transfer of DNA to nylon membranes

The method of capillary transfer of DNA to a solid support was based on the method developed by Southern (1975). Following electrophoresis the gel was stained with 10 µg/ml EtBr in 1 x TAE for 45 min and photographed under UV illumination. The gel was inverted and soaked in 800 ml 0.25 M HCl for 10 min shaking at approx. 30 rpm. This

treatment of brief depurination nicks DNA strands thereby enhancing the transfer of large fragments to the membrane. The 0.25 M HCl solution was removed and the gel was rinsed in briefly in d.H_2O. Following this the gel was soaked in 800 ml of denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 45 min shaking as before, to denature the DNA *in situ*. The solution was removed, the gel rinsed briefly in d.H_2O and placed in 800 ml of neutralisation solution (1 M Tris, pH 7.4, 1.5 M NaCl) for 30 min with gentle agitation.

The blot was constructed as follows. A piece of Whatman 3 MM paper was wrapped around a perspex support and placed inside a larger plastic container. The container was filled with 10 x SSC (20 x SSC is 175.3 g NaCl, 88.2 g Na.citrate in 1 l, adjusted to pH 7.0) to a depth of 2 cm and the 3 MM paper was allowed to wet by capillary action. Any air bubbles were removed using a glass rod. A sheet of Hybond™ N nylon membrane (Amersham, UK) was cut so that it was slightly larger than the gel and immersed in a dish of d.H_2O for 5 min, prior to it being soaked in 10 x SSC. The inverted gel was placed on the support and any air bubbles between it and the 3 MM paper were removed using a glass rod; overhanging sheets of parafilm were then placed on the gel edges to prevent the blot from short-circuiting. The nylon membrane was placed on top on the gel, with its edge aligned with the top of the gel or the wells if the portion of the gel above the wells had been removed. As before, any air bubbles were removed. Two pieces of 10 x SSC wetted 3 MM paper were placed on top of the membrane, and air bubbles removed. An 8 cm stack of dry paper towels surmounted by a glass plate was placed on top and the whole assembly was weighed down with a 500 g weight. Blotting was allowed to proceed for between 4 and 16 h after which the blot was dismantled, the position of the gel wells marked on the filter and the filter rinsed briefly in 2 x SSC. The filter was allowed to air-dry and the DNA was fixed by

exposing the filter DNA side up to UV irradiation at a total energy dose of 0.4 J/cm^2(λ = 312).

2.14.5 Probe hybridisation

Hybridisation of a radioactive probe to the immobilised DNA followed the procedure described in section 2.15.

2.14.6 Washing hybridised filters of immobilised DNA

Following hybridisation the probe solution was removed and stored at - 20°C. The filter was then washed in the hybridisation bottle using a series of washes going from low to high stringency.

(A) 2 x 30 min in 4 x SSC + 1% SDS at 65°C

(B) 2 x 30 min in 2 x SSC + 0.5% SDS at room temperature

(C) 1 x 30 min in 1x SSC at room temperature

The volumes used in the washes were for A, 100 ml for each wash; B and C, 200 ml for each wash.

After washing the filter was air-dried and sealed in Saran™ wrap. Autoradiography (section 2.16) was performed and in certain cases phosphoimagery (section 2.17).

2.15 Radioactive probing of immobilised nucleic acids

Handling and disposal of radioactive isotopes was according to the regulations and advice of the Radiation Committee of the University of Edinburgh.

2.15.1 Probe synthesis using the random primer labelling method

The protocol used was based on the method for radiolabelling DNA restriction endonuclease fragments to high specific activity (Feinberg and Vogelstein, 1983, 1984). Approx 200 ng of apoaequorin cDNA isolated from the plasmid pAEQ1 (see section 3.2.1.2) was added to 19 µl of s.d.H_2O so the final volume of the reaction would be 30 µl, and was boiled for 5 min to denature the DNA. Six µl of 5 x oligonucleotide-labelling buffer (oliB) (250 mM Tris.Cl, pH 8.0, 25 mM MgCl, 5 mM ß-mercaptoethanol, 2 mM of dATP, dTTP, dGTP, 1 M HEPES, pH 6.6, 1 mg/ml random hexamers) was then added followed by 0.5 µl of 20 mg/ml BSA, 1.5 µl of Klenow (5 U/µl) and finally 30 µCi (3 µl) of [α^{32}P] dCTP (3 Ci µmole^{-1}). The reaction was incubated at 37 °C for 1 hour and stored at -20 °C until use.

2.15.2 Probe purification

The radioactive probe was purified by running the reaction mix through a column of G-50 Sephadex (Pharmacia, UK), which retains the unincorporated nucleotides but allows the newly synthesised probe to pass through. The column was set up in a 1 ml syringe barrel, with a small piece of glass paper to retain the Sephadex at the bottom. TE, pH 7.6, was added into the column using a Pasteur pipette until it passed through the paper. A slurry of G50-sephadex in TE was added to the syringe barrel until it was 0.5 cm from the top. TE was then added to stop the column from running dry. The column was fixed in a clamp stand. The radioactive probe mix was loaded into the top of the column followed by the continued addition of TE. The two fractions of the column were monitored using a Geiger counter. The highly radioactive probe fraction (approx 500 µl) was collected in an Eppendorf tube.

2.15.3 Hybridisation of the probe to immobilised nucleic acids

Hybridisation was carried out in a Hybaid HB-OV-BL hybridisation bottle (Hybaid, UK). When more than one filter was subjected to identical hybridisation, a piece of nylon mesh (20 cm x 20 cm, pore size 100 μM) was placed between the filters.

Filters were prehybridised to prevent non-specific binding in 35 ml of hybridisation buffer (4x SSC, 20 mM Tris.Cl, pH 7.5, 10% dextran sulphate, 2Dehardts solution) (Denhardt's solution contains 1% polyvinylpyrrolidone, 1% bovine serum albumin, and 1% Ficoll in s.d. H_2O) for 1 h at 65 °C. The probe was denatured by boiling for 5 min and then added to 15 ml of hybridisation buffer. This immediately replaced the pre-hybridisation solution and the filter and probe were incubated together overnight rotating at 65 °C in a Hybaid dual hybridisation oven (Hybaid, UK).

2.16 Autoradiography

The probed filter sealed in Saran™ wrap was placed in a light-tight developing cassette with an image intensifying screen (Dupont, USA). A piece of Dupont Cronex 4 medical X-ray film (Dupont, USA) was placed on top of the filter and exposed at -70 °C for between 4 and 24 h depending upon the level of radioactivity, to determine the optimal exposure time. Following exposure the film was processed using an automated X-ray developer.

2.17 Phosphoimagery

Quantification of bands of radioactive nucleic acids was carried out using the Molecular Dynamics phosphoimager and Image Quant™ software package

2.18 Northern Analysis of Total RNA

2.18.1 Total RNA extraction

All procedures involving formaldehyde were carried out in a fume hood.

Forty-three hour-old mycelium was washed and weighed as in the Southern protocol (section 2.14.1). The mycelium was then ground in liquid nitrogen using a pre-cooled pestle and mortar and immediately transferred into a 50 ml polypropylene tube which contained RNA extraction buffer (150 mM LiCl, 5 mM EDTA, 50 mM Tris. Cl, pH 9.0) so that the w/v ratio was 1:5. This was vortexed briefly and 10% sodium dodecyl sulphate (SDS) was added until the SDS:buffer ratio was 1:3. The suspension was mixed gently. The sample was snap-frozen in liquid nitrogen and stored at -70 °C. Samples were thawed rapidly in a 65 °C water bath Two phenol:chloroform extractions were performed to remove contaminating protein followed by a chloroform extraction to remove any phenol. To precipitate the RNA, 8 M LiCl was added so the final concentration in each extract was 2 M, and extracts were incubated overnight at 4 °C. The RNA was pelleted by centrifugation at 3600 rpm for 10 min, washed in 70% EtOH and dried under vacuum at -1 bar. The lyophilized RNA pellet was resuspended in Northern loading buffer (1 ml contained, 200 µl s.d.H_2O, 500 µl formamide, 200 µl formaldehyde, 100 µl 10x running buffer). Running buffer (10x strength) contained 200 mM MOPS, 50 mM NaAc, 10 mM EDTA, pH 7.0.

Total RNA from an apoaequorin transformed *N. plumbaginifolia* line was extracted in the same way and used as a positive control.

The concentration and purity of RNA was analysed spectrophotometrically (section 2.5.3) and its integrity examined by gel

electrophoresis (section 2.6). RNA was stored at -70 °C until use. Before electrophoresis known concentrations of RNA were lyophilised and then resuspended in Northern loading buffer which included 400 µg/ml EtBr.

2.18.2 Gel electrophoresis of total RNA

The gel tank was positioned so that each end was on a magnetic stirrer to keep the buffer in the electrode compartments mixed while the gel was running. The tank was filled with 1x Northern running buffer and a 150 ml 0.7% agarose gel containing 37% formaldehyde and 1x running buffer was placed inside (gel dimension 10 cm x 16 cm x 0.6 cm). For each sample 40 µg of RNA in a total volume of 20 µl was heated at 85 °C for 10 min and loaded onto the gel directly from the hot block. RNA size markers were treated in the same way. The gel was electrophoresed in the fume hood at 6.0 V/ cm until the samples had moved into the gel. A Saran™ wrap covered perspex sheet of similar dimensions to the gel was placed over the upper gel surface to minimise formaldehyde leakage into the buffer, and the gel was electrophoresed at 6.0 V/ cm for 3-4 hours. For the estimation of molecular weight, 4 µg (1 mg/ml) kb RNA ladder (Gibco BRL, UK) in Northern loading buffer (including EtBr) was electrophoresed alongside the samples. Following electrophoresis the gel was photographed under UV illumination.

2.18.3 Capillary transfer of RNA to nylon membranes

The gel was soaked in d.H_2O for 10 min and blotted (see section 2.14.4) using x 10 SSC as the transfer buffer. Following overnight transfer the blot was dismantled and the filter was washed in 2 x SSC and allowed to dry. The RNA was fixed to the nylon membrane by exposing it to UV irradiation of 0.4 J/cm^2. The membrane was stored between two sheets of Whatman 3MM paper prior to probing.

2.18.4 Probe hybridisation and washing

The filter was probed as described in section 2.15.3. After the probe was removed the filter was washed twice for 5 min in 100 ml 2 x SSC and 0.1% SDS. The filter was allowed to air dry and sealed in Saran™ wrap.

2.18.5 Autoradiography and Phosphoimagery

The procedures were identical to that described in section 2.16 and section 2.17.

2.19 Assay for glutamate dehydrogenase (GDH)

The activity of GDH can be accurately determined by measuring the formation of its reaction product NADPH which shows a large absorbance peak at 340 nM in the reaction shown below (see Sheffield, 1994):

$$\text{L-Glutamate} + NADP^+ + H_2O \rightarrow \alpha\text{-ketoglutarate} + NH_4^+ + NADPH + H^+$$

Protein extract (50 µl in AR buffer) was added to 2.8 ml of 0.16 M L-glutamate in 0.1 M Tris.Cl, pH 8.5 (maintained at 35 °C) in a 3 ml plastic cuvette with a 1 cm light path. To this 200 µl $NADP^+$ was added, the contents were mixed by inversion and the cuvette placed in the cell-holder of a UV spectrophotometer (Pie Unicam, UK) maintained at 35 °C. The accumulation of NADPH was recorded at 15 sec intervals over a two min period starting from the addition of $NADP^+$. Control reactions were run in the absence of glutamate.

The change in concentration of NADPH was calculated using the following equation.

$\Delta C = \Delta O.D/(\varepsilon.L)$

ε = extinction coefficient of NADPH (6.22×10^6)

C = concentration of NADPH

L = light path (cm)

This allows the change in concentration of NADPH to be expressed as a specific activity for GDH which is related to the protein concentration of each sample.

2.20 Simultaneous alive - dead staining of *N. crassa*

The viability of germinated macroconidia and spheroplasts was assessed using simultaneous staining with fluorescein diacetate (FDA)(Sigma, UK) and propidium iodide (PI)(Sigma, UK). FDA is non-polar and is rapidly accumulated in cells. Those cells which possess esterase activity, a criterion of viability cleave the molecule yielding fluorescein which is fluorescent (Oparka and Read, 1994). Propidium iodide stains nucleic acids but can only enter the cell if the membrane is damaged and the cell is effectively dead (Oparka and Read, 1994). Stock solutions of 10 mg/ml FDA (in DMSO) and 10 mg/ml PI (in $d.H_2O$) were maintained at 4 °C and a working concentration of 2 µg/ml each made up immediately before use in $d.H_2O$ (germinated conidia) or 1 M sorbitol (spheroplasts). Five µl of a cell suspension was placed on a microscope slide and one drop of the FDA/ PI solution from a Pasteur pipette was applied to the cells. A coverslip was placed over this and the cells were immediately examined using blue light excitation under x40 magnification on a Polyvar photo-microscope (Reichert-Jung, Austria).

Chapter Three

3. The Construction of Apoaequorin Expression Vectors and Transformation of *Neurospora*

3.1 Introduction

Aequorin can be introduced into cells in five different ways as discussed in section 1.3. However, only one method ensures that aequorin can be present in all cells for measurement of $[Ca^{2+}]_c$ throughout growth and development. This is the stable and heritable incorporation of the apoaequorin gene into the genome by transformation. It allows the formation of aequorin simply by incubating the transformed host in coelenterazine (Knight *et al.*, 1991a and b; Nakajima-Shimada *et al.*, 1991a and b).

To express a heterologous gene such as apoaequorin in *Neurospora*, a suitable DNA construct must be introduced and stably maintained as an integrated part of the genone. For maximum expression it is generally desirable for the foreign gene to be joined to promoter and terminator sequences from highly expressed native genes. Depending on the choice of promoter, one may aim at either high-level constitutive control or inducible expression. In many filamentous fungal species including *N. crassa*, the range of promoters and terminators which have been cloned and characterised is quite small. Even fewer, are the expression signals which have been tested in transforming DNA linked to reporter genes (see table 1.4). Fortunately this potential limitation has been circumvented in many cases by the use of effective expression signals across species barriers. For example the hygromycin B phosphotransferase gene flanked by the expression signals of the *A. nidulans trpC* gene has conferred hygromycin resistance on at least ten filamentous fungal species (van den Hondel *et al.*, 1991).

In addition to the chimeric gene for the foreign protein, a transforming construct must contain a native or foreign gene for the selection of transformants. Selection may be for protrophy (repair of an auxotrophic mutant) or for some new function, usually antibiotic resistance. Finally the construct must also contain the bacterial plasmid sequences necessary for selection and propagation in *E. coli*.

For successful transformation of the host organism (i.e. *N. crassa*), there are three steps which must be performed: the preparation of competent cells, induction of those cells to take up DNA and the application of selective pressure to identify those cells carrying the plasmid sequences. The first routine transformation of a filamentous fungus was achieved by treating qa^{2-} *N. crassa* spheroplasts with a plasmid carrying the qa^{2+} gene in the presence of PEG 4000 and $CaCl_2$; transformants were selected on the acquired ability to grow on minimal plates (Case *et al.*, 1979). This method was later optimised by Vollmer and Yanofsky (1986) yielding 1-2 x 10^4 transformants per µg DNA and has been applied to a number of other species (Ballance *et al.*, 1983; Binninger *et al.*, 1987). Other methods of transformation which have been applied to the filamentous fungi include bombardment of the organism with DNA coated particles and application of an electrical pulse to permeablise the cells to transforming DNA (Herzog *et al.*, 1996; Chakraborty and Kapoor, 1990; data presented here).

This chapter presents the initial stage in the development of aequorin as an indicator for measuring $[Ca^{2+}]_c$ in *N. crassa*, the introduction of apoaequorin into the cells to be studied. This was achieved through the construction of suitable expression vectors and their introduction into *N. crassa* using an optimised transformation protocol.

3.2 Design and construction of vectors for apoaequorin production in *N.crassa*

Two integrative apoaequorin expression vectors were constructed, pNCAEQ1 and pNCAEQ3. As the aim of this research was to examine $[Ca^{2+}]_c$ regulation throughout growth and development the promoters were selected that had previously been shown to exhibit both constitutive expression and a high level of protein production.

3.2.1 pNCAEQ1

3.2.1.1 Component Sequences

The 588 bp apoaequorin A cDNA *aeq 1* (Prasher *et al.*, 1986) encoded the 189 amino acid (a.a) polypeptide. The promoter fused upstream of *aeq 1* was a truncated 800 bp version of the *N. crassa* malate synthase (*ms*) promoter (3.3 kb) (Sheffield, 1994). Malate synthase is a glyoxylate cycle enzyme which shows strong repression by sucrose and induction when acetate is the sole carbon source. Deletion analysis of the promoter (3.3 kb) showed that this 800 bp fragment did not contain the *creA* (carbon catabolic repression) and *amdI9* (acetate induction) consensus sequences (Mizote *et al.*, 1996) and directed constitutive expression when fused with the glutamate dehydrogenase (*am*) gene (Sheffield, 1994). The level of glutamate dehydrogenase (GDH) produced in some of these transformants was comparable to wild type levels, approximately 0.25% total soluble protein (Sheffield, 1994). This promoter fragment has also been used to direct the expression of several heterologous genes in *N. crassa* (I. Connerton, pers. comm.). The transcriptional terminator originated from the *am* gene (Kinnaird *et al.*, 1982; Kinnaird and Fincham, 1983). It possessed a single transcription termination site and generated an untranslated region of 224 bp (I. Connerton, pers. comm.). Like a number

of other *Neurospora* genes, *am* has no polyadenylation consensus sequence (5'AAUAAA3')(Gurr *et al.*, 1987).

The vector pMAT (4.31 kb) contained the *ms* promoter and *am* terminator separated by a short polylinker. The bacterial plasmid sequences were from pBluescriptSK$^-$.

For the selection of transformants hygromycin B was used. Hygromycin B is an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus*. It inhibits protein synthesis by interfering with translation (Cabañas *et al.*, 1978). The *E. coli* plasmid gene encoding hygromycin B phosphotransferase confers resistance to hygromycin and has been used successfully in a wide range of filamentous fungi, including *N. crassa* (Staben *et al.*, 1989). The vector used by Staben *et al.* (1989), pCSN43, provided the expression cassette encoding hygromycin B phosphotransferase flanked by the expression signals of the *A. nidulans trp C* (Mullaney *et al.*, 1985) gene.

3.2.1.2 Construction

Ligation of the apoaequorin cDNA into the vector pMAT used the *Nde*I (CATATG) site of the linker (*Nco*I and *Hin*dIII cut *aeq1*). As *aeq*1 did not possess terminal *Nde*I sites it was necessary to incorporate these using PCR, with synthetic oligonucleotide primers which included the *Nde*I recognition sequence (underlined) as a 5' "add on".

aeq 1	5'			**ATG**	ACC	AGC	GAA	CAA	TAC	TCA
initiation end	3'			TAC	TGG	TCG	CTT	GTT	ATG	ACT
Primer 721	5'	GC	CAT	ATG	ACC	AGC	GAA	CAA	TAC	T

aeq 1	5'	GTC	CCC	**TAA**	GAA	ACT	CTG	CGG		
termination end	3'	CAG	GGG	ATT	CTT	TGA	GAC	GCC		
Primer 722	5'	G	GGG	ATT	CTT	TGA	GAC	GCC	GTA	TAC CG

Although two additional bases were included at the 5' end of the primers to aid subsequent *Nde*I digestion, direct ligation of the PCR product into pMAT proved unsuccessful. An additional cloning step in which the termini of the apoaequorin PCR product were flush-ended using T4 polymerase and ligated into pBluescript (KS^-) at the *Eco*RV site, yielded the plasmid pAEQ1 (see Fig. 3.1, track 2). The apoaequorin coding sequence was then digested with *Nde*I and the resulting fragment ligated into the *Nde*I site of pMAT to give the plasmid pMSAEQAM. A *Bam*HI digest was conducted to assess the orientation of the apoaequorin cDNA with respect to the promoter (see Fig. 3.1, track 7).

From the sequencing information available the junction between the promoter and the apoaequorin cDNA was:

TAT TTC ACC ATG GAT ATT CCC GGG AAG CTT CAT ATG ACC AGC
ms promoter polylinker *aeq1*

From the sequence it can be seen that there are two potential translation initiation sites, one originating from the *ms* gene and the other provided by the apoaequorin cDNA. Ribosome binding is favoured at the *ms* initiation site, as it is first in line and flanking bases show a perfect Kozak consensus sequence (AXXAUGG, where X denotes any nucleotide)(Kozak, 1981). The second AUG does not show this consensus, and in addition the presence of a pyrimidine at position -3 to the AUG, is frequently observed flanking non functional AUG triplets (Kozak, 1981). Bearing this in mind, it was possible that the apoaequorin polypeptide would possess an additional seven amino acids (Asp, Ile, Pro, Gly, Lys, Leu, His). The influence that the additional sequence or the two start codons may have on the efficiency of transcription, translation or protein function is unclear. The nucleotide and protein sequence does not contain any signal sequences so far identified. The 5' fusion of proteins to

apoaequorin, without loss of aequorin activity (Casadei *et al.*,1990; Badminton *et al.*, 1995) suggests the effect on protein structure and function would be minimal. pNCAEQ3 (section 3.2.2) possesses a single AUG originating from apoaequorin.

The hygromycin B phosphotransferase expression cassette was removed from pCSN43 using *Sal*I and ligated into the *Sal*I site adjacent to the *am* transcription terminator of pMSAEQAM, thereby producing the complete plasmid for *N. crassa* transformation pNCAEQ1. Fig.3.1, track 3, shows pNCAEQ1 digested with *Nde*I which releases apoaequorin (588 bp) and two other fragments due to the presence of an *Nde*I site in the hygromycin cassette.

3.2.2 pNCAEQ3

3.2.2.1 Component sequences

The apoaequorin PCR product used to construct pNCAEQ1 was again used here. Apoaequorin gene expression was directed by the 1.4 kb glucose-repressible gene (*grg-1*) promoter (McNally and Free, 1988; Wang *et al.*, 1994). The *grg-1* gene shows strong induction under glucose-deprived conditions and is analogous to the clock-controlled gene (*ccg-1*) (Loros *et al.*, 1989) with levels of expression changing 5-10 fold during the circadian cycle. Under de-repressing conditions, as during growth on sucrose the *grg-1* promoter was shown to direct calf preprochymosin gene expression in *N. crassa* (Nakano *et al.*, 1993).

The dominant selectable marker used in this study was phosphoinothricin (PPT) resistance. PPT is a potent inhibitor of glutamine synthetase causing the rapid accumulation of ammonia which leads to plant cell death (Tachibana and Kaneko, 1986). A PPT acetytransferase gene (*bar*) isolated from *Streptomyces hygroscopicus*

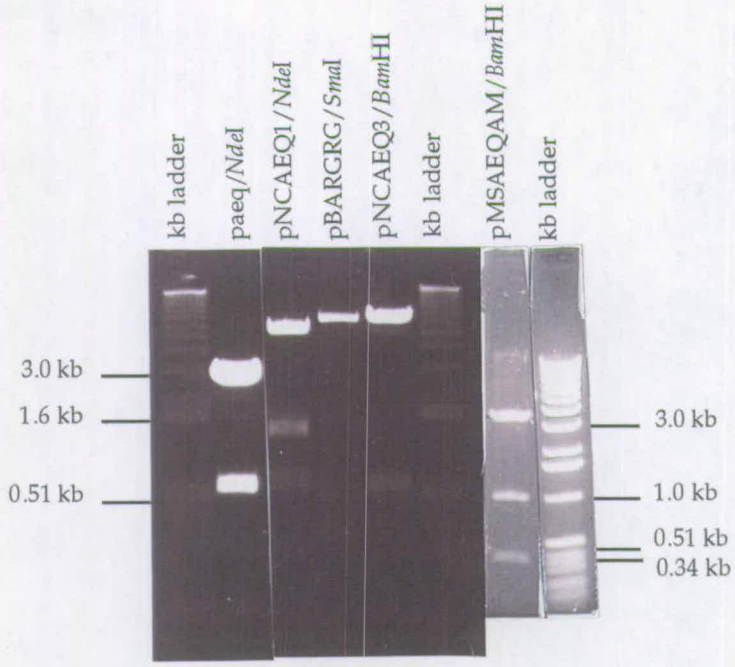
confers resistance to PPT (Avalos *et al.*, 1989). Selection using the *bar* gene has been shown to be effective in *N. crassa* in nitrogen-free Vogel's medium N when either nitrate or proline was the sole nitrogen source (Pall, 1993a and b). The bacterial sequences were from a pUC plasmid (Pall and Brunelli, 1994).

3.2.2.2 Construction

The pUC-based plasmid pBARGRG1 (6.35 kb)(see Fig. 3.1, track 4) was kindly donated by Dr. I. Connerton (BBSRC, Reading, UK). This vector contains an expression cassette of the *N. crassa grg-1* promoter and the *A. nidulans trpC* terminator, flanking a short polylinker. The *grg-1* promoter does not possess the start codon of the *grg-1* gene, and so translation of a coding sequence introduced into the polylinker will be governed by its own ATG. pBARGRG1 also carries the *bar* gene under the control of the *A. nidulans trp C* promoter.

The apoaequorin cDNA was removed from pNCAEQ1 using *Nde*I, the 5' overhangs were "filled in" using Klenow and the blunt-ended fragment was ligated into the *Sma*I site of the polylinker. The orientation of apoaequorin was assessed using a *Bam*HI digest (see Fig. 3.1, track 5).

Figure 3.1: Diagnostic restriction enzyme digests in the construction of the *N. crassa* expression vectors, pNCAEQ1 and pNCAEQ3. Approximately 0.5 µg plasmid DNA was digested with the appropriate restriction enzyme, prior to gel electrophoresis. A sample of 0.4 µg of kb ladder was used as the size marker. Digests: paeq/*Nde*I, the digest releases the apoaequorin cDNA (following PCR) (588 bp) from pBluescriptSK$^-$ (2.95 kb); pNCAEQ1/*Nde*I, the digest releases three fragments including the apoaequorin cDNA; pBARGRG1/*Sal*I, the plasmid is linearised (6.35 kb); pNCAEQ3/*Bam*HI, the digest shows the correct orientation of the apoaequorin cDNA after it was ligated into pBARGRG1 (i.e. correct orientation of apoaequorin with the *grg-1* promoter should yield two bands, at approximately 6.35kb and 550 bp); pMSAEQAM/*Bam*HI, the digest shows the correct orientation of the apoaequorin cDNA in pNCAEQ1 (i.e. correct orientation of apoaequorin with the *ms* promoter should yield three bands at approximately 4.5 kb, 1.1 kb and 330 bp).



3.3 Transformation of *N. crassa*

Initial PEG + $CaCl_2$ transformation experiments with pNCAEQ1 followed a modified method of Vollmer and Yanofsky (1986)(Dr. J. Bond, pers. comm.) To assess each transformation the following treatments and controls were always performed.

(A) Efficiency of Novozyme 234 treatment: Untransformed spheroplasts were added to 15 ml of molten (55 °C) 1 x Vogel's medium N, 0.5% glucose, 0.5% fructose, 2% sorbose, 2% agar. This mixture was overlaid onto 1 x Vogel's salts, 0.5% glucose, 0.5% fructose, 2% sorbose, 2% agar plate.

(B) Viability of spheroplasts surviving transformation: Transformed spheroplasts were added to 15 ml of molten 1 x Vogel's medium N, 1 M sorbitol, 0.5% glucose, 0.5% fructose, 2% sorbose, 2% agar and poured onto 1 x Vogel's salts, 0.5% glucose, 0.5% fructose, 2% sorbose, 2% agar plate

(C) Transformation efficiency: Transformed spheroplasts were overlaid in 1 M sorbitol agar (as for B) onto plates (as for B) which were supplemented with 200 µg/ml hygromycin B.

Using this method after 3 days at 25 °C numerous colonies grew on the plates and the serial dilution of transformation experiments was essential to resolve their frequency. The majority were less than 2 mm in diameter and were dismissed as abortive transformants. Other "dominant" transformants, which showed stronger growth on transformation plates were inoculated onto slants containing 1 x Vogels medium N, 2% sucrose, 200 µg/ml hygromycin B (VSH slants). However these colonies repeatedly failed to grow. The high level of abortive transformants required a series of transformation optimisations to be made, and resulted in the

development of electro-transformation of spheroplasts, a more effective method for the introduction of DNA into *N. crassa*. All optimisation experiments used the integrative circular plasmid pNCAEQ1 and 74*A* *N. crassa*.

Table 3.1: Inhibition of *N. crassa* Sta 74*A* by increasing concentrations of hygromycin B. Growth measurements were recorded at 2 day intervals. The data represent the mean of two replicate plates.

hygromycin concentration µg/ ml	Colony diameter at 2 days	Colony diameter at 4 days
0	1500 mm	n.a
50	300 mm	1500 mm
100	75 mm	105 mm
200	n.g	n.g
500	n.g	n.g
750	n.g	n.g
1000	n.g	n.g

n.a= growth was not continued until day 4

n.g= no growth was visable

Staben *et al.* (1989) tested 15 strains of *N. crassa* for sensitivity to hygromycin and determined that in general strains were completely inhibited by 150 µg/ml hygromycin B. Due to the high number of abortive transformants present in initial transformation experiments the wild type 74*A* was checked for hygromycin sensitivity. A conidial inoculation was made directly onto 15 cm diameter 1x Vogel's medium N, 2% sucrose, 2% agar plates, which contained between 0 and 1000 µg/ml

hygromycin B (Boehringer Mannheim) and plates were incubated in darkness at 25 °C for up to four days.

Table 3.1 shows that hygromycin concentrations over 200 µg/ml completely inhibit *N. crassa* Sta 74*A*, when germinated conidia are applied directly to the surface. This is in agreement with the data of Staben *et al.*, (1989). To increase the stringency of antibiotic selection the overlay agar was reduced in volume from 15 ml to 8 ml; however this did not eliminate the very high level of abortive transformants. Additionally, the levels of fructose and glucose were reduced to 0.05% this improved the sorbose-induced colonial growth.

To determine the origin of abortive transformants two additional controls were included in the transformation experiments: Firstly, to determine if untransformed cells were inhibited by hygromycin B using the overlay method, untransformed spheroplasts were overlaid onto plates with and without 200 µg/ml hygromycin B. Secondly, the ability of spheroplasts, which had been exposed to the transformation procedure but without DNA, to grow on hygromycin B was tested. In addition, spheroplasts were plated onto 200 - 500 µg/ml hygromycin B.

Table 3.2: Viability of spheroplasts following various treatments. The effect of PEG + $CaCl_2$ transformation and hygromycin on colony regeneration. Number of spheroplasts per treatment was 1×10^7. The data were calculated from duplicate platings of appropriate spheroplast dilution, 3 days after treatment.

Treatment	µg/ml hygromycin B			
	0	200	300	500
Untransformed spheroplasts, no osmotic protectant	0	nt	nt	nt
Transformation procedure with DNA	3.08×10^5	1.76×10^5	9.35×10^4	1.03×10^5
Untransformed spheroplasts with osmotic protectant	$2.31 \times 10^{7*}$	6.6×10^6	nt	nt
Transformation procedure without DNA	nt	1.54×10^5	1.32×10^5	1.1×10^5

* = dilution error figure should not exceeded 1×10^7

nt= not tested

The controls included in the transformation experiment presented in table 3.2 showed that a large number of untransformed colonies were able to grow on transformation plates. Indeed the plate dilutions required for good colony density would substantially reduce the chance of identifying stable transformants if they occurred at a very low frequency. By incubating transformation plates for over eleven days a reliable morphological distinction between stable and abortive transformants could be made. Compact, domed growth was seen on plates which did not include hygromycin and made up a small population of colonies on transformation plates. The number of domed colonies decreased as hygromycin concentration increased (i.e. 200 µg/ml = 649 domed; 300 µg/ml = 451 domed; 500 µg/ml 88 domed). Diffuse growth was seen in untransformed colonies on hygromycin plates and made up virtually all the colonies growing on the transformation plates. When colonies were inoculated onto VSH slants, virtually all the domed colonies grew, none of the diffuse colonies grew under these conditions. The morphological

distinction allowed accurate calculation of the efficiency of stable transformation using the PEG + $CaCl_2$ procedure (see table 3.4).

Electroporation of spheroplasts was developed as an alternative to PEG + $CaCl_2$ transformation as it was considered that the direct plating used would ease the isolation of stable transformants. In *N. crassa* electro-transformation has been achieved using germinated conidia (Chakraborty and Kapoor, 1990; Chakraborty *et al.*, 1991). It was thought that the lack of cell wall on spheroplasts may promote the uptake of DNA and increase the transformation efficiency. The methodology of this procedure was based upon protocols for *N. crassa* germinated conidia (Chakraborty and Kapoor, 1990) and *S. cerevisiae* (available from Biorad, UK) transformation and from studies on spheroplast regeneration (Selitrennikoff and Bloomfield, 1984).

Spheroplasts were produced as in section 2.10.1. Following an additional wash in cold 1 M sorbitol the cells were resuspended in 1-5 ml of cold 1 M sorbitol. An aliquot of 6×10^6 chilled spheroplasts (approx 50 µl) was mixed with 1 µl (2 µg) of chilled pNCAEQ1 and transferred to a sterile chilled 0.2 cm width electroporation cuvette. The cuvette was placed in the safety chamber of the Biorad Gene Pulser and a square electrical pulse administered (resistance 200 Ω, capacitance 25 µF, time constant 4-5 msec, field strength 12.5 kV). The electroporated cells were immediately transferred to 1 ml of 1x Vogel's medium N, 1 M sorbitol, 0.5% glucose, 0.5% fructose and incubated at 30 °C shaking at 100 rpm for 4 hours to allow cell wall regeneration. A 300 µl aliquot of the cell suspension was then plated directly on to 300 µg/ ml hygromycin B underlay plate (see section 2.10.2) and incubated at 25 °C for 6 days. Table 3.3 shows the results from a typical electroporation experiment.

Table 3.3: Transformation by electroporation. The data represents the number of colonies, following correction for any dilutions made. 6×10^6 spheroplasts were used in each treatment.

Treatment	µg/ml Hygromycin B	
	0	300
no DNA, electroporated	1.33×10^5	63
DNA, no electroporation	9.17×10^7	0
DNA, electroporated	not tested	77

The limited number of untransformed colonies present in the hygromycin control showed the diffuse morphology of abortives in the PEG + $CaCl_2$ procedure. Transformation plates exhibited a high frequency of domed colonies. When these domed colonies were inoculated onto 300 µg/ml hygromycin B VS slants all showed vigorous growth and conidiation.

Table 3.4: Comparison of PEG + $CaCl_2$ transformation and electro-transformation.

Calculation	Method of Transformation	
	PEG + $CaCl_2$	Electroporation
Spheroplasts surviving treatment	3.08% [a]	2.22% [b]
Spheroplasts surviving treatment which are untransformed	87.5% [c]	0.047% [d]
Stable transformants of spheroplasts surviving treatment	0.027% [e]	0.058% [f]
Stable transformants per 1 x 10^6 spheroplasts	45 [g]	12.8 [h]
Stable transformants per µg DNA	225.5 [i]	38.5 [i]

Abbreviations: UT denoted untransformed

[a] from table 3.2.: 100 x (colonies on DNA, transformation, no hygromycin plate at 3 days ÷ total number of spheroplasts transformed)

[b] from table 3.3. : 100 x (colonies on no DNA, electroporation, no hygromycin plate at 6 days ÷ total number of spheroplasts electroporated)

[c] from table 3.2: 100 x (colonies on no DNA, transformation, 200 µg/ml hygromycin plate at 3 days ÷ colonies on plus DNA, transformation, no hygromycin plate at 3 days).

[d] from table 3.3: 100 x (colonies on no DNA, electroporation, hygromycin plate ÷ colonies on no DNA, electroporation, no hygromycin plate)

[e] 100 x (colonies on plus DNA, transformation, 200 µg/ml hygromycin plate at day 11÷ colonies on plus DNA, transformation, no hygromycin plate at day 11) i.e. 100x (649÷(1.65x10^6))

[f] from table 3.3: 100 x (domed colonies on plus DNA, electroporation, hygromycin plate at 6 days ÷ colonies on no DNA, electroporation, no hygromycin plate at 6 days)

[g] domed colonies on plus DNA, transformation, 300 µg/ml hygromycin plate at 11 days ÷10 i.e 450÷10

[h] from table 3.3: domed colonies on plus DNA, electroporation, hygromycin plate at 6 days ÷ 6 i.e. 77÷6

[i] domed colonies on plus DNA, 300 µg/ml hygromycin plates ÷2

3.4 Transformation using apoaequorin expression vectors

3.4.1 pNCAEQ1

Thirty-six 74*A* and 10 *BdA* stable transformants were produced using the PEG + $CaCl_2$ procedure. Electroporation yielded another 15 stable transformants in a single experiment.

In an attempt to generate high copy number transformants the concentration of pNCAEQ1 was increased from 2 µg to 15 µg per transformation. This experiment only produced colonies which grew poorly or not at all on selective slants and no further work was pursued with them.

3.4.2 pNCAEQ3

pNCAEQ3 was introduced into *N.crassa* using electro-transformation. Stable transformants were isolated on 2% agar plates of nitrogen free Vogel's medium N supplemented with 0.5% proline, 2% sorbose, 0.05% fructose, 0.05% glucose, and 200 µg/ml phosphoinothricin (Sigma, UK). The low levels of abortive transformants, and clear morphological distinction between abortive and stable transformants was repeated here. Thirty 74*A* stable transformants were obtained.

3.5 Discussion

Both apoaequorin expression vectors were successfully transformed into *N. crassa,* and transformants were selected on the basis of acquired antibiotic resistance to either hygromycin B (pNCAEQ1) or phosphoinothricin (pNCAEQ3). Chapters 4 and 5 detail the analysis of 60 of these transformants with respect to apoaequorin expression. Comparison of apoaequorin yields in pNCAEQ1 and pNCAEQ3 transformants will be expected to reveal any deleterious effects caused by

the presence of two translation initiation sites in pNCAEQ1 and may also give an insight into the relative strengths of these promoters.

The production of spheroplasts from germinated macroconidia was generally very efficient, greater than 99% when assessed by plating without an osmotic protectant (table 3.2). Simultaneous alive/dead staining of spheroplasts (see Chap 2, Fig. 2.2) showed that viability was consistently greater than 95%, indicating that the transformation procedure itself killed a large proportion of the spheroplasts (table 3.4). The efficiency of transformation is commonly expressed as transformants per μg DNA, a calculation that does not reflect the number or viability of the spheroplasts used. Using this calculation, a comparison with other studies showed that the optimised PEG + $CaCl_2$ protocol (table 3.4) was within the range of that obtained by other researchers (147 per μg DNA using 4 x 10^7 cells; Buxton and Radford, 1984) while the results obtained for electro-transformation was less efficient (1,100-1750 μg DNA using 6 x 10^6 cells; Chakraborty and Kapoor, 1990). An increase in electroporation efficiency may be achieved by extending the outgrowth period. Selitrennikoff and Bloomfield (1984) showed that cell wall regeneration increased from 45% to 80% between 3 and 8 hours.

In comparing the two techniques the percentage stable transformants of the cells surviving transformation (table 3.4) provides the best estimation of stable transformant production. This shows that electroporation yields at least twice as many stables as PEG + $CaCl_2$. It is possible that electroporation is more effective in breaching the plasma and nuclear membranes, thereby reducing exposure of the plasmid to external and cytosolic nucleases. A general observation from transformation experiments and also witnessed here is the very low proportion of spheroplasts that yield stable transformants. This could be due to the

procedures used being very inefficient or that only a very small population of the spheroplasts are truly competent. Recent evidence has suggested the latter and indicated that it is some property of the nucleus which determines transformation competence. Transformation of forced heterokaryons followed by re-isolation to their homokaryotic states showed that in the heterokaryotic spheroplasts transformation commonly occurred in only one nucleus (Pandit and Russo, 1992). This was supported further by cotransformation experiments where a high proportion of homokaryons contained both plasmids when only one was selected for (Grotelueschen and Metzenberg, 1995).

The ability of untransformed cells to grow on hygromycin plates was alarming (table 3.2) and it is thought that this was a flaw of the overlay technique. The fast growth rate of *N. crassa* permitting significant colony formation before the antibiotic had diffused entirely through the top agar. An interesting comparison would have been to direct-plate a PEG + $CaCl_2$ transformation following an outgrowth period to see if results were similar to those obtained by electroporation. It is considered that the "dominant" transformants in the initial transformations were the true abortives and the smaller colonies untransformed. Plating on different concentrations of hygromycin showed an inverse relationship between hygromycin concentration and stable transformant frequency. This suggests that resistance to higher hygromycin concentrations requires either multiple integrations or integration at special high expression loci. This could be investigated by Southern Blotting.

From this work, the preferred method of transformation for future use is electroporation. It is rapid, a higher proportion of the cells surviving are stable transformants, direct plating eliminates background growth and

the need for extensive dilution series', and stable colonies appear more quickly.

Chapter Four

4. Apoaequorin in Transformed *Neurospora*

4.1 Introduction

To introduce aequorin into *N. crassa* for the measurement of $[Ca^{2+}]_c$, two expression vectors which contained apoaequorin flanked by native expression signals were constructed and transformed into the wild type and the *Band* mutant (chapter 3). Active aequorin could then be formed in the organism by incubation with coelenterazine and methods for the measurement of $[Ca^{2+}]_c$ developed (chapter 5).

The integration of foreign DNA into the *N. crassa* genome is generally ectopic, and the site of transgene integration may influence expression greatly. Transformation with circular DNA requires breakage of the plasmid prior to integration, and the now linear plasmid may also be eroded by exonuclease; in both cases this may lead to disruption of the apoaequorin gene. As a result of these variables, stable transformants selected on the basis of a marker gene carried by the plasmid will vary in the expression of the desired transgene, and a screen for this gene and its product will be necessary. Screening of transformants can be carried out at any of three levels: probing for the integrated transgene (Southern blots), mRNA production (Northern blots) and protein yield, if a suitable assay is available. To characterise apoaequorin expression in *N. crassa* it was necessary to quantify apoprotein production from a large number of transformants, and show that the apoprotein synthesised was similar to that obtained from other sources, most importantly in its ability to form active aequorin on incubation with coelenterazine.

This chapter presents the analysis of apoaequorin expression in *N. crassa*. Western blotting and then *in vitro* constitution and luminometry,

a luminescent assay for active aequorin, were used for initial screening of transformants. The primary transformants were probably mostly heterokaryotic, and homokaryotic derivatives were isolated in the hope of increasing apoaequorin production.

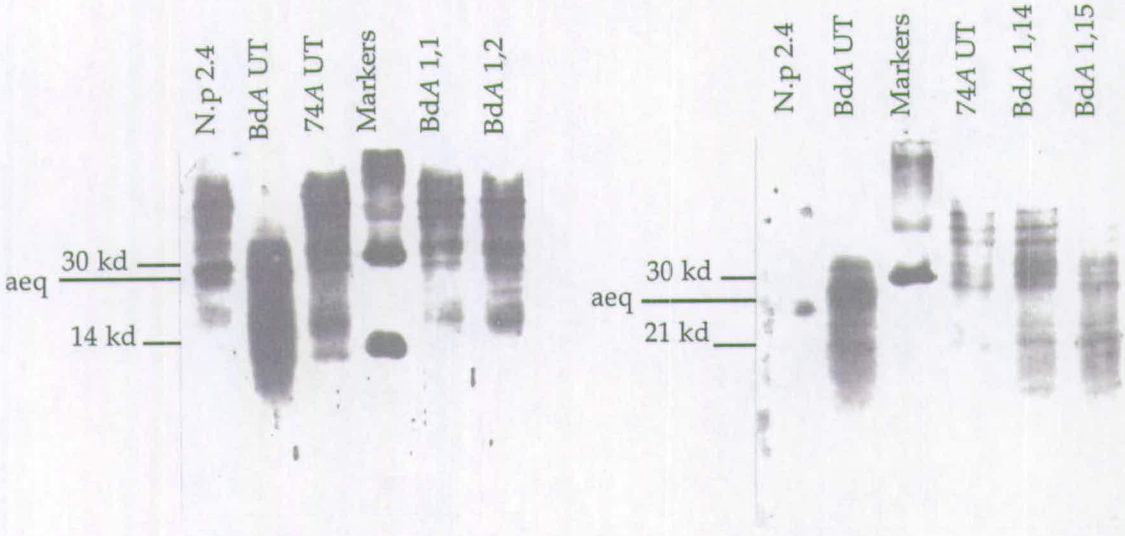
4.2 Western blotting of apoaequorin-transformed *N. crassa*

Western blotting of *N. crassa* extracts employed the ECL detection system which is stated as being capable of detecting approximately 1 pg of antigen (Amersham, UK). The primary anti-apoaequorin antibody used was polyclonal (described in section 2.11.9). Initially large and then mini-protein gels were used for Western blotting, and detection followed the manufacturers protocol. The images obtained showed a very high level of non-specific binding of the horseradish peroxidase (HRP)-labelled secondary antibody and a band corresponding to *N. crassa* apoaequorin was not discerned (Fig. 4.1a). Possible causes for the high background were inefficient blocking of non-specific sites on the nitrocellulose membrane, and/or a lack of specificity in antibody binding (primary and/or secondary). Two blocking reagents were tested (5% low fat dried milk, 0.1% Tween 20, in Tris buffered saline, pH 7.6, and 2% Tween in Tris buffered saline, pH 7.6), but neither improved the specificity of the procedure. In subsequent experiments using minigels, 5% low fat dried milk, 0.1% Tween 20, in Tris buffered saline, pH 7.6 was used. The specificities of the two antibodies were tested in a dot blot using the SDS-7 molecular weight markers as a source of non-specific protein. This showed that the non-specific binding originated from cross-reactivity of the anti-apoaequorin antibody.

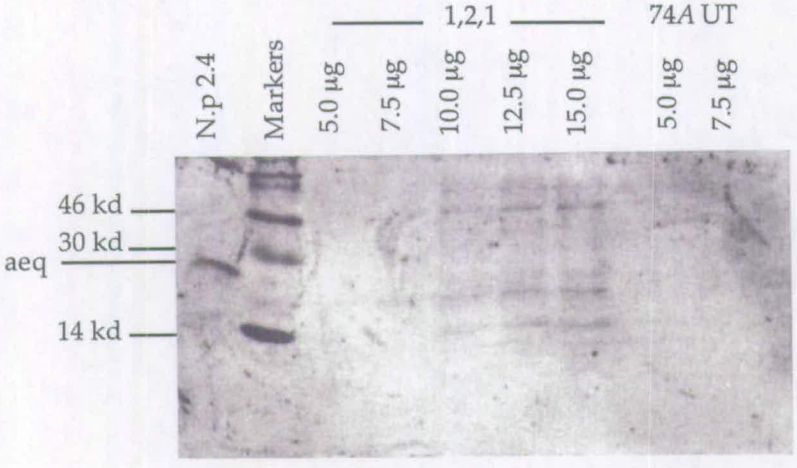
Incubating filters in a 1:1000 dilution of primary antibody followed by a 1:5000 dilution of HRP-labelled secondary antibody revealed a clear apoaequorin band in extracts of transgenic *N. plumbaginifolia.*

Figure 4.1: Western analysis of protein extracts from transformed (Bd*A*1,1; 1,2; 1,14; 1,15) and untransformed (Bd*A* UT; 74*A*UT) *N. crassa,* and apoaequorin transformed *N. plumbaginifolia* (N.p2.4). Aeq denotes *N. plumbaginifolia* apoaequorin.

A: Four *N. crassa* primary transformants. 15 μg of *N. crassa* protein and 3 μg *N. plumbaginifolia* protein was electrophoresed prior to blotting and detection.



B: Increasing the concentration of electrophoresed protein using the homokaryon 1,2,1 (derived from the primary transformant Bd*A* 1,2); and the untransformed 74*A*. 0.45 μg of *N. plumbaginifolia* protein was used as a positive control.



No such band showed up in either transformed or untransformed *N. crassa* extracts. A sample of 0.45 μg of protein yielded a single band corresponding to apoaequorin in transgenic *N. plumbaginifolia* extracts (Fig. 4.1b).

Even at approximately 30 times this loading there was no apoaequorin band detected in transgenic *N. crassa* extracts (Fig. 4.1b). As the protein concentration was increased a variety of bands appear similar in position to those seen in wild type *N. crassa* extracts in other gels (Fig. 4.1a); none matches in position the apoaequorin band from transgenic *Nicotiana*. It was therefore decided to discontinue Western blotting of *N. crassa* extracts, and to look for transgenic apoaequorin by a more sensitive method.

4.3 A luminescent assay for *N. crassa* aequorin

The light emission from recombinant aequorin in saturating Ca^{2+} is 4.30-5.16 x 10^{15} photons/mg (Shimomura, 1991). By using a luminometer (described in section 2.12.1) it is possible to determine aequorin luminescence at quantities as low as 10^{-18} g. This method will be much more sensitive in detecting apoaequorin expression (as active aequorin) than ECL western blotting. *In vitro* constitution of aequorin from apoaequorin, coelenterazine and oxygen is optimal in a high salt, high EGTA buffer supplemented with ß-mercaptoethanol (Inouye *et al.*, 1986). Extracts from apoaequorin-transformed organisms have been assayed using this technique (Knight *et al.*, 1991a and b; Nakajima-Shimada *et al.*, 1991a and b). Optimisation of *in vitro* constitution is essential for extracts from different transformed organisms to ensure that aequorin constitution is maximal and light emission is not effected in any way.

Preliminary *in vitro* constitution of extracts from eight *N. crassa* transformants followed the optimised procedure for *N. plumbaginifolia*

(section 2.12.3.2). On the addition of $CaCl_2$ luminescence was detected from some of these samples, but at a much lower level than seen in *N. plumbaginifolia* (data not shown). It was not clear whether this was a real difference in expression or due to the unoptimised assay for *N. crassa* protein extracts. To determine the optimal conditions for *in vitro* constitution in *N. crassa* extracts, and to compare the reaction kinetics of *Neurospora* transgenic aequorin against others known to be functional, a number of tests were carried out. Mycelial extracts from the homokaryotic strain 1,2,1 (Fig. 4.12d) were used to determine the relationship between coelenterazine dose and aequorin luminescence response, and the time course of aequorin formation. In addition, to detect any influence of the protein extract on aequorin luminescence, the effect of extract concentration was investigated, along with apoaequorin and aequorin stability in the extract.

Certain procedures and conditions were identical in all experiments (unless stated otherwise). They were as follows. Protein extracts were obtained from 41-44 hour old *N. crassa* standing liquid cultures in aequorin constitution buffer (AC buffer) (section 2.12). After the addition of coelenterazine, the reaction was aerated by expelling a 300 µl volume of air into it. The final volume of all reactions was 100 µl. All reactions were incubated in darkness at 25 °C. Light emission was measured by making a 1:50 dilution of the reaction in TE pH 7.0 (final volume 500 µl), and measuring luminescence on the addition of an equal volume of 100 mM $CaCl_2$. As aequorin has an estimated half life of 8 msec in the presence of Ca^{2+}, light yield integrated over a 10 second period was more than sufficient to measure the total luminescence from a sample.

Recombinant aequorin from *E. coli* (Molecular Probes, USA) was used as a comparison in some experiments and for the calibration of aequorin light emission. This recombinant aequorin is a different isozyme to that

which has been introduced into *N. crassa* and *N. plumbaginifolia*. The two forms differ slightly in molecular weight and light emitting capacity (Shimomura, 1991). Reconstitution of recombinant aequorin was necessary following prolonged storage; aliquots of recombinant apoaequorin were incubated with 1.25 μM coelenterazine in AC buffer, prior to use (see section 2.12.2.2).

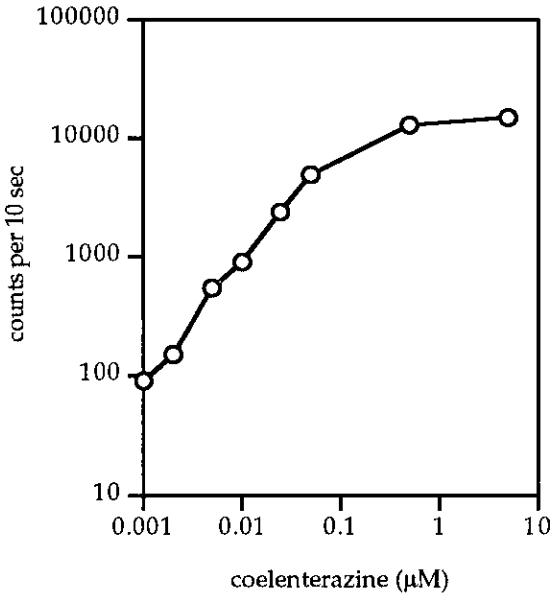
4.4 Optimising aequorin *in vitro* constitution for use in *N. crassa*

4.4.1 Coelenterazine dose-response

To determine the concentration of coelenterazine required to saturate apoaequorin present in the *N. crassa* extract, individual aliquots of 200 μg protein were added to AC buffer in separate cuvettes followed by the addition of 0 to 5 μM coelenterazine from a 25 μM stock solution (dissolved in 100% methanol:AC buffer, 1:9). The methanol concentration in all tubes was standardised at 1%. Samples were incubated for 3 h prior to the measurement of light emission by luminometry.

Fig 4.2 shows that the constitution reaction is virtually saturated at concentrations of 0.5 μM and over, it was decided that in subsequent experiments 2.5 μM coelenterazine would be used (excluding experiment on the time course, Figure. 4.3). The graph is similar to those obtained for *E. coli* recombinant aequorin *in vitro* (Inouye *et al.*, 1986) and aequorin constitution in *E. coli* cells (Knight *et al.*, 1991a). The yield from *N. crassa* aequorin appears to be far lower than that obtained from saturating coelenterazine concentrations in *E. coli* cells.

Figure 4.2: Coelenterazine dose-response curve. Each point represents the mean of three separate mycelial extracts each of which was tested three times. The standard errors were calculated on all nine readings and are too small to show in the graph.



4.4.2 Time course of *N. crassa* aequorin formation

To determine the optimal *in vitro* constitution incubation time for maximal luminescence of *N. crassa* aequorin; individual aliquots of 200 μg protein were incubated with 1.25 μM coelenterazine in AC buffer for between 0 and 24 h. A time course of recombinant aequorin formation in the presence of 200 μg of 74*A* protein, with 1.75 x 10^{-12} M apoaequorin and 2.5 μM coelenterazine, was determined so the time course of *N. crassa* aequorin formation could be compared with that of an aequorin standard, measured under similar conditions.

Figure 4.3: Time course of the formation of *N. crassa* aequorin and recombinant aequorin. Each point represents the mean of nine replicas from one mycelial extract. Luminescence values are presented as a percentage of the maximum luminescence counts for that aequorin type (i.e. 24 h time point).

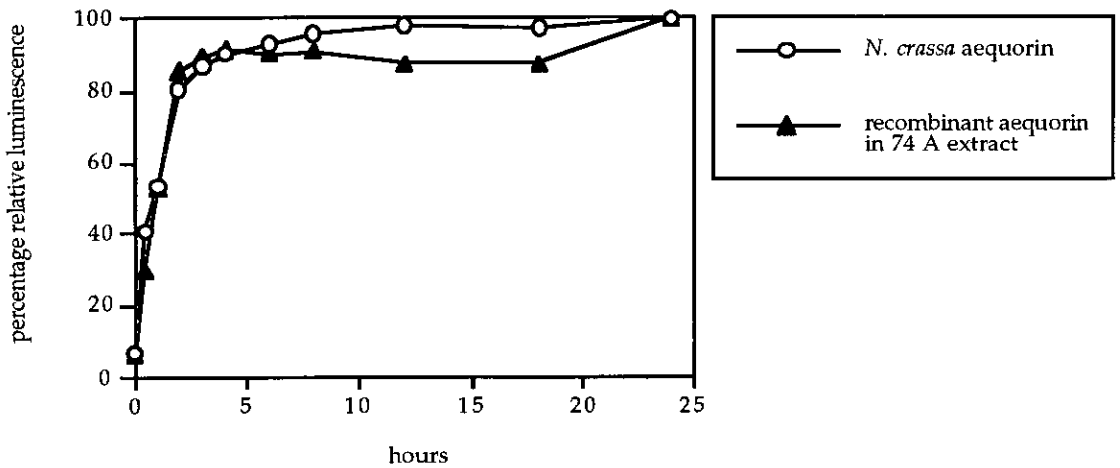


Fig. 4.3 shows that the time course of *N. crassa* aequorin formation does not differ from that of the recombinant aequorin, used as a standard. The reaction was 50% complete at 52 min and 57 min for *N. crassa* aequorin and recombinant aequorin respectively. For subsequent experiments, *in vitro* constitution proceeded over a 4 h period, at which point *N. crassa* aequorin formation was 90% complete.

4.4.3 Effect of extract concentration on *N. crassa* aequorin luminescence

The influence of mycelial extract concentration on aequorin was examined. Individual mycelia of the homokaryon 1,2,1 (Fig. 4.12d) were extracted in AC buffer at either a 5%, 10%, 20% or 30% tissue : buffer ratio. From these protein extracts aliquots of 200 µg protein were taken and incubated in 2.5 µM coelenterazine for 4 hours prior to the measurement of aequorin luminescence.

Figure 4.4: *N. crassa* aequorin luminescence from 20 µg aliquots of the constitution mixes of protein extracted with different mycelium : buffer ratios. Each bar represents the mean of 5 individual mycelia which were each tested three times, standard errors were calculated using all 15 values, and are indicated.

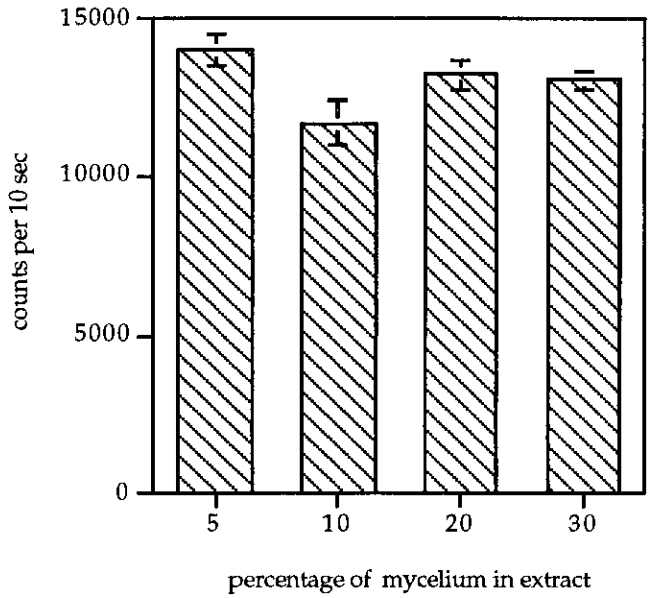


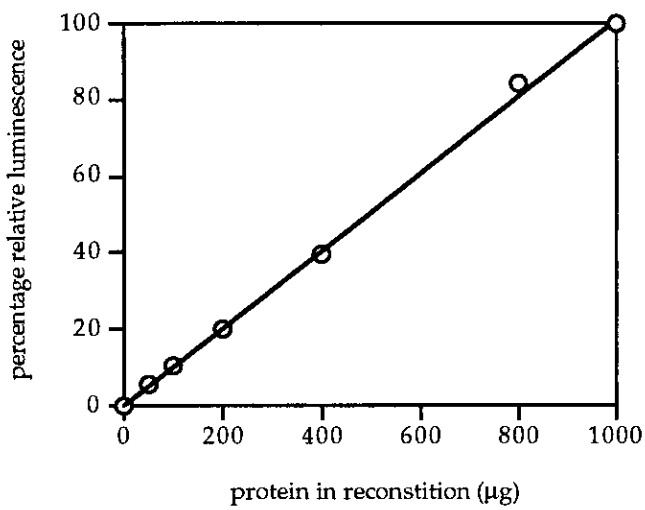
Fig. 4.4 confirms that aequorin luminescence is not affected by the initial concentration of the extract, as it might have been had apoaequorin been subjected to degradation by proteases present in the extract or buffering was insufficient. In subsequent experiments mycelia weighing under 100 mg were extracted at 5% w:v; mycelia over this weight were extracted at 20% w/v.

4.4.4 Effect of protein concentration in the *in vitro* constitution reaction

The effect of protein concentration in the constitution assay was investigated to check the possibility that some component tended to reduce aequorin luminescence. Such an effect has been observed in transgenic *N. plumbaginifolia* extracts in *in vitro* constitution reactions

containing greater than 200 µg protein, due to the high levels of Ca^{2+} present (M. Knight pers. comm.). Individual aliquots of 0 to 1000 µg protein from the homokaryon 1,2,1 (Fig.4.12d) were incubated with 2.5 µM coelenterazine for 4 hours. Ten µl of each sample was assayed in TE in the usual way.

Figure 4.5. *N. crassa* aequorin luminescence from *in vitro* constitutions containing between 0 - 1000 µg protein from the homokaryon 1,2,1. The y-axis represents the counts obtained at different protein concentrations expressed as a percentage of the maximum (i.e. that obtained from a 1000 µg *in vitro* constitution). The x-axis represents the protein present in an *in vitro* constitution reaction. Each point on the graph represents the mean of three separate reactions each of which were tested three times.



Two observations can be made from Fig. 4.5. Firstly, under the conditions tested light emission is directly proportional to the amount of extract and no reduction of the signal occurs in *N. crassa* extracts, unlike in *N. plumbaginifolia*. Secondly, the linearity of the graph confirms that coelenterazine was in excess over the whole range of apoaequorin concentrations. In subsequent experiments, *in vitro* constitution reactions

each contained 200 µg of transformant protein, an amount easily obtained from the extraction protocol.

4.4.5 Protein stability during *in vitro* constitution

The time course of recombinant and *N. crassa* aequorin formation (Fig. 4.3) shows that light emission is over 90% complete from 4 hours incubation onwards. The plateau in light emission over this time may represent the stability of the aequorin complex or a steady state between aequorin break-down and formation. To determine if aequorin did break-down in *N. crassa* extract, reconstituted recombinant aequorin (1.17×10^{-12} M) was incubated in the presence of 200 µg protein from untransformed 74*A* extract or in AC buffer only. Luminometry was performed on samples at time intervals up to 24 hours.

Figure 4.6: Stability of aequorin incubated with and without *N. crassa* extract. Each point represents the mean of nine replicas, standard errors were calculated for nine replicas and are shown for those points where agreement of replicas was not very close. The reason for the slightly higher readings at 24 h is not understood.

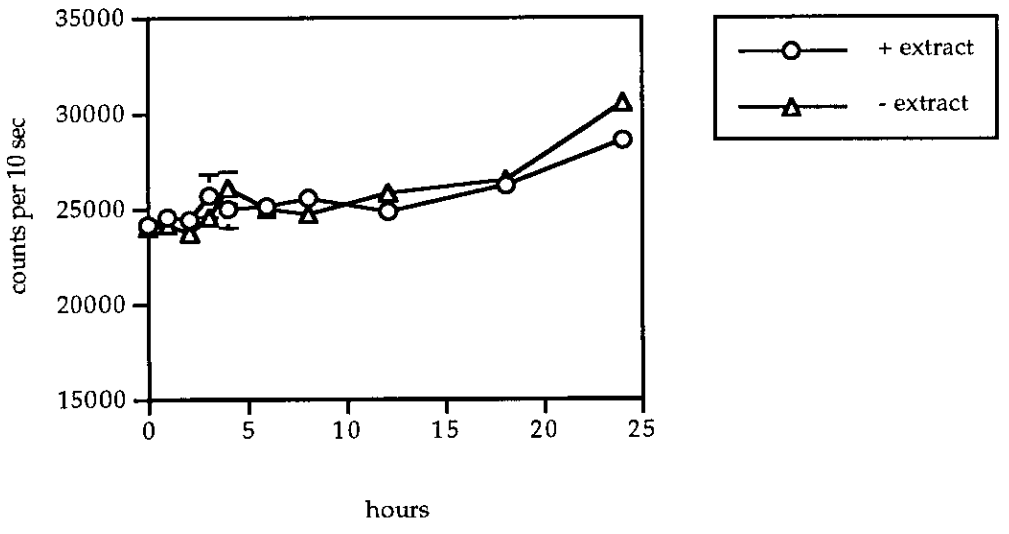


Fig. 4.6 shows that *N. crassa* extract does not influence aequorin luminescence, and the photoprotein is clearly stable under these conditions, so aequorin breakdown is not the cause of the plateau observed in the constitution time course.

A possible cause of the low level of light emission in the *in vitro* constitution reaction could be rapid degradation of the apoprotein before it was stabilised as aequorin. To determine the stability of apoaequorin in *N. crassa* extracts; 90 µl samples containing 200 µg protein from the homokaryon 1,2,1, (Fig. 4.12d) in AC buffer extract, were incubated in darkness at 25 °C for between 0 and 27 hours prior to the addition of coelenterazine to a final concentration of 2.5 µM. Constitution proceeded for 4 hours.

Figure 4.7: Apoaequorin stability in *N. crassa* protein extract. The y-axis represents the percentage relative luminescence of aequorin i.e counts per 10 sec expressed as a percentage of the maximum, obtained at 0 h. The x-axis represent the period of apoaequorin incubation without coelenterazine. Each point represents the mean of 9 replicas from a single protein extract, the standard errors each calculated on the nine readings were too small to show on the graph.

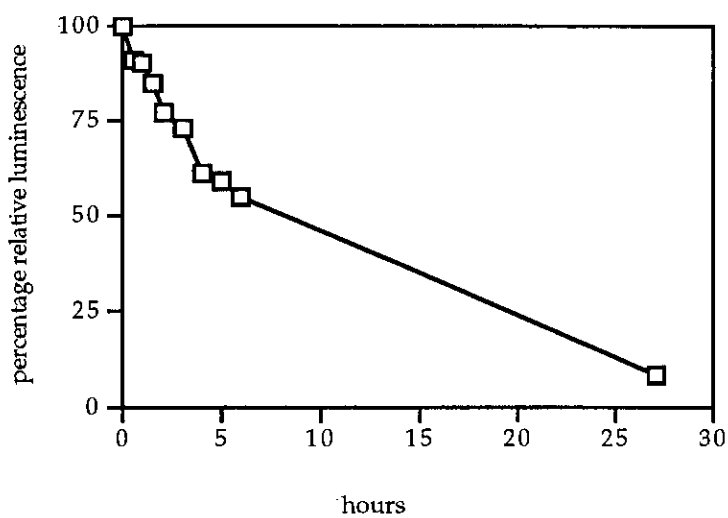


Fig. 4.7 shows that apoaequorin is much less stable than aequorin in *N. crassa* protein extract ($t_{0.5}$ = 7-8 hours). In the *in vitro* constitution two reactions will be occurring simultaneously, aequorin formation and apoaequorin degradation. As aequorin formation occurs much more quickly i.e. 54% in one hour as opposed to 10% degradation in this time, the degradation reaction will only have a slight effect on the total amount of aequorin measured in transformed extracts.

4.4.6 Summary of *in vitro* constitution and luminometry assay

It can be concluded that *in vitro* constitution of aequorin from transformed *N. crassa* extracts provides a reasonably accurate measurement of apoaequorin expression.

The coelenterazine dose-response and time course of *N. crassa* aequorin formation show similar characteristics to recombinant aequorin from other species, indicating that the apoprotein is fully functional. Aequorin is stable in *N. crassa* protein extract and apoaequorin break-down will not effect the final aequorin yield greatly.

Now that the optimal conditions for *in vitro* constitution of *N. crassa* aequorin had been established a screen of all transformants could be conducted. In the transformant screen, extracts were obtained from mycelia at either 5% w:v or 20% w:v ratios, 200 µg of transformant protein was included in the reaction with 2.5 µM coelenterazine and the reaction was incubated for 4 hours at 25 °C prior to the detection of luminescence.

4.5 Screening stable transformants by *in vitro* constitution and luminometry of aequorin

4.5.1 Transformant Screens

N. crassa 74*A* transformed with pNCAEQ1 (Fig 4.8).

Apoaequorin synthesis is expected to be constitutive due to its regulation by the truncated malate synthase promoter (section 3.2.1). Prior to extraction transformants were grown for 41-44 h at 25°C in standing liquid culture, supplemented with 200 µg/ ml hygromycin B.

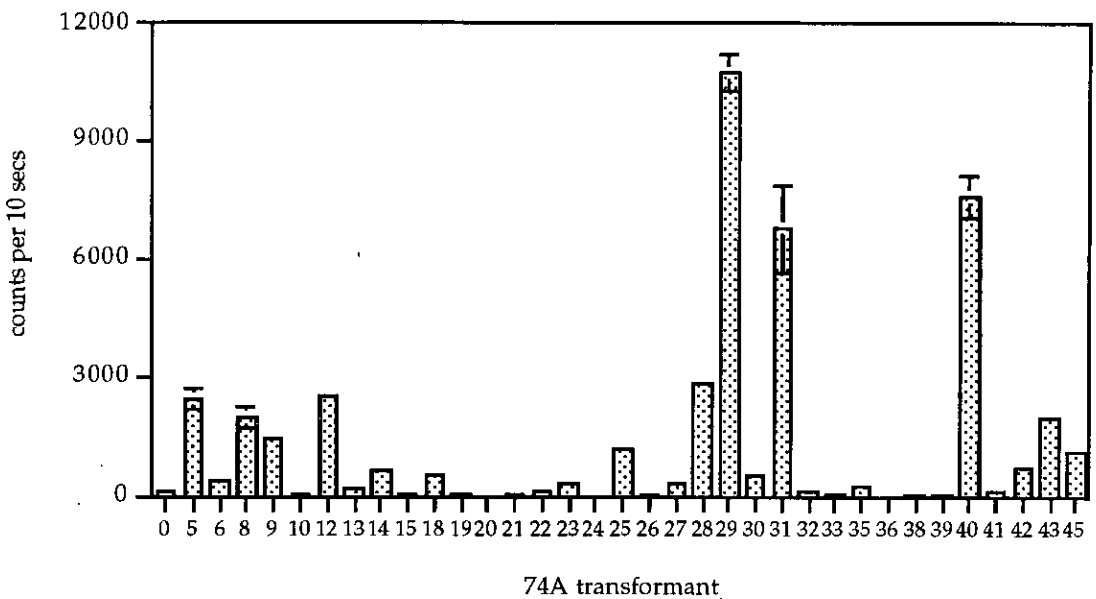
N. crassa BdA transformed with pNCAEQ1 (Fig. 4.9)

Transformants were grown in the same way as pNCAEQ1 transformed *N. crassa* 74*A*.

N.crassa 74*A* transformed with pNCAEQ3 (Fig. 4.10)

The screen for apoaequorin expression which was regulated by the *grg-1* promoter followed the method of Nakano *et al.* (1993). Stable primary transformants were inoculated into 25 ml standing liquid culture (Vogels without a nitrogen source, 0.5% proline, 2% sucrose) supplemented with 200 µg/ ml phosphinothricin (stock solution 50 mg/ml in s.d.H_2O) and grown for 41 hours at 25 °C.

Figure 4.8: Aequorin luminescence in extracts of pNCAEQ1 transformed *N. crassa* 74*A*. 200 µg of protein was reconstituted and light emission detected from a 20 µg protein aliquot. Each bar represents the data obtained for 20 µg protein from three mycelia, each tested three times. Standard errors each calculated on all nine readings are shown only where they are large enough to be drawn. Transformant numbers which are missing from the x-axis denoted slow growing colonies and were discarded from the screen. Zero denotes untransformed 74*A*.



In vitro constitution and luminometry of aequorin from *N. crassa* transformant extracts has provided a sensitive test for determining apoaequorin expression in different transformants. Both apoaequorin expression cassettes function in *N. crassa* transformants. Interestingly, there was little or no overall difference as might have been expected between the two different promoters. McNally and Free (1988) showed that the *grg-1* gene was derepressed up to 50-fold when glucose was removed from the medium. No glucose derepression of apoaequorin was observed using the transformant 74*A* 3,5 (data not shown).

Figure 4.9: Aequorin luminescence in extracts of pNCAEQ1 transformed *N. crassa BdA*. Other details as for Fig. 4.8.

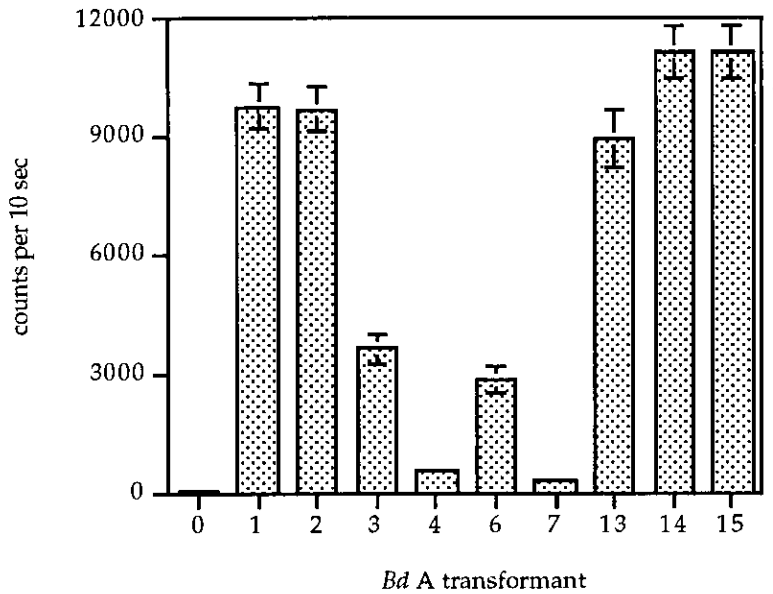
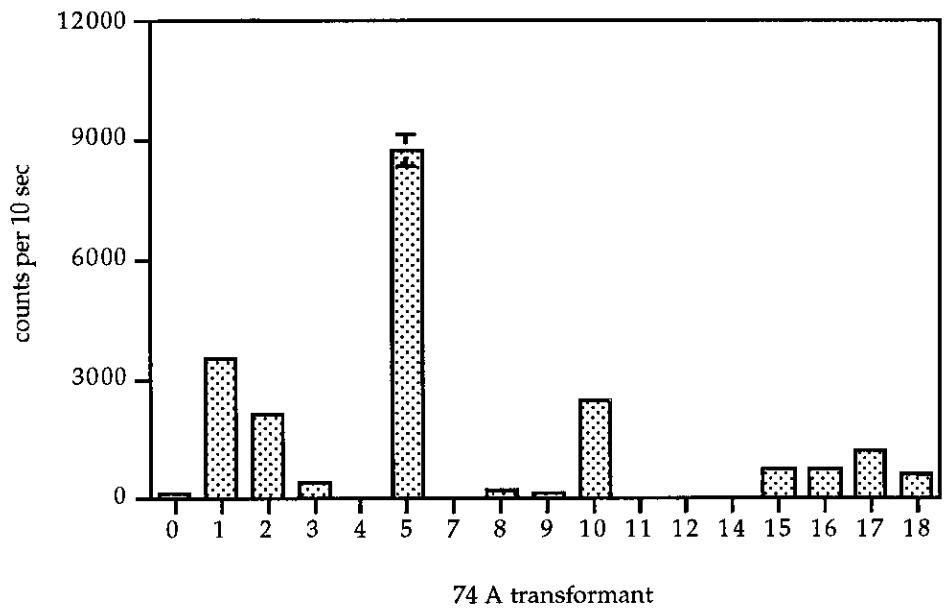


Figure 4 .10: Aequorin luminescence in extracts of pNCAEQ3-transformed *N. crassa 74A*. Each bar represents the data obtained from three mycelia, each mycelium was tested three times. Transformant numbers which are missing from the x-axis denoted slow growing colonies and were discarded from the screen. Zero denotes untransformed *74A*.



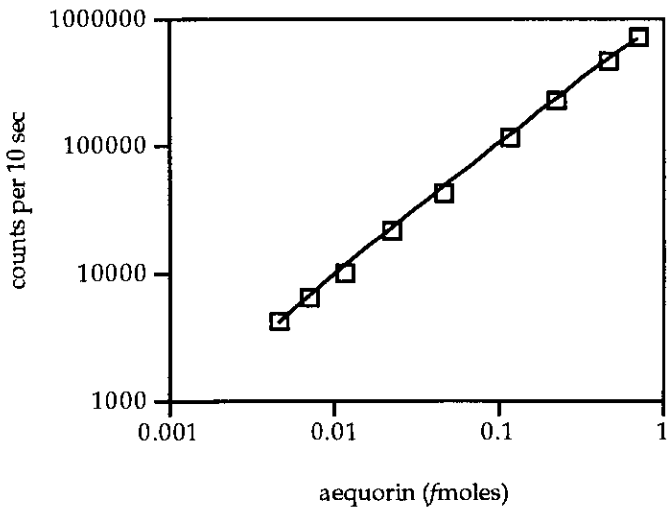
The different levels of expression with the same promoter could reflect the influence of the surrounding host sequences at different sites of apoaequorin gene integration. They may also be due in part to the heterokaryotic nature of many of the primary transformants, with different proportions of transformed nuclei. Controls using untransformed, wild type extracts showed a negligible level of light emission.

4.5.2 Calibration of apoaequorin expression in *N. crassa*

Calibration of the aequorin signal from transformed *N. crassa* was performed to enable comparison with aequorin levels in other transformed organisms and to compare the level of expression of this heterologous gene with other transgenes in *N. crassa.* The amount of apoaequorin in *N. crassa* extracts was estimated by comparing the light yield of *N. crassa* aequorin following *in vitro* constitution, with the light yield from known amounts of recombinant aequorin in the presence of 200 µg of untransformed 74*A* protein. Aliquots containing 20 µg protein from this were assayed in TE pH 7.0, by luminometry.

The standard curve in Fig. 4.11 allowed the quantification of aequorin from *N. crassa* transformants (see Table 4.1). An equivalent standard curve was produced for apoaequorin expressed in *Nicotiana plumbaginifolia. N. plumbaginifolia* leaf tissue was used for the protein extract *in vitro* constitution followed the method in 2.12.3.2. The line of best fit for recombinant aequorin in *N. plumbaginifolia* extract was y = 1013711.271x + 20811.137 (graph not shown).

Figure 4.11: Standard curve of aequorin light emission. Each point represents the mean of nine replicas, standard errors are too small to be visible on the y-axis scale. The line of best fit is plotted (y = 995395.351x - 577.914).



From table 4.1 it can be seen that the level of apoaequorin expressed in *N. crassa* is very low. Whilst these concentrations are well within the range of aequorin detection by luminometry they are evidently too low for adequate detection by Western blotting. The best-yielding *N. crassa* transformant produced 365 times less apoaequorin than equivalent *N. plumbaginifolia* extracts. It is considered that any differences between the aequorin forms present in *N. crassa* and *N. plumbaginifolia* extracts and that of recombinant aequorin (Molecular Probes, USA) will not affect this calibration significantly.

Table 4.1: Quantification of aequorin from extracts of transformed *N. crassa* and *N. plumbaginifolia*.

Trans-formant[a]	counts per 10 sec for 20 µg protein	*f*moles aequorin in 20 µg protein	aequorin *f*g / µg protein[b]
74A 1,29	10756	0.0114	12.5
74A 1,40	7615	0.0082	9.0
BdA 1,1	9745	0.0103	11.3
BdA 1,2	10527	0.0112	12.3
BdA 1,13	8941	0.0096	10.6
BdA 1,14	11145	0.0118	13.0
BdA 1,15	11153	0.0118	13.0
74A 3,5	8734	0.0094	10.3
N.p 2.4	4,390,773	4.31	4740

Notes:

[a] *N. crassa* transformants: the strain is given, followed by the plasmid (i.e. 1= pNCAEQ1, 3=pNCAEQ3) and then the number of the transformant within the series.
N.p. 2.4: denotes a *N. plumbaginifolia* apoaequorin transformant (Knight *et al.*, 1991b). Leaf tissue was used for the protein extract; *in vitro* constitution followed the method described in section 2.12.3.2

[b] One femto-mole of aequorin weighs 2.2×10^{-11}g.

4.5.3 Summary of transformant screens and calibration

The very low level of apoaequorin in the *Neurospora* transformants is a source of concern, as it may limit the measurement of $[Ca^{2+}]_c$ using aequorin; factors contributing to the low yield are investigated in sections 4.7 to 4.10.

The variation in apoaequorin yield, between transformants may have more than one cause. One inescapable source of this variation will be the effect of different integration sites on transgene expression (Sheffield, 1994). Also, those showing a higher level of expression may contain multiple transgene copies. Another complication is that, since most of the macroconidia used to make spheroplasts are multinucleate, and that only a single nucleus tends to be transformed (Grotelueschen and Metzenberg, 1995; Pandit and Russo, 1992), the primary transformant mycelium is expected to be heterokaryotic. It is clearly desirable to obtain homokaryotic transformants, in which all nuclei will be contributing to the transformed phenotype. There are two standard ways of producing homokaryons: by sexual crossing to produce pure haploids carrying the transgene, and repeated re-isolation from single conidia.

Sexual crossing has the disadvantage in *Neurospora* that gene sequences which are artificially repetitive are subjected to disruption by RIP (repeat-induced point mutations) immediately prior to meiosis (Selker, 1990). Some transformants may have more than one copy of the transgene, and all transformants should have a duplication of the malate synthase or glucose-repressible gene promoter, since there was one copy of each already present in the untransformed wild type. Thus, some transformants made homokaryotic by outcrossing may lack transgene activity. However, except where repeated sequences are closely linked

(where RIP may affect nearly 100% of meiotic products), 50% or more of the duplicated sequences usually escape RIP.

Repeated re-isolation of conidia provides an alternative method where outcrossing fails. Experience shows that six cycles of single-conidium isolation are virtually guaranteed to produce homokaryosis, and three-cycles do so with high probability. This procedure avoids the problem of RIP but transgene expression per mycelium may not necesssarily be improved. The primary transformant may have been homokaryotic in the first place, and also duplicated transgene copies may be subjected to the poorly understood process of asexual gene silencing, known as quelling (Cogoni *et al.*, 1996; Pandit and Russo, 1992).

4.6 The production of homokaryons from *N. crassa* primary transformants

Homokayons were made from primary transformants that expressed the highest levels of apoaequorin, using both sexual crossing and repeated re-isolation from a single conidium. Mycelia for protein extraction was obtained from 41-44 hour old standing liquid cultures. Apoaequorin expression was determined using *in vitro* constitution and luminometery following the optimised protocol.

Figure 4.12: Analysis of homokaryons from primary transformants. Each bar represents the mean light emission from 20 µg aliqouts of the constitution mixes of three individual mycelial extracts which were each tested three times. Standard errors calculated on the nine readings are shown for those points where agreement of replicas was not very close.

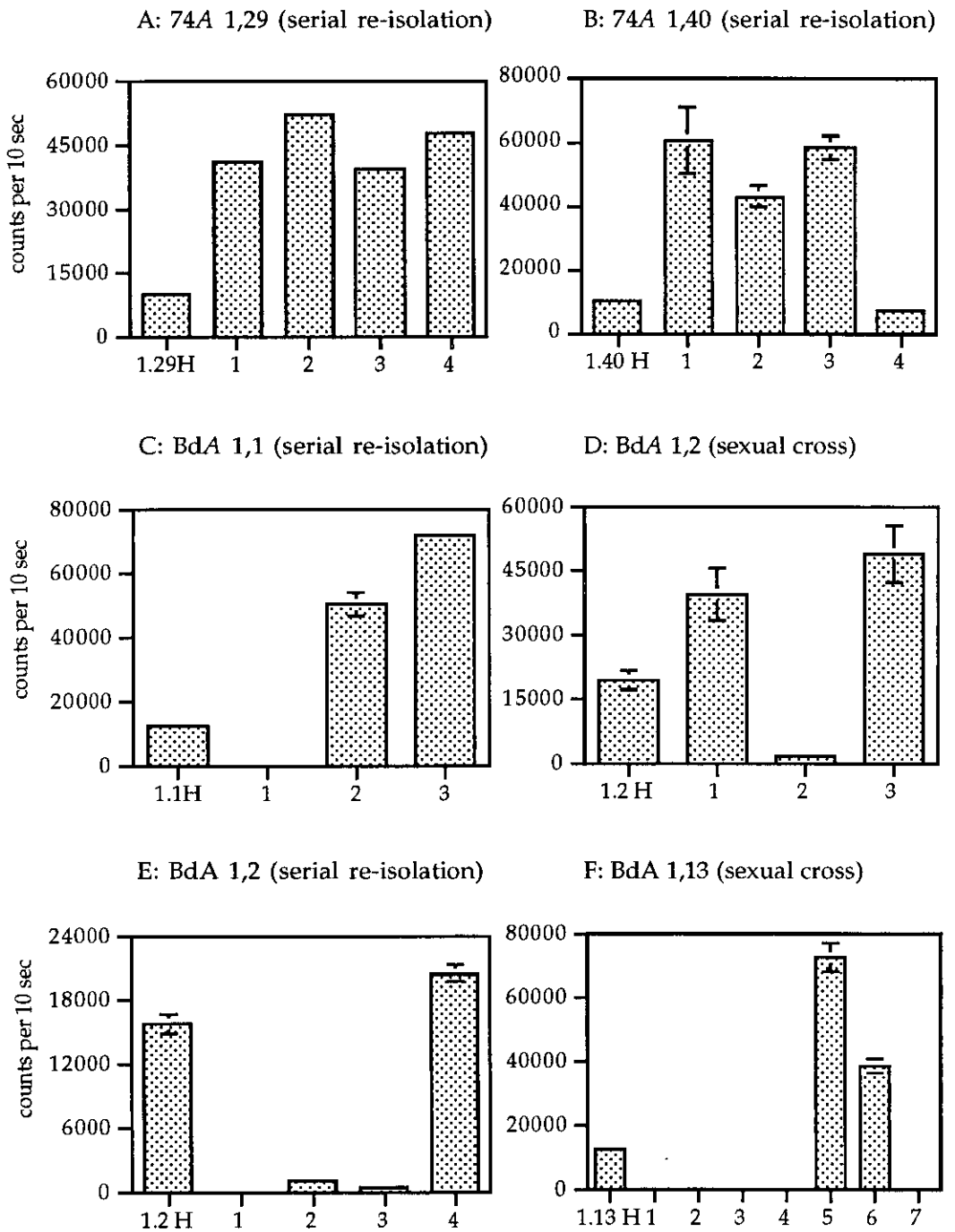


Figure 4.12 continued:

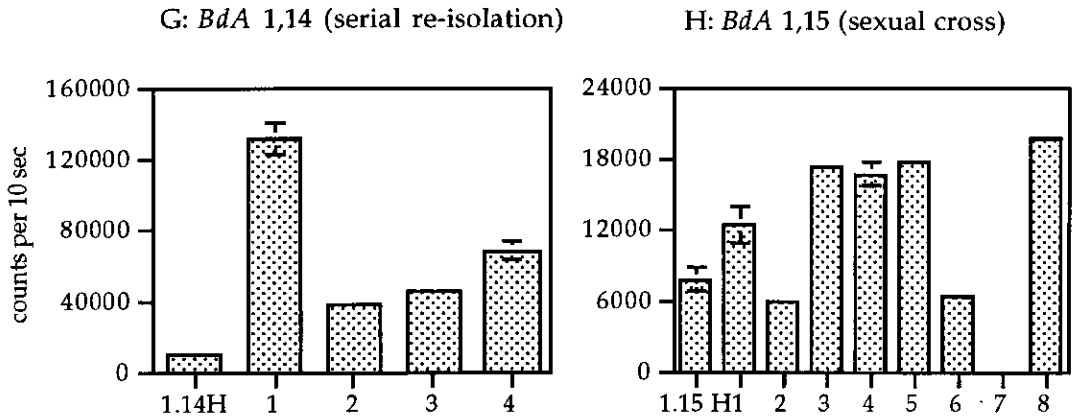


Fig. 4.12 presents the estimated apoaequorin yield from homokaryotic strains derived from primary transformants which expressed the highest levels of apoaequorin. The results obtained were very variable. All outcrosses of transformants produced hygromycin-resistant derivatives with the exception of the sexual cross of *BdA* 1,1 (data not shown). All the outcrossed transformants gave at least one hygromycin-resistant derivative with virtually no apoaequorin activity, as if RIP was occurring; one cross (*74A*, 1,40), gave no apoaequorin producers at all (data not shown). However the same transformant did yield significantly improved levels of apoaequorin production through conidial isolation. The apparently non-RIP crossed derivatives showed up to a 7- fold (1,13,5) increase in apoaequorin production over that obtained for the presumably heterokaryotic primary transformant. A puzzling feature from the sexual crosses was that different non-RIP derivatives of the same cross sometimes showed quite different apoaequorin expression levels (see *BdA* 1,2; *BdA* 1,13 and *BdA* 1,15). It is hard to explain this purely as the result of RIP which is normally an all-or-nothing phenomenon. It could be due to the occurrence of quelling in these transformants, or possibly to transformation of more than one independent nucleus in the spheroplast.

In the derivatives of primary transformants obtained by the serial re-isolation of conidia in most cases apoaequorin yield was increased over that of the primary transformant. Some derivatives from the primary transformants showed drastic reductions in yield (*BdA* 1,1 and *BdA* 1,2 series). As with the homokaryons derived by sexual crossing, derived strains differed considerably in the expression of apoaequorin (74A 1,40; *BdA* 1,1 series). Such inconsistent results may be due to different degrees of quelling, but there is also the small chance that these re-isolates, unlike those from the sexual crosses, are not homokaryotic.

The presumed homokaryon *BdA* 1,14,1, showed the greatest increase in apoaequorin expression, some 11-12 times greater than the primary transformant; this re-isolate produced the highest level of apoaequorin of all the transformants and their derivatives.

It would have been of interest to screen some of the homokaryons and relevent heterokaryons by Southern blotting for methylation of the apoaequorin gene which would be expected to accompany either RIP or quelling. However, time did not permit. The *in vivo* work presented in chapter five utilized the homokaryon 1,13,5 which contained 81.2 *fg* apoaequorin per µg protein and *BdA* 1,14,1 which contained 146 *fg* apoaequorin per µg protein.

These levels, though increased as compared with the primary transformants, are still extremely low, and the following sections explore the possible reasons for this poor transgene expression. The most obvious possibilities are incomplete integration of the coding sequence and its promoter, very low levels of apoaequorin mRNA (due to either poor transcription or instability), and *in vivo* instability of apoaequorin.

4.7 Southern analysis of transgene integration

Southern blotting was conducted to analyse plasmid integration into the genome in transformants which gave a reasonable signal in the *in vitro* constitution screen. For all transformants, two restriction digests were carried out on the genomic DNA; one used an enzyme which did not cut within the transforming DNA, so that the number of integration sites could be determined, and the other removed the complete apoaequorin expression cassette in a fragment of predictable size so that any truncation of the expression cassette could be detected. Untransformed *BdA* and *74A* DNA was used as a control where appropriate. The radioactive probe was synthesised from apoaequorin cDNA which had been isolated from a plasmid in which *N. crassa* sequences were absent. This was to avoid contamination by radiolabelled DNA which could bind to host sequences.

For pNCAEQ1 transformants *Bgl*II was used to determine the number of integration sites and *Cla*I to release the intact expression cassette. *Cla*I digestion of pNCAEQ1 releases a band of approximately 1.8 kb; 750 bp of the malate synthase promoter, 588 bp apoaequorin cDNA and 500 bp of the *am* terminator.

For the analysis of pNCAEQ3 transformants, DNA was digested with *Sac*I to determine the number of integration events. *Eco*RI digestion of pNCAEQ3 releases a fragment of approximately 1.95 kb, composed of the 1.4 kb *grg-1* promoter and 588 bp apoaequorin cDNA.

Figure 4.13: Autoradiograph of apoaequorin probed *Bgl* II digested genomic DNA, from selected pNCAEQ1 primary transformants (Bd*A* 1,2; 1,13; 1,14; and 74 A 1,5; 1,12; 1,28; 1,29; 1,31; 1,40) and homokaryons derived from Bd*A* 1,2 (1,2,1; 1,2,2; 1,2,3). UT denotes untransformed.

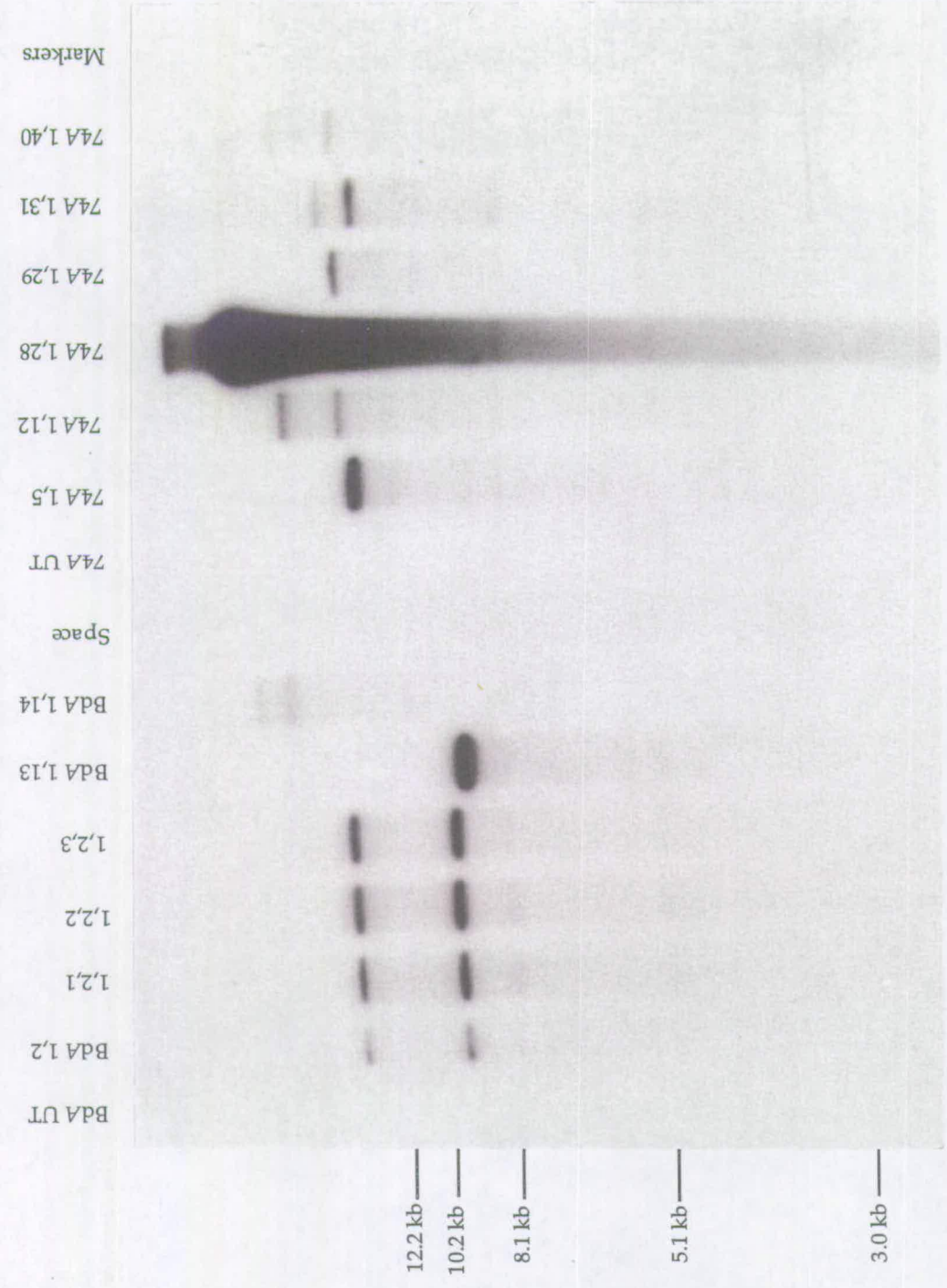


Figure 4.14: Autoradiograph of apoaequorin probed *Cla*I digested genomic DNA, from selected pNCAEQ1 primary transformants (Bd*A* 1,2; 1,13; 1,14; and 74 *A* 1,5; 1,12; 1,28; 1,29; 1,31; 1,40) and homokaryons derived from Bd*A* 1,2 (1,2,1; 1,2,2; 1,2,3). UT denotes untransformed; aeq denotes the released apoaequorin expression cassette (approximately 1.8 kb).

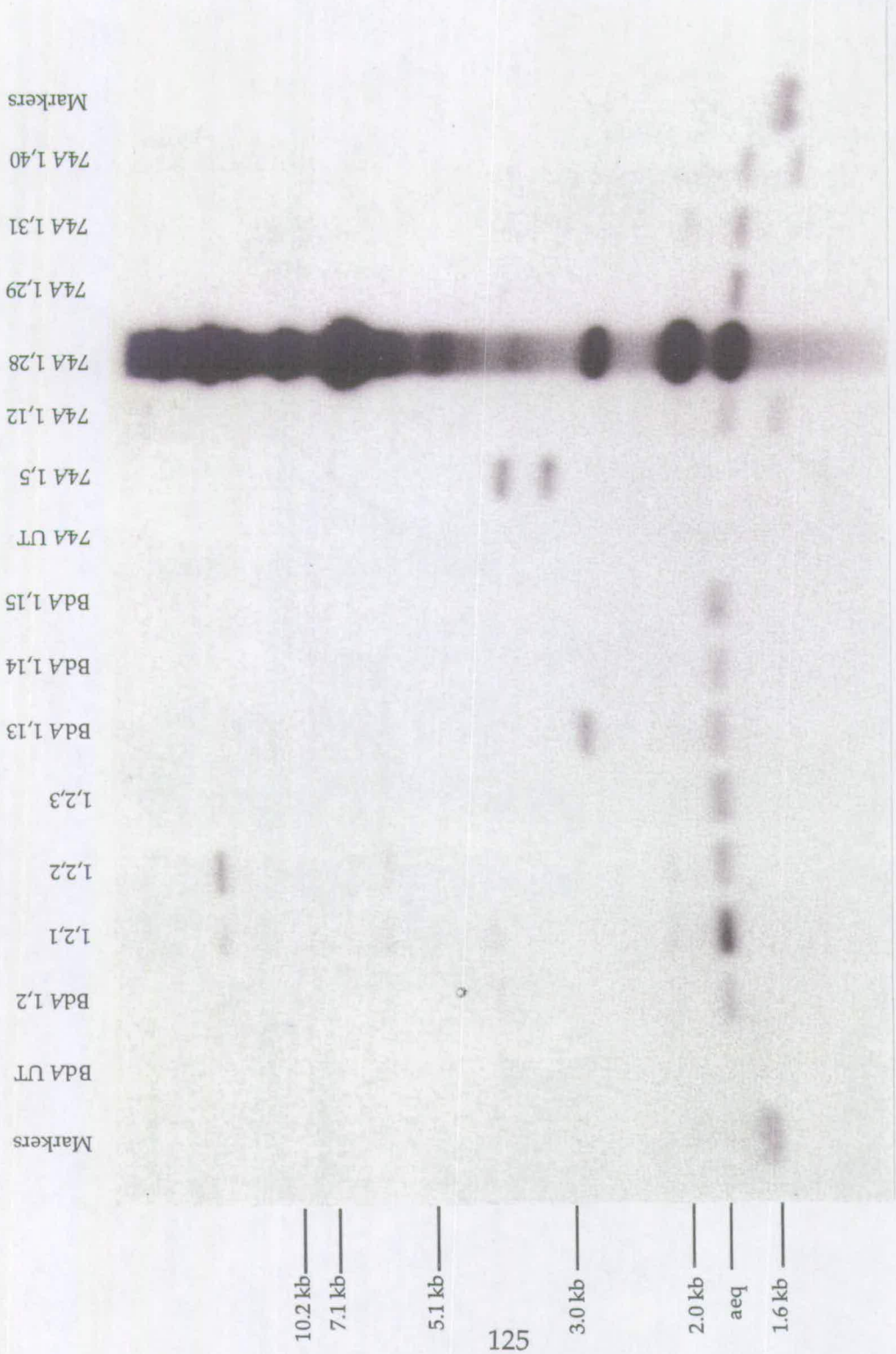


Figure 4.15: Autoradiograph of apoaequorin probed *Sac* I and *Eco* RI digested genomic DNA from 74*A* pNCAEQ3 transformants (3,1; 3,2; 3,5), and *Bgl* II digested genomic DNA from pNCAEQ1 transformants (Bd*A* 1,15, 74*A* 1,28). UT denotes untransformed; aeq denotes the intact 1.4 kb grg-1 promoter and 550 bp apoaequorin cDNA.

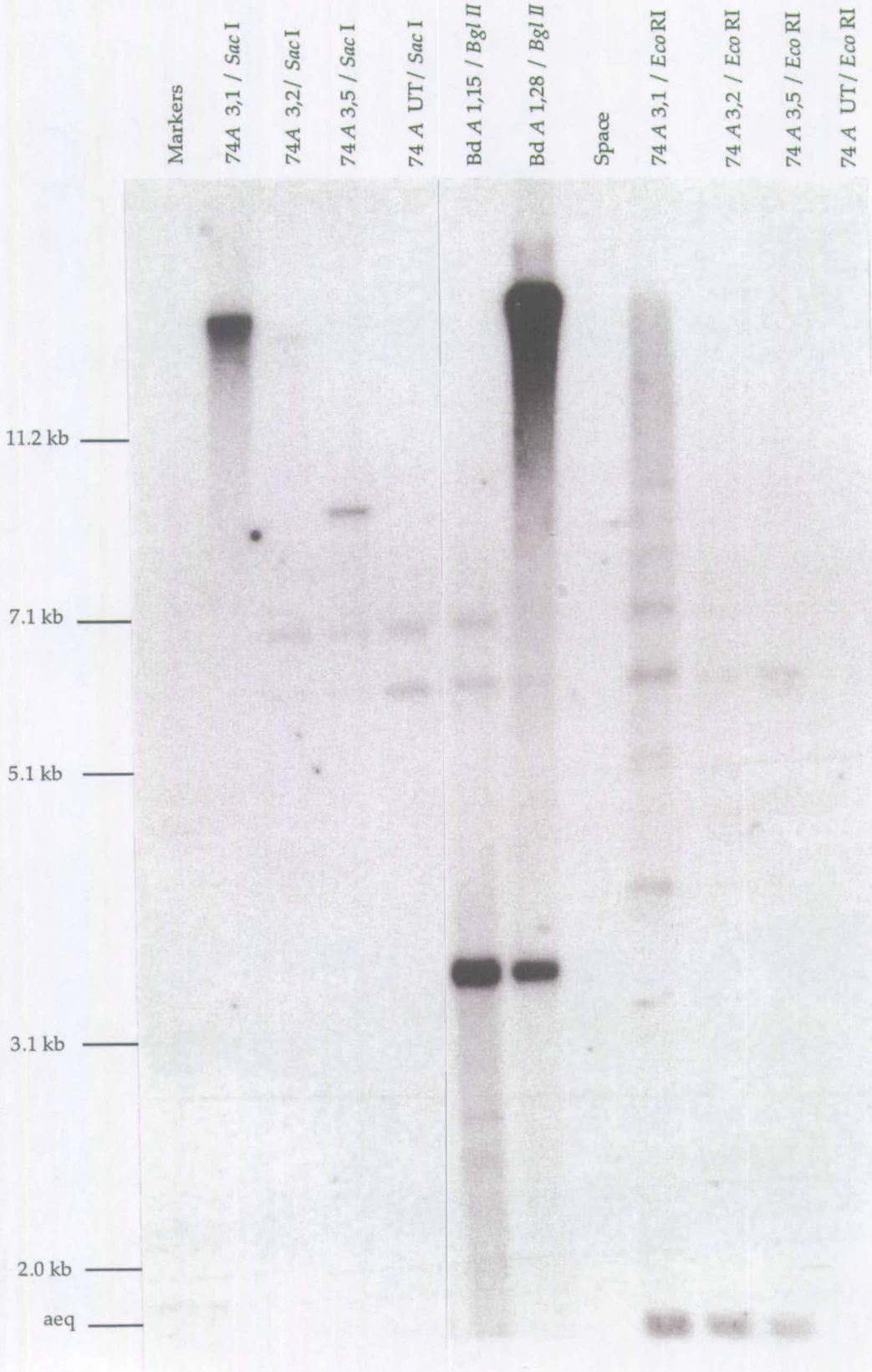


Table 4.2: Characteristics of selected transformants determined by Southern analysis.

Transformant	Integration events [a]	Estimation of copies integrated[b]	Unmodified expression cassette[c]	Modified expression cassette[d]
BdA 1,2	2	1 + 1	yes	no
BdA 1,13	1	14-15	yes	at 3.06 Kb
BdA 1,14	2	1* + 1*	yes	no
BdA 1,15	1	not determined	yes	no
74*A* 1,5	1	8-9	no	at 3.74 and 4.47 Kb
74*A* 1,12	2	1*+ 1*	yes	at 1.55 Kb
74*A* 1,28	many	numerous	yes	at 2.32, 3.2, 8.4, 10.5 and 11.5 Kb
74*A* 1,29	1	1-2	yes	no
74*A* 1,31	2/3	1-2 + 1*	yes	no
74*A* 1,40	2	1 *+ 1*	yes	at 1.51 kb
74*A* 3,1	many	numerous	yes	at 4.55 kb
74*A* 3,2	1	not determined	yes	no
74*A* 3,5	1	"	yes	no

notes:

a: number of hybridised bands from transformant genomic DNA digested with *Bgl*II (pNCAEQ1) or *Sac*I (pNCAEQ3).

b: from phosphoimagery of the filter represented in Fig. 4.13

+ denotes more than one integration site

* denotes a faint band, assumed to be a single copy

c: hybridised band corresponding to the expected size of the intact expression cassette

d: additional bands not corresponding to the expected size of the intact expression cassette

Estimates of apoaequorin gene copy number were based mainly on the number of *Bgl*II fragments for pNCAEQ1 (Fig. 4.13) transformed *N. crassa*, and *Sac*I fragments for pNCAEQ3 (Fig. 4.15) transformants. Both restriction enzymes cut only outside the transforming DNA, and the number of integrations should equal the number of fragments. As a second less certain measure, some comparisons of band intensities were made by phosphoimaging of the pNCAEQ1 transformed *N. crassa* genomic DNA digested with *Bgl*II (Fig. 4.13) , using the lower band in *BdA* 1,2 as a "single copy" standard. This method would reveal multiple copies at the same site, as might result from the integration of tandem repeats. The conclusions are summarised in table 4.2.

The *Cla*I digests of pNCAEQ1 transformants (Fig. 4.14 and Fig 4.15), and the *Eco*RI digests of pNCAEQ3 transformants (Fig. 4.15), revealed whether at least one complete expression cassette has been integrated in each transformant. In all but one transformant (*BdA* 1,5) this was clearly the case. This general observation shows clear evidence that the low level of apoaequorin production was not due to the loss of essential promoter sequences during integration.

In several of the transformants (e.g. 74*A*1,12; 74*A*1,40; *BdA*1,13; 74*A*1,5; 74*A*1,31; 74*A*3,1) it was clear that the transforming DNA was quite extensively deleted or rearranged before or during integration. This could be due to recombination between plasmids and/or nuclease activity. More extensive restriction site analysis would be necessary to show what may have happened in these cases.

Analysis of the transformant *BdA* 1,2 and derivatives, obtained by sexual crossing, showed the expected increase in gene dose in the

derivatives. It also showed that the site of integration is not the only reason for different apoaequorin yields.

74*A* 1,28 (see Fig. 4.14 and Fig. 4.15) shows enormous gene amplification which was not understood and time did not permit further investigation. It included many copies of the intact expression cassette, but this was not reflected in increased apoaequorin yield, which suggests gene silencing in all copies but one.

In summary, all but one of the transformants tested contained at least one intact expression cassette known to function from the transformant screens.

4.8 Northern analysis of selected *N. crassa* transformants

Northern analysis was performed on six pNCAEQ1 primary transformants and two associated homokaryons. Two of the transformants showed a low level of aequorin in *in vitro* constitution assay (*BdA* 1,4; 74*A* 1,6), the others showed a higher level (*BdA* 1,2; *BdA* 1,15; 74*A* 1,5; 74*A* 1,40) and were also included in the Southern analysis. As for the Southern analysis, the radioactive probe was made from apoaequorin cDNA obtained from a source devoid of *N. crassa* sequences.

If the apoaequorin gene is transcribed correctly a single band approximately 1 kb should be visible. This will be composed of a leader sequence of 50-70 bp from the malate synthase promoter (I. Connerton, pers. comm.), 588 bp apoaequorin cDNA, 220 bp of 3' untranslated sequence from the *am* terminator and a poly A tail which is commonly about 200 bp in length.

Figure 4.16: Autoradiograph of total RNA from pNCAEQ1 primary transformants (Bd*A* 1,2; 1,4; 1,15 and 74A 1,5; 1,6; 1,40) and derived homokaryons (1,2,1; 1,15,8). 40 µg of RNA from each *N. crassa* strain and 8 µg of RNA from apoaequorin transformed *N. plumbaginofolia* (N.p2.4) was separated by electrophoresis prior to blotting and detection. Plasmid denotes 10 ng of pAEQ1 which contains the apoaequorin cDNA but is devoid of any *N. crassa* sequences. UT denotes untransformed; aeq denotes *N. crassa* apoaequorin mRNA (approximately 950 bp).

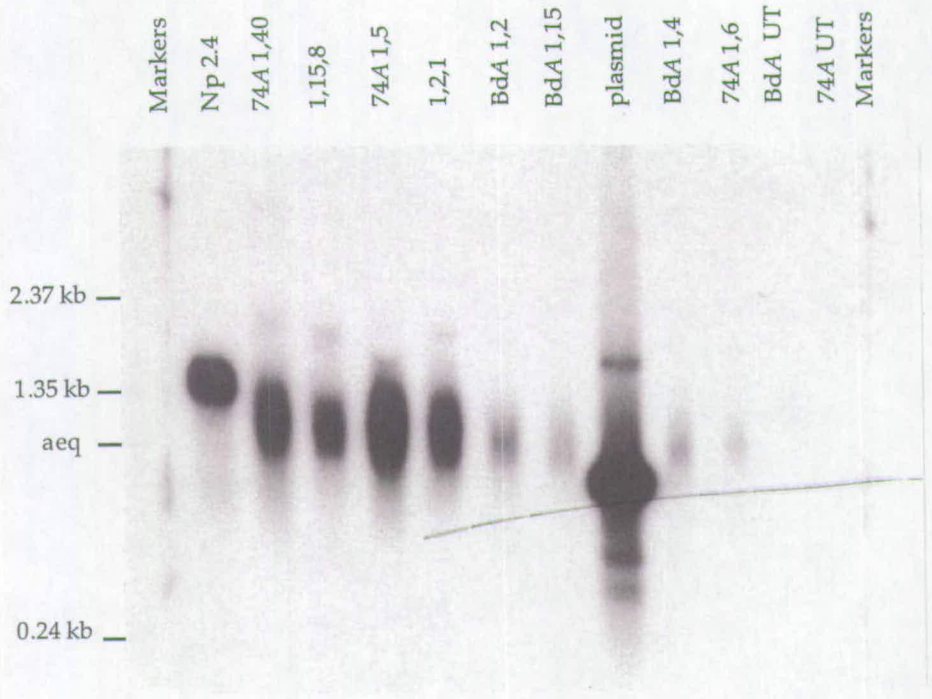


Fig. 4.16 showed that in all transformants the apoaequorin probe revealed mRNA approximately 950 bp in size (size assessed at the bottom of dense band). This corresponds well with the expected size of the transcript from the apoaequorin expression cassette. The *N. plumbaginifolia* apoaequorin mRNA is approximately 1.4 kb. As expected the probe did not hybridised to any mRNA in untransformed *74A* and *BdA*. Little breakdown product of the mRNA is visible.

Among the *Neurospora* transformants there is a general correlation between mRNA level and aequorin. Transformants which yield a low light signal (i.e. *BdA*1,4; *74A*1,6) exhibit less hybridised mRNA. However, no such correlation is apparent in transformants which give a higher aequorin signal (i.e. *BdA*1,15; 74*A*1,5). In the two cases investigated (*BdA*1,2 and *BdA*1,15) the derived homokaryons seemed to perform better than the primary transformants.

Taking into account the different RNA loadings, the apoaequorin mRNA seems somewhat more abundant in *N. plumbaginifolia* (8 µg total RNA loaded) than in the *N. crassa* transformants (40 µg total RNA loaded). The difference as determined by phosphoimaging, shows approximately 2-fold more mRNA in *N. plumbaginifolia* than in the *N. crassa* transformant 74*A*1,5. This is clearly not sufficient to account for the almost 400-fold difference in apoaequorin yield.

Northern analysis showed that the transgene was efficiently transcribed. Thus the block acting on aequorin yield from transformants must be at translation or beyond. Two important factors effecting these steps will be; at translation, codon requirements of the transgene and beyond this, apoprotein stability in the new cellular environment. The contribution of both of these factors was examined.

4.9 Possible effects of incompatible codon usage

One possible explanation for translational inefficiency of a transgene is that it contains too many codons that are only rarely used in the host species. Here, a codon was designated as rare if it was utilised 11% or less for a specific amino acid. Fortunately, the recent transformation of two other fungal species, *Candida albicans* and *Saccharomyces cerevisiae* (Prof. N.A.R. Gow, pers. comm.) allowed a direct comparison of apoaequorin transgene expression in three fungal species, to be made by aequorin *in vitro* constitution and luminometry. The results from which could then be discussed with respect to the codon usage of the individual species.

Apoaequorin transformed *C. albicans* and *S. cerevisiae* were extracted in AC buffer, following the protocol developed for *N. crassa* (kindly donated by T. Perera University of Aberdeen, UK). *In vitro* constitution and luminometry used the optimised procedure for *N. crassa* extracts.

The codon usage in both *N. crassa* and *C. albicans* shows a strong bias against apoaequorin codon usage (table 4.3), whereas in *S. cerevisiae* and *N. plumbaginifolia* there is very little. Fig. 4.17 shows that the expression of the apoaequorin transgene was very much better in *S. cerevisiae* than in *C. albicans* (approximately 30 times better), and this may well be due to more compatible codon usage. However, the *C. albicans* apoaequorin yield was itself about seven times higher than that of the best *N. crassa* transformant, in spite of the apparently similar degrees of codon incompatibility.

Unfortunately only general observations can be drawn from the comparisons of codon usage in different species and the influence that this may have on apoaequorin expression.

Figure 4.17 Apoaequorin expression in transformed *S. cerevisiae* (SC) and *C. albicans* (CA). On the x-axis, UT denotes untransformed, numbers (1-3) denote different transformants. Each bar represents the mean of three 20 μg protein aliquots from separate *in vitro* constitutions, from a single protein extraction. The highest expressing apoaequorin transformed *N. crassa* protein extract yields approximately 130,000 counts under identical conditions.

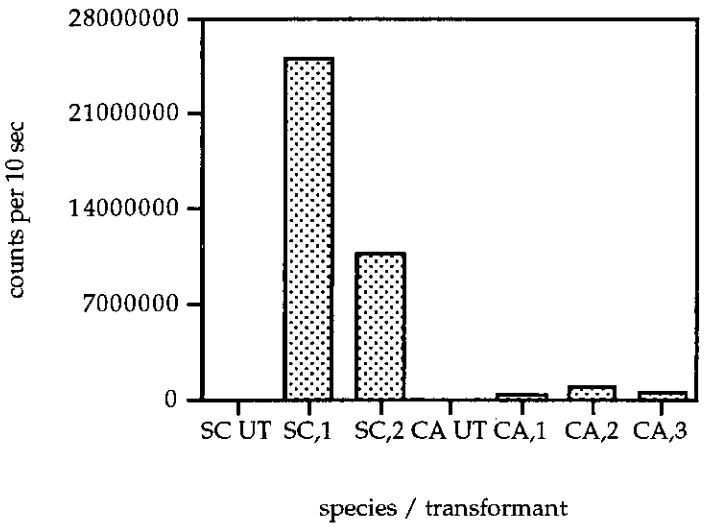


Table 4.3: The occurrence and distribution of codons in apoaequorin which are used in the host organism at a frequency of 11% or less.

Rare codons	Transformed species		
	N. crassa (aeq1)	*C. albicans (AQ440)*	*S. cerevisiae (AQ440)*
total	44	49	10
doublets	9	10	0
triplets	1	1	0
quadruplets	0	1	0

notes:
Doublets, triplets, quadruplets: 2, 3,or 4 rare codons occuring in succession.
Determination of rare codons was based on the following codon usage tables, *N. crassa* based on 85 genes (Edelmann and Staben, 1994), *C. albicans* based on 27 genes (Wada *et al.*, 1992) and *S. cerevisiae* based on 575 genes (Sharp and Cowe, 1991).

4.10 Apoaequorin stability in *N. crassa*

An attempt was made to assess the *in vivo* stability of apoaequorin in *N. crassa* mycelium by monitoring its rate of disappearance when further protein synthesis was inhibited. To achieve this, 41 hour standing liquid cultures of the homokaryon 1,2,1 were supplemented with 2 μg/ml cycloheximide (CHX). This concentration causes a 90% inhibition of protein synthesis within 1 hour in *N. crassa* (Aisemberg *et al.*, 1989). Control cultures were supplemented with an equal volume of sterile distilled H_2O. Cultures were incubated at 25 °C rotating at 100 rpm to ensure that all the mycelial mat was be exposed to CHX. At appropriate time intervals cultures were removed, washed and frozen in liquid nitrogen prior to extraction, as described in section 2.14.1. Extracts were subjected to *in vitro* constitution and luminometry in the usual way.

Figure 4.18: Stability of apoaequorin in *N. crassa* mycelium determined by treating cultures with cycloheximide. +CHX data points represent the mean of protein extracts from three individual mycelial pads each tested three times, standard errors are shown for those points where agreement of replicas was not very close.

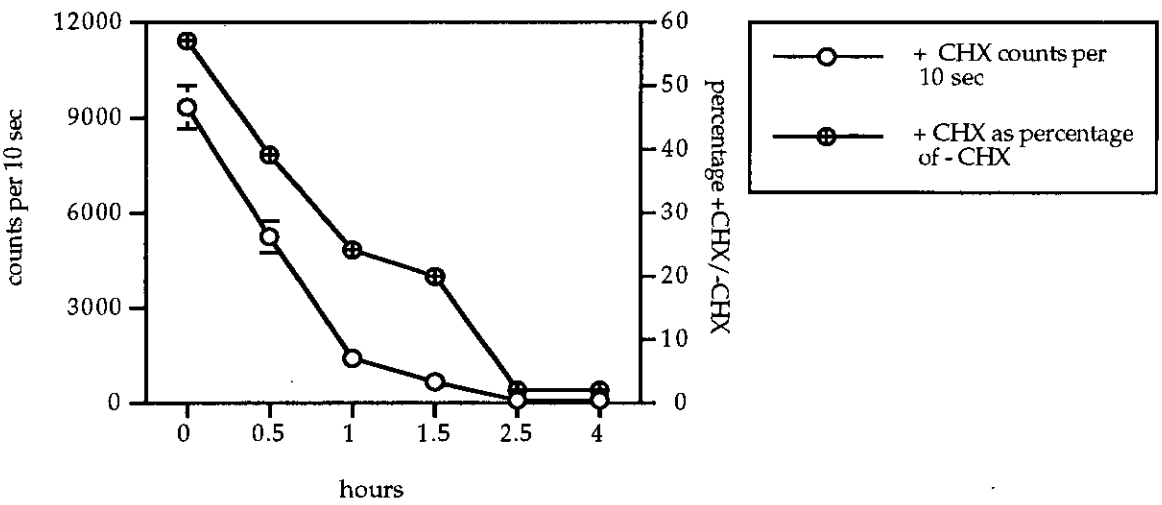


Fig. 4.18 shows that treatment of cultures with CHX resulted in a steep decline in apoaequorin, to what was effectively zero at 2.5 h. The half life

for apoaequorin in *N. crassa* mycelium was approximately 45 min. The data presented in Fig. 4.19 represent one experiment of three all of which showed the same effect of cycloheximide.

The initial decline in apoaequorin was very fast, with a drop of 41% between +CHX and -CHX cultures at time zero. In practise, time zero was actually 30 and 90 seconds of exposure to CHX and it was unexpected that this short exposure would cause such a large reduction. It is conceivable that apoaequorin breakdown continued as the mycelium was processed after removal from the culture medium and prior to freezing in liquid nitrogen. The processing required 10 min.

An unexpected effect seen in this experiment was the decline in aequorin light emission from the -CHX control. The growth of cultures prior to the experiment was in constant darkness at 25 °C. On setting up the experiment, all cultures were exposed to daylight, slight variations in temperature, and during the experiment, to gentle movement on a rotary shaker. It is possible that the introduction of such environmental variables caused this decline by increasing apoaequorin breakdown, e.g. by up-regulating protease activities or increasing transport to the protease-rich compartments. Whether these changes are occurring in the +CHX treatment cannot be determined, since protein synthesis is inhibited. Thus unfortunately it was not possible to determine whether the +CHX treatment demonstrates the effect of CHX only or a combination of CHX and certain environmental variables.

The rapid loss in apoaequorin on treatment with CHX was compared with that of a native *N. crassa* protein, glutamate dehydrogenase (GDH), to gain an insight into the specificity of protein breakdown. GDH showed virtually complete stability, remaining unchanged in both the +CHX and -

CHX treatments for up to 2.5 hours, at which point a slight decrease was seen in both treatments (data not shown).

The interpretation of the cycloheximide experiment must be made with caution as its effect on protein breakdown systems is unknown. Superficially the data indicates a half life of apoaequorin in *N. crassa* of about 0.75 hours. This degree of instability would certainly decrease the steady-state level of the protein, but this alone cannot really explain the very low levels of apoaequorin actually found.

When the truncated malate synthase promoter, used here, was fused to the *N. crassa am* (glutamate dehydrogenase) gene, the level of GDH expression was almost that of the wild type (approximately 0.25% of total soluble protein). This is many thousands of times that obtained for apoaequorin from the best homokaryotic transformant. GDH is virtually stable, and if the rates of synthesis of the two proteins were equal, the rate of degradation of apoaequorin plus the rate at which it is diluted by growth would have to equal the rate at which GDH is diluted by growth. This would require that the half life of apoaequorin was many thousands of times shorter than the doubling time of the mycelium, which under good growing conditions, is hardly more than 12 hours. The cycloheximide experiment, if taken at face-value, shows that even though apoaequorin seems somewhat unstable *in vivo*, its instability alone cannot account for its poor yield. Unfortunately, since the effects of cycloheximide on protein degradation systems are undetermined, the above conclusion is far from certain.

4.11 Discussion

The most disappointing observation from the *N. crassa* transformant screens was the low level of apoaequorin expression, when compared to that obtained for transgenic *N. plumbaginifolia* (4730 *f*g/μg protein). In pNCAEQ1 transformants, the yield seems even lower when compared with the performance of the truncated malate synthase gene promoter when joined to the native *N. crassa am* gene, encoding glutamate dehydrogenase (Sheffield, 1994).

The Western analysis was insufficiently sensitive to detect the apoaequorin present in the primary transformants though, with hindsight it might have been more successful with some of the highest-yielding homokaryotic derivatives. Because of the failure of the Western blots it was not possible to confirm that the transgenic apoaequorin had the expected molecular weight, but its successful constitution with coelenterazine and luminescent response to calcium showed that it was functionally normal.

Variation between transformants were probably, mainly due to the different sites of integration, some locations giving more transcription than others. The transformants could be broadly categorised into three groups: high expressing transformants which yield aequorin luminescence over 7000 counts, mid-range transformants which yielded between 1000 and 4000 counts, and low expressing transformants which gave less than 1000 counts. But even in the most productive primary transformants, i.e. *BdA* 1,14 and *BdA* 1,15, apoaequorin expression did not exceed 13 *f*g /μg protein. Recovering the transformed nucleus in a homokaryon increased apoaequorin yield to a maximum of 146 *f*g/μg protein (1,14,1). The increase in apoaequorin yield above that of the primary transformant presumably reflected the proportion of transformed

nuclei which were present in the heterokaryotic mycelium, which would depend on the number of nuclei present in the transformed spheroplast, the number of untransformed nuclei which could be supported on antibiotic media, and any limitations placed on the rate of division of the transformed nucleus by the integrated DNA. The presumably homokaryotic derivatives that showed reduced or even zero levels of apoaequorin could be due to either RIP (Selker, 1990), in the case of the cross-derived strains, or to the poorly characterised phenomenon of quelling (Pandit and Russo, 1992).

The maximal level of aequorin in *N. crassa* pNCAEQ1 primary transformants was 365 times less than that quantified from transgenic *N. plumbaginifolia*, by the isolation of homokaryons this was reduced to 32 times less. Even in transformed *N. plumbaginifolia* the level of apoaequorin expressed is much lower than expected with the 35S cauliflower mosaic virus expression cassette (M. Knight pers. comm.). Sheffield (1994) used the truncated malate synthase promoter present in pNCAEQ1 to express the native *N. crassa am* gene. The transformants, homokaryotic for the *am* gene showed levels of glutamate dehydrogenase which were comparable to that obtained in wild type cultures, approximately 0.25% soluble protein (i.e. 2.5 ng per μg soluble protein). Thus, it can be seen that expression of the heterologous apoaequorin gene directed by the truncated malate synthase promoter is very drastically reduced, approximately by 133,000 times. The sensitivity of the luminometer is a most fortunate advantage, for although the level of aequorin is very low, luminescence from homokaryon 1,14,1 extract is still 600 to 700 times above background. Sheffield (1994), attempted to express two heterologous genes, papain and hen egg white lysozyme in *N. crassa* under the control of the acetate inducible promoter for isocitrate lyase. Neither papain or hen egg-white lysozyme was detected in mycelial

extracts of appropriate transformants, indicating that the expression of these heterologous genes may be affected in the same way as apoaequorin.

To confirm that the very low level of expression of the pNCAEQ1 transformants was not due to any problem with the expression cassette, a second vector was constructed and transformed into *N. crassa*. The *grg-1* promoter had been used previously to express bovine preprochymosin in *N. crassa*. A long *grg-1* promoter (1563 bp), 160 bp longer than the version used in this study, yielded between 0.9 and 1.2 µg of enzymatically active bovine chymosin per ml culture medium from the best transformants (Nakano et al., 1993). A shorter version (837 bp) yielded approximately 0.3 to 0.5 µg/ml. The best *grg-1* apoaequorin primary transformant yielded 10.3 pg apoaequorin per mg protein, indicating that this heterologous gene is less favourably expressed than preprochymosin and suggests that the same detrimental factors act on the expression of both pNCAEQ1 and pNCAEQ3.

Examples of heterologous gene expression in *N. crassa* are few and, with the exception of Nakano *et al.* (1993), are not quantitative. Systematic studies in other species have found similar results to those obtained from the transformant screen presented here (e.g. Harrki *et al.*, 1989). In virtually all cases production of a foreign protein is many times lower than that of the protein product of the native gene from which the promoter originates or an ectopically integrated native transgene.

Southern analysis of transformants showed that, in most of the transformants tested, the apoaequorin expression cassette was incorporated intact into the *N. crassa* genome. The presence of apoaequorin in transformant protein extracts indicates that it was transcribed correctly. Some transformants also showed additional

apoaequorin gene fragments; whether these are transcriptionally active is unknown.

The completeness of the integrated copies was investigated by digestion with the enzyme *Cla*I for pNCAEQ1 transformants and *Eco*RI for pNCAEQ3, such a digestion released the expression cassette from the rest of the plasmid. In *BdA*1,5 both copies of the expression cassette had lost at least one *Cla*I site. Other transformants showed both an intact copy and an altered copy in which the expression cassette had either gained (74*A*1,12; 74*A*1,40) or lost (*BdA*1,13) one or more *Cla*I sites. The remarkably high level of hybridisation in *BdA*1,28 (repeated isolations gave the same result) indicates that the *N. crassa* genome is capable of integrating very large stretches of repetitive DNA which is mitotically stable; a similar observation was made in *A. niger* (Verdoes *et al.*, 1993). *BdA*1,28 shows a mid-range level of expression which does not correlate with the apparently huge number of gene copies, indicating that many of these copies must be incomplete or silenced.

The influence of the integration site on transgene expression is problematic as most transformants contain more than one gene copy, but it is suggested in 74*A*3,2 and 74*A*3,5. Both transformants appear to have a single gene copy but 74*A*3,5 yields approximately three times more light.

Northern analysis suggests that the low level of apoaequorin is due to an effect at translation or beyond; mRNA levels seem not to be limiting. In all transformants tested, a hybridised band of about the expected molecular weight was identified. The absence of band smearing to lower fragment sizes shows that the mRNA produced was stable. Probing the filter with β-tubulin allowed a visual estimation of RNA loading to be made. This confirmed that mRNA in the two homokaryotic strains (1,2,1

and 1,15,8) was improved several times over that of the respective primary transformant.

A very interesting observation obtained from phosphoimaging is that the ratio of mRNA production (2:1 using 74*A*1,5) in *N. plumbaginifolia* and *N. crassa* was not nearly as extreme as the ratio at the protein level (365:1). This strongly suggested the presence of a factor or factors which act post-transcriptionally, and which have a much more debilitating effect in *N. crassa* than in *N. plumbaginifolia*. A possible candidate is the difference between the codon usage of the organism and that of the heterologous gene.

Indirect evidence for the involvement of codon usage was obtained by comparing codon usage in apoaequorin with that in different host organisms, *N. crassa, S. cerevisiae* and *C. albicans*. The apoaequorin gene contains 44 codons that are rare (i.e. with less than 11% usage) in *N. crassa* but only one that is rare in *N. plumbaginifolia*. In *N. crassa* expression may be particularly disadvantaged by the high frequency of codons in apoaequorin which have a third position adenine. These include 11 GGA, 10 AAA and 5 ACA codons; the percentage use of these codons in *N. crassa* are 10.8%, 10.4 % and 10.7% respectively. These considerations and, even more, the very different yields of transgenic apoaequorin in *S. cerevisiae* and *C. albicans* broadly suggest that the closer the codon usage of a heterologous gene is to the optimal codon usage of the host, the higher protein production will be. A surprising observation was that *C. albicans* produced almost seven times more apoaequorin than *N. crassa*, although the codon usage of the apoaequorin gene appears to be about equally incompatible with both species.

Where codon usage of the heterologous gene is divergent from the optimal codon usage of the host organism, as is the case for *N. crassa* and

C. albicans, it may be the arrangement of now rare codons within the gene which will limit translation. Low-usage codons have been suggested to be much more effective in blocking ribosome movement on the mRNA when arranged in clusters than when dispersed. In *N. crassa* the introduction of two and three adjacent rare codons with third position adenine caused a 50% and 70% loss in protein production respectively (Kinnaird *et al.*, 1991). The arrangement of *N. crassa* and *C. albicans* rare codons in apoaequorin was examined, to determine if their arrangement could provide an insight into the difference in apoaequorin production in the two species. Translation rates in both species were presumed to be limited by the longest stretch of rare codons in the message. Ribosome density downstream of this cluster would be much reduced and subsequent rare codons may not limit translation, unless their relative rarity coupled with their arrangement exceeded the 'pause' of the longest cluster. In the context of *N. crassa*, the apoaequorin gene has one doublet before reaching three consecutive rare codons. In *C. albicans* it has three doublets before reaching a series of four adjacent rare codons at the same position in the message. On this basis one might expect apoaequorin expression in *C.albicans* to be more handicapped than in *N. crassa*. Determination of the abundance of transcript or protein stability in both species would provide an insight in the observed difference in apoaequorin yield. In summary, the effects, if there are any, of codon usage, are far from well understood.

Although differences in codon usage may affect protein production, work with gene fusions suggests that other factors are involved in its determination. A number of studies have shown that the inclusion of native protein sequences, making a hybrid protein, can improve foreign protein yield (Nyyssönen *et al.*, 1993, Jeenes *et al.*, 1994). The levels obtained are still considerably lower than those obtained in the expression of transgenes in their own species. Obviously the codon requirements of

the foreign gene have not changed, but it has been provided with additional properties that improve translation and/or protein stability. It would be of great interest to examine the expression of a heterologous gene, which has been synthesised to correlate with the optimal codon usage of the host, and examine its expression alone and as part of a fusion protein.

The low level of apoaequorin present in transformants may be due in part to the differences in codon usage between the gene and *N. crassa* but there are other factors which are also exerting an effect. Very few studies on heterologous expression in filamentous fungi have looked at the stability of the foreign protein in the new cellular environment. Fig. 4.19 presents the data obtained on supplementing transformed cultures with the protein synthesis inhibitor cycloheximide. In *N. crassa* cycloheximide (2μM/ml) causes 90% inhibition of protein synthesis in the first hour followed by an adaptive recovery to about 50%. Early on during inhibition several proteins are synthesised (Aisemberg *et al.*, 1989). By inhibiting apoaequorin synthesis it was possible to monitor the durability of apoaequorin in the cytosol. Apoaequorin was fairly unstable under these conditions, with a half life of approximately 45 min. Glutamate dehydrogenase is virtually stable under the same conditions. The instability of the protein as the main cause of low apoaequorin yield in *N. crassa* has not been ruled out, but interpretation of the data is complicated by the uncertainty of what cycloheximide does to the protein degrading systems.

The stability of transgenic apoaequorin in a mammalian cell line was estimated as $T_{0.5}$=20 min (Badminton *et al.*, 1995). A gene fusion of luciferase (N-terminus) to apoaequorin produced a hybrid protein with increased stability ($T_{0.5}$= 80-90 min)(Badminton *et al.*, 1995). These results suggest that the N-terminal fusion proteins expressed in the filamentous

fungi, produce higher levels of the foreign protein by protecting the N-terminus of the foreign protein from degradation. One reason for this increase in stability is the probable change in the N-terminal residue. It has been shown that the N-terminal amino acid of a protein is important in determining its half life (Varshavsky, 1995). The stabilisation of apoaequorin as a fusion protein, in *N. crassa* should increase the amount of apoaequorin available for $[Ca^{2+}]_c$ detection within the cell. It has been shown that N-terminal protein fusions do not affect the formation of active aequorin (Casadei *et al.*, 1990).

In summary, this chapter has presented the development of a method to analyse apoaequorin transformed *N. crassa*, analysis of primary transformants using this method, the formation of homokaryons which may dramatically improve apoaequorin production by the transformed mycelium, and an analysis of the steps in apoaequorin protein production. The following chapter presents the development of techniques to measure $[Ca^{2+}]_c$ using the homokaryons which showed the highest level of apoaequorin production.

Chapter Five

5. Towards the Measurement of Cytosolic Free Calcium in *Neurospora*

5.1 Introduction

In chapter three, strains of *N. crassa* were obtained which had been transformed with the apoaequorin gene, controlled by either the native *ms* or *grg-1* promoter. In chapter four, the yield of apoaequorin from these strains was assessed, and transgene expression investigated by analysis of DNA integration, mRNA production, influence of codon usage and apoprotein stability. This chapter presents the development of methods whereby these transformant strains may be able to report changes in their $[Ca^{2+}]_c$.

When developing transgenic aequorin as an intracellular Ca^{2+}-indicator, some general considerations need to be taken into account. First, it is important to show that the presence of aequorin or its constituents do not effect the organism. Second, non-aequorin sources of light emission should be minimised. Finally, one needs a method for translating the measurements of light intensity into absolute Ca^{2+} measurements.

The range of Ca^{2+} concentrations measured using aequorin can be extended by using semi-synthetic aequorin. This is obtained by the incubation of transformed cells with selected synthetic coelenterazines (Knight *et al.*, 1993). Semi-synthetic aequorins of the *e*-type show a bimodal luminescence spectrum thereby simplifying the calibration of $[Ca^{2+}]_c$ concentration.

When applying a putative 'stimulus' of a Ca^{2+}-regulated response to cells containing transgenic aequorin, initially it is important to apply a well characterised agent, such as an ionophore, which facilitates Ca^{2+} uptake into cells. This can then be followed by stimuli in which a role for Ca^{2+} has been indicated from studies in this or other species.

5.2 Assessing the impact of aequorin components on *N. crassa*

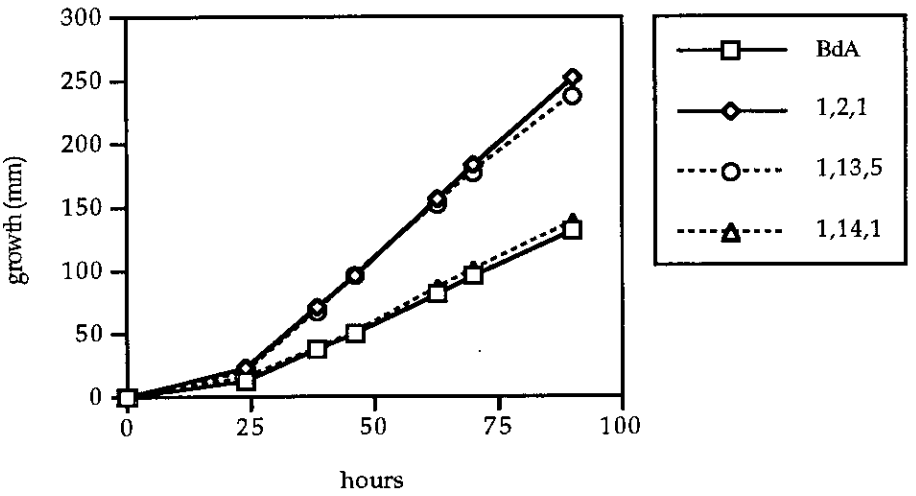
5.2.1 Apoaequorin

The effect of possessing an actively expressing apoaequorin gene was examined by monitoring the growth rate of three transformed strains and an untransformed control. Differences in growth rate may reflect the integration site of the apoaequorin expression cassette or the burden placed on the cell by apoaequorin production.

Conidia ($5x10^6$) were inoculated at one end of a race tube (section 2.2.1.3.2.5) and incubated in darkness at 25 °C. Growth was monitored at regular intervals.

The Fig. 5.1 shows that there are two distinct growth rates. The two growth rates correlated with the morphology of the strain. Slower-growing colonies (*BdA* untransformed and 1,14,1) showed clear bands of conidiation. The fast growing strains (1,13,5 and 1,2,1) show sparse conidiation, and no banding. 1,13,5 and 1,2,1 are homokaryons produced from sexual crosses with the strain *73a*, it is suggested that 1,2,1 and 1,13,5 are Bd^+. Unfortuately no Bd^+ control was included. Comparing untransformed *BdA* with 1,14,1, showed that neither the integration event, nor apoaequorin expression affected growth rate.

Figure 5.1: Growth rate of apoaequorin transformed *N. crassa* strains. Each data point represents the mean of three race tube measurements. Standard errors were too small to show in the graph.



5.2.2 Coelenterazine

The effect of coelenterazine concentration on *N. crassa* cells was assessed. Spheroplasts (5×10^6) from the strain 1,13,5, were incubated in 100 µl of 1 M sorbitol, supplemented with between 0 and 50 µM coelenterazine (MeOH concentration 1% or less). Each sample was incubated for 1 hour at 25 °C, prior to simultaneous staining by FDA and PI (section 2.20).

The results showed that neither coelenterazine nor methanol had an effect on cell viability, which exceeded 95% in all treatments. The experiment also showed that the formation of active aequorin does not effect cell viability. Active aequorin was detected by luminometry, in spheroplasts incubated in 30 µM and 50 µM coelenterazine, on lysis with HPLC H_2O. In subsequent experiments, 50 µM coelenterazine was used for aequorin formation in *N. crassa*.

5.3 Refining the *in vivo* assay system

5.3.1 Controlling background luminescence

To determine the source and level of any background luminescence in the cell suspension, the light emitting properties of chemical components present were investigated. It had previously been noted that mixing coelenterazine and Ca^{2+} can cause a low level of light emission; this is termed the "coelenterazine effect" (M. Knight, pers. comm.). Light emission was measured in the luminometer as follows: to 100 µl of a 50 µM coelenterazine solution (in HPLC H_2O), was added, 500 µl of either d.H_2O, HPLC H_2O or 100 mM $CaCl_2$. HPLC H_2O only was used as a no coelenterazine control.

Figure 5.2: Light emission from solution components present during aequorin detection in *N. crassa* cells. Light emission was measured continuously over 100 sec; water or $CaCl_2$ was injected directly into the coelenterazine (or control) after the first 20 sec. Each point represents the mean of three replicas. Standard errors were calculated on all points, but are only visible in this representation for certain treatments.

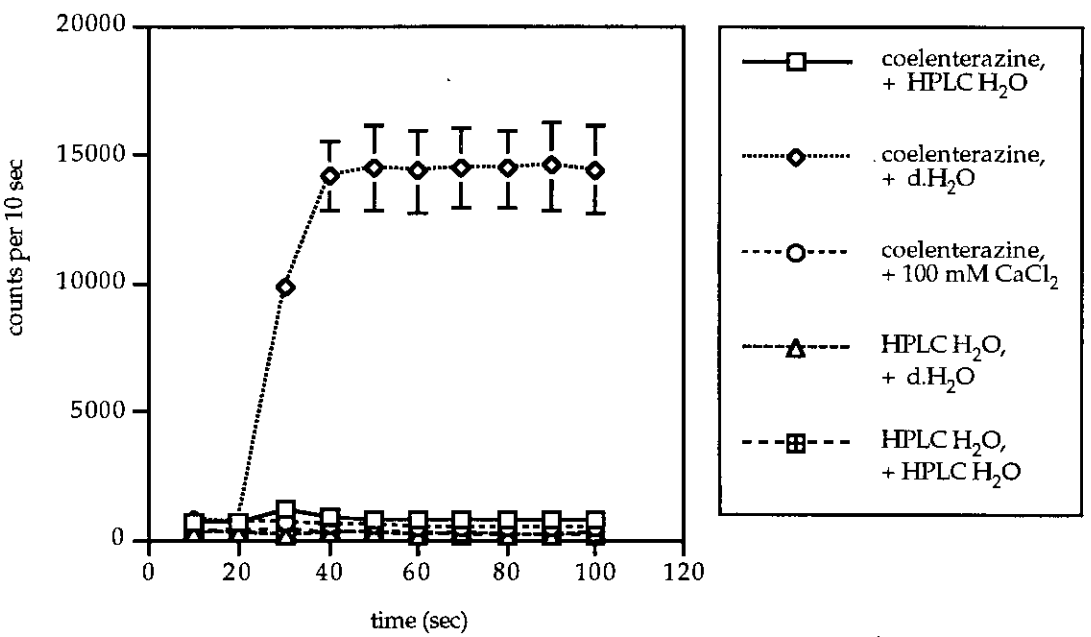


Fig 5.2 shows that there was no appreciable "coelenterazine effect". On the addition of d.H_2O to the coelenterazine solution, a continuous, constant light signal was detected, and a similar, but a much lower signal was obtained on adding HPLC H_2O. The light signal obtained from mixing coelenterazine and d.H_2O suggests that the d.H_2O contains some component which causes coelenterazine to luminesce. In further tests, HPLC H_2O was used throughout.

5.3.2 Possible use of synthetic coelenterazine

The use of synthetic coelenterazines with apoaequorin, yielding semi-synthetic aequorins (see table 1.3), can extend the range of Ca^{2+} measurement. In addition, *e*-aequorin will permit the quantitation of Ca^{2+} concentration without knowledge of how much aequorin is present. To determine if various semi-synthetic aequorins could be of use for Ca^{2+} measurement in *N. crassa*, an *in vitro* time course of aequorin formation was performed to assess regeneration time and stability.

In vitro constitution of 200 µg of protein from the homokaryon 1,2,1 was performed in the usual way, with the exception that 2.5 µM *e*-coelenterazine, *f*-coelenterazine, *h*-coelenterazine or *hcp*-coelenterazine was used. Normal coelenterazine (2.5 µM), and no coelenterazine (MeOH only) were used as controls. Light emission was measured from a 20 µg protien aliquot of the constitution mix diluted in TE (1:50).

Figure 5.3: Time course of semi-synthetic aequorin formation *in vitro*. Each point represents the mean of three replicas. Standard errors were calculated but are too small to be seen for some time points.

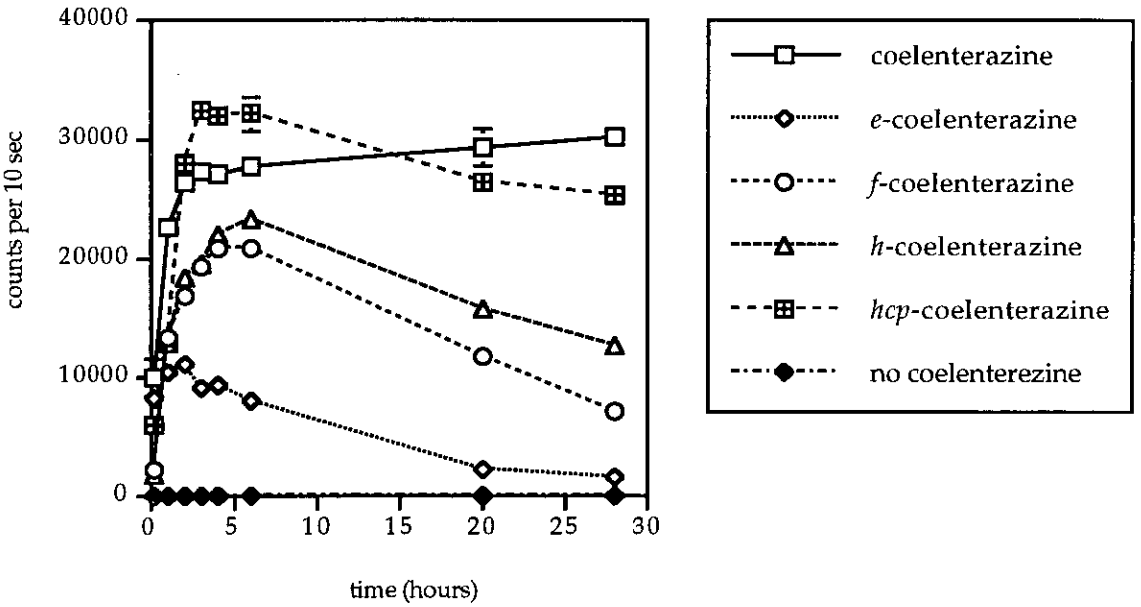


Fig 5.3 shows that functional *N. crassa* semi-synthetic aequorin is formed with all the different coelenterazine analogues. The semi-synthetic aequorins differ in stability; the slightly Ca^{2+} sensitive forms (*f* and *h*) and the highly Ca^{2+} sensitive form (*hcp*) shows maximum constitution at 6 hours after this, the rate of breakdown exceeds that of formation. Normal coelenterazine makes the only stable aequorin. *e*-Aequorin shows faster regeneration than the other aequorins, but it possesses a low relative luminescence capacity (on the basis of equal amounts of apoprotein) and is the most unstable. This may preclude the use of *e*-coelenterazine from experiments of long duration.

An *in vivo* time course in *N. crassa*, is needed to test the usefulness of these semi-synthetic aequorins. In particular, it would be of interest to test the *in vivo* stability of the highly Ca^{2+}-sensitive *hcp*-aequorin; this shows a

good luminescence capacity and high relitive light intensity when compared with normal aequorin (Shimomura *et al.*, 1993).

Note: Due to an error at the manufacturers, the data on *hcp*-coelenterazine should be treated with some caution as it may now relate to *cp*-coelenterazine.

5.3.3 Development of an agent for lysing *N. crassa* cells

The measurement of $[Ca^{2+}]_c$ depends on a calibration curve relating Ca^{2+} concentration to the proportion of the aequorin which is reacting with it (Cobbald and Rink, 1987). This proportion depends only on Ca^{2+} concentration and not on the absolute quantity of aequorin present. Thus, to estimate $[Ca^{2+}]_c$, the light emission from a change in $[Ca^{2+}]_c$ needs to be expressed as a proportion of the maximum light emission when all the aequorin present is saturated with Ca^{2+}. To determine this maximum it is necessary to obtain total release of aequorin from the cells, and so a procedure for efficient lysis of cells that will not interfere with aequorin light emission is required.

To be able to convert aequorin luminescence from *N. crassa* cells into actual changes in $[Ca^{2+}]_c$, it was necessary to find a reagent which would permeabilise the plasma membrane, allowing Ca^{2+} to flood in. It was important that this agent of lysis did not affect aequorin light output.

A range of detergents (NP40 and CHAPS) and solvents (acetone, DMSO and ethanol) mixed with 100 mM - 500 mM $CaCl_2$, and HPLC H_2O were tested on germinating conidia and spheroplasts. The ability to damage the membrane of the cells was assessed by simultaneous alive/dead staining, the effect of lysis agents on aequorin luminescence was determined, and agents which lysed the membrane and did not substantially quench

aequorin luminescence, were tested on cells which contained active aequorin.

5.3.3.1 The effect of putative agents of lysis on cell viability

Aliquots of cells (either germinated conidia or spheroplasts) were mixed in five times that volume of the lysis agent/$CaCl_2$ mix. Cell viability was assessed by FDA/PI staining (section 2.20), immediately after addition and again after a 5 min incubation.

Figure 5.4: Survival of germinated conidia after treatment with the lysis agent / $CaCl_2$ mix. Each column represents the mean of three viability counts of 100 conidia. Standard errors were calculated but are only visible in this represention for certain treatments. X-axis: A = water control; B = 1 M $CaCl_2$ control; C = 10% acetone + 100 mM $CaCl_2$; D = 20% acetone + 200 mM $CaCl_2$; E = 50% acetone + 500 mM $CaCl_2$; F = 10% DMSO + 100 mM $CaCl_2$; G = 50% DMSO + 500 mM $CaCl_2$; H = 1% NP40 + 100 mM $CaCl_2$; I = 2.5% NP40 + 250 mM $CaCl_2$; J = 5% NP40 + 500 mM $CaCl_2$.

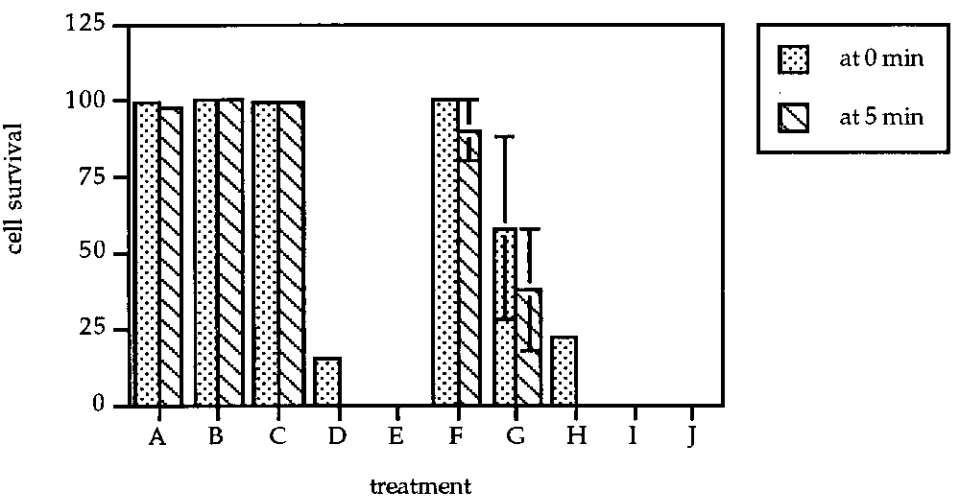


Fig. 5.4 shows that suitable lysis agents for *N. crassa* germinated conidia were 20% and 50% acetone, and 1%-5% NP40. Treating cells with 1 M

$CaCl_2$ has no effect on cell viability, indicating that it is the solvent or the detergent itself which is damaging the cells.

Figure 5.5: Survival of spheroplasts after treatment with the lysis agent / $CaCl_2$ mix, or HPLC H_2O. Each column represents the mean of three viability counts of 100 spheroplasts. Standard errors were calculated but are only visible in this represention for certain treatments. X-axis: A = 1 M sorbitol control; B= 1 M $CaCl_2$ control; C = HPLC H_2O; D = 10% EtOH + 100 mM $CaCl_2$; E = 1% NP40 + 100 mM $CaCl_2$; F = 5% CHAPS + 100 mM $CaCl_2$; G = 10% acetone + 100 mM $CaCl_2$; H = 50% acetone + 500 mM $CaCl_2$; I = 10% DMSO + 100 mM $CaCl_2$; J = 50% DMSO + 500 mM $CaCl_2$.

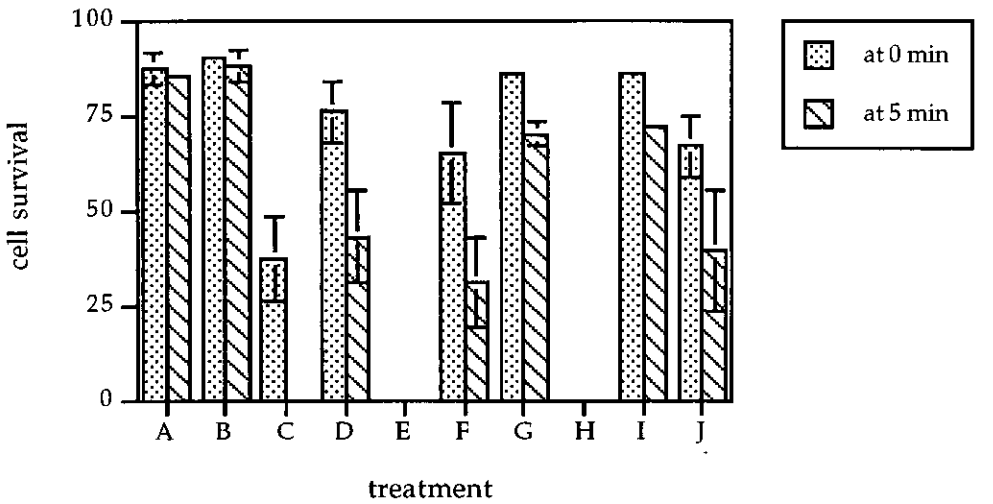


Fig. 5.5 shows that the agents suitable for lysis of *N. crassa* spheroplasts are HPLC H_2O, 1% NP40 and 50% acetone. 1 M $CaCl_2$ is hypertonic to the osmotic protectant used for the spheroplasts (1 M sorbitol), and does not exert a toxic effect.

5.3.3.2 The effect of lysis agents on aequorin luminescence *in vitro*

To determine the effect of successful lysis agents on aequorin luminescence, 200 µg protein aliquots from the transformant 1,2,1, and the untransformed 74*A* were used for *in vitro* constitution. Light emission

from a 20 µg protein aliquot of the constitution mix in TE was measured on addition of the lysis agent/$CaCl_2$ mix.

Figure 5.6: Light emission from reconstituted 20 µg protein extracts in TE (final volume 100 µl), on the addition of 500 µl of the lysis agent / $CaCl_2$ mix. Each column represents the mean of three individual *in vitro* constitutions of aequorin. Standard errors were calculated but are only visible in this represention for certain treatments. Any values of under 100 counts per 10 sec are indistinguishable from the background and were treated as zero. X-axis: A = 100 mM $CaCl_2$ control; B = 1M $CaCl_2$ control; C = 1% NP40 + 100 mM $CaCl_2$; D = 2.5% NP40 + 250 mM $CaCl_2$; E = 20% acetone + 200 mM $CaCl_2$; F = 50% acetone + 500 mM $CaCl_2$; G = extract without coelenterazine mixed with 100 mM $CaCl_2$.

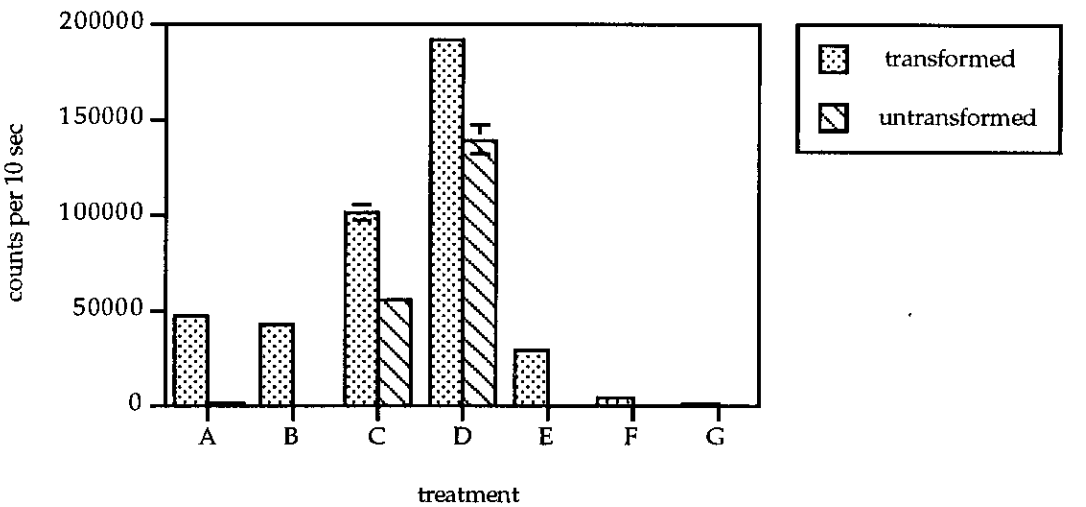


Fig. 5.6 shows that NP40 is not suitable for use as a lysis agent for determining total aequorin in cells, since its interaction with coelenterazine results in a strong light signal. The best lysis agent for germinated conidia is 20% acetone and will be tested *in vivo*. Unfortunately in the *in vitro* assay 20% acetone quenches aequorin luminescence by approximately 37% when compared with light emission obtained for 100 mM $CaCl_2$. For spheroplasts HPLC H_2O and 20% acetone were tested *in vivo*.

5.3.3.3 Aequorin luminescence during cell lysis

To determine how well the selected lysis agents released total aequorin from cells, both germinated conidia (in VS growth medium) and spheroplasts (in 1 M sorbitol) were incubated for 1 hour in 50 μM coelenterazine, to reconstitute aequorin *in vivo*. Five time this volume of lysis agent/$CaCl_2$ mix was added to the cells, and light emission measured simultaneously.

Figure 5.7: Light emission from 5 x 10^6 germinated conidia, preincubated for 1 hour in 50 μM coelenterazine, on the addition of 20% acetone/200 mM $CaCl_2$. Two background counts were recorded prior to the addition of the lysis mix at time=0. Each point on the graph represents the mean cumulative counts over the previous 10 sec for three replicas.

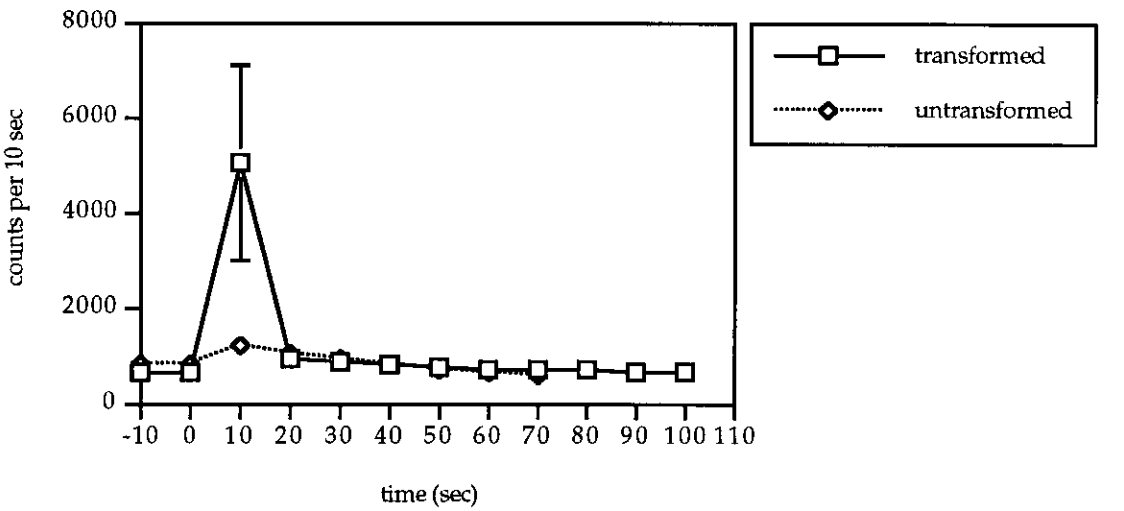


Fig 5.7 shows that a mix of 20% acetone/100 mM $CaCl_2$ rapidly discharged aequorin from germinated conidia. The peak light emission shows a lot of variation between samples; this may result from variable coelenterazine loading, or variable mixing on addition of the lysis agent. The small increase in light emission in the untransformed sample may be

due to the mixing of coelenterazine with HPLC H_2O present in the lysis agent mix (section 5.3.1).

Figure 5.8: Light emission from 5 x 10^6 spheroplasts in 1 M sorbitol, preincubated for 1 hour in 50 µM coelenterazine, on the addition of 500 µl HPLC H_2O. Two background counts were recorded prior to the addition of the HPLC H_2O at time=0. Each point on the graph represents the mean cumulative counts over the previous 10 sec for three replicas.

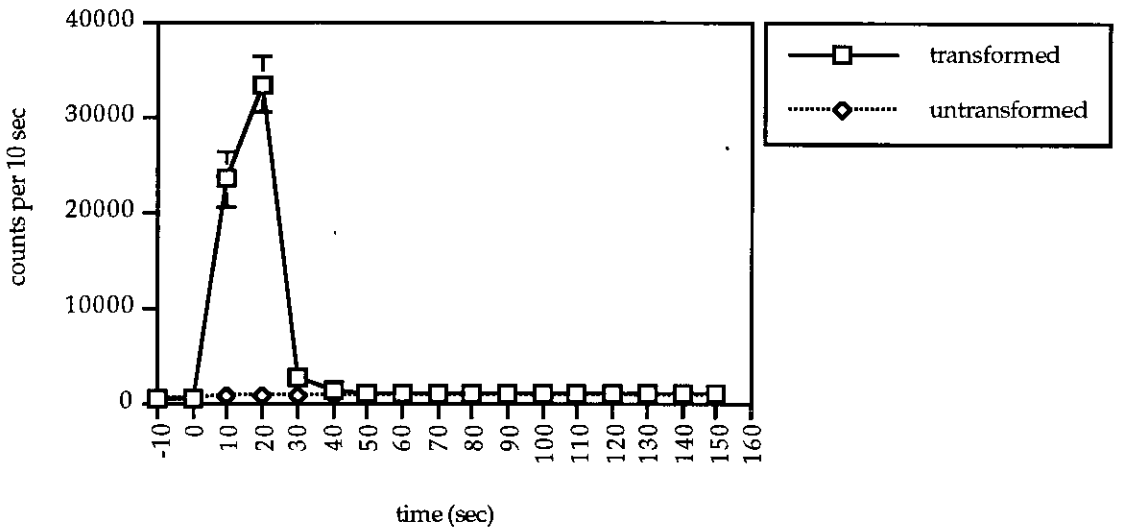


Fig. 5.8 shows that spheroplasts are rapidly lysed by HPLC H_2O and that there is sufficient Ca^{2+} present, presumably from its release from intracellular stores and trace amounts in the water, to discharge all the aequorin within the cells. No further light was detected from samples on the addition of 500 µl of 100 mM $CaCl_2$, indicating that all the aequorin had been discharged. An additional control, where coelenterazine was added to cells immediately prior to lysis showed that aequorin luminescence was not the result of rapid formation *in vitro*, after the plasma membrane had been breached. When the osmotic protectant was later changed from 1 M sorbitol to 1 M sorbitol, 10 mM Tris.Cl, pH 7.6, lysis of spheroplasts with HPLC H_2O showed that this change did not effect aequorin discharge.

Figure 5.9: Light emission from $5x10^6$ spheroplasts in 1 M sorbitol, preincubated for 1 hour in 50 µM coelenterazine, on the addition of 500 µl of 20% acetone/200 mM $CaCl_2$ mix. Two background counts were recorded prior to the addition of the lysis mix at time=0. Each point on the graph represents the mean cumulative counts over the previous 10 sec for three replicas, the standard errors were very small.

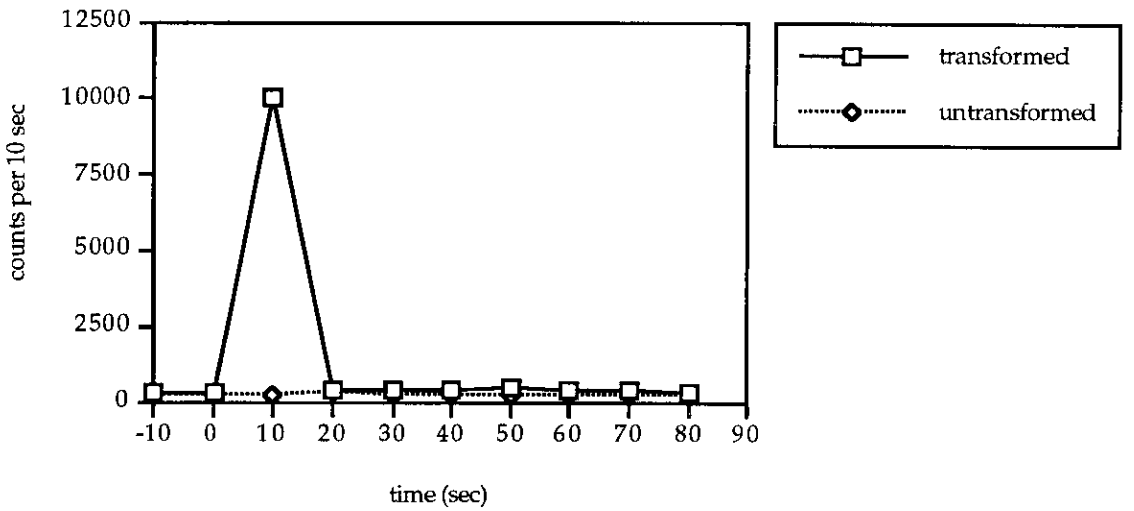


Fig. 5.9 shows that 20% acetone/100 mM $CaCl_2$ discharges aequorin present in the spheroplasts. However, comparison with the light yield from spheroplasts lysed with HPLC H_2O (Fig 5.8), acetone quenches aequorin luminescence by 82%, much greater than the 37% quenching obtained *in vitro* (Fig.5.6).

In summary, two lysis agents have been tested for their release of aequorin from cells, to permit the calibration of an unknown light signal in terms of Ca^{2+} concentration. HPLC H_2O is the preferred method for lysis of spheroplasts containing aequorin. For germinating conidia 20% acetone + 200 mM $CaCl_2$ is effective but allowance must be made to account for acetone quenching of luminescence.

5.4 Tests of the *in vivo* assay

5.4.1 Effect of ionophore and of added Ca^{2+} on *N. crassa* containing transgenic aequorin

Ionophores, such as 4-bromo-A23187 (Br-A23187) are used to facilitate the rapid equlibration of Ca^{2+} across cell membranes. By forming a liposoluble complex with Ca^{2+} (and other divalent cations to a lesser extent) the permeability of the membrane for Ca^{2+} is increased. In *N. crassa* application of A23187 was shown to dissipate the tip high gradient in membrane-bound Ca^{2+} and resulted in the emergence of multiple branches from the hyphal apex (Schmid and Harold, 1988). In *S. cerevisiae* containing transgenic aequorin, application of 10 µM A23187 and 10 mM $CaCl_2$ resulted immediately in a detectable light signal (Nakajima-Shimada *et al.*, 1991b).

Br-A23187 was applied to *N. crassa* spheroplast populations containing aequorin to determine if an increase in $[Ca^{2+}]_c$ could be detected. Spheroplasts ($5x10^6$) for all treatments were incubated in 100 µl of 1 M sorbitol supplemented with 50 µM coelenterazine, for 1 hour at 25 °C. 200 µl of 10 µM Br-A23187 was added, and light emission was measured simultaneously and for a further 80 sec, at which point 500 µl of 416 mM $CaCl_2$ (slightly hypertonic to the osmotic protectant) was added, and light measurement continued for a further 80 sec. After treatment a 200 µl aliquot of the treated cells was placed in a clean tube and light emission measured on lysis by 1 ml of HPLC H_2O. This signal was adjusted to represent the entire spheroplast population which had been treated with Br-A23187 and $CaCl_2$.

Figure 5.10: The effect of exogenous Br-A23187 and $CaCl_2$ on *N. crassa* spheroplasts containing aequorin. Two background counts were recorded followed by Br-A23187 at time = 0, and 416 mM $CaCl_2$ at 80 sec. Each point on the graph represents the cumulative counts for the previous 10 sec. Each line represents the mean of three separate cell populations, with the exception of untransformed, + ionophore which was obtained from a single cell population.

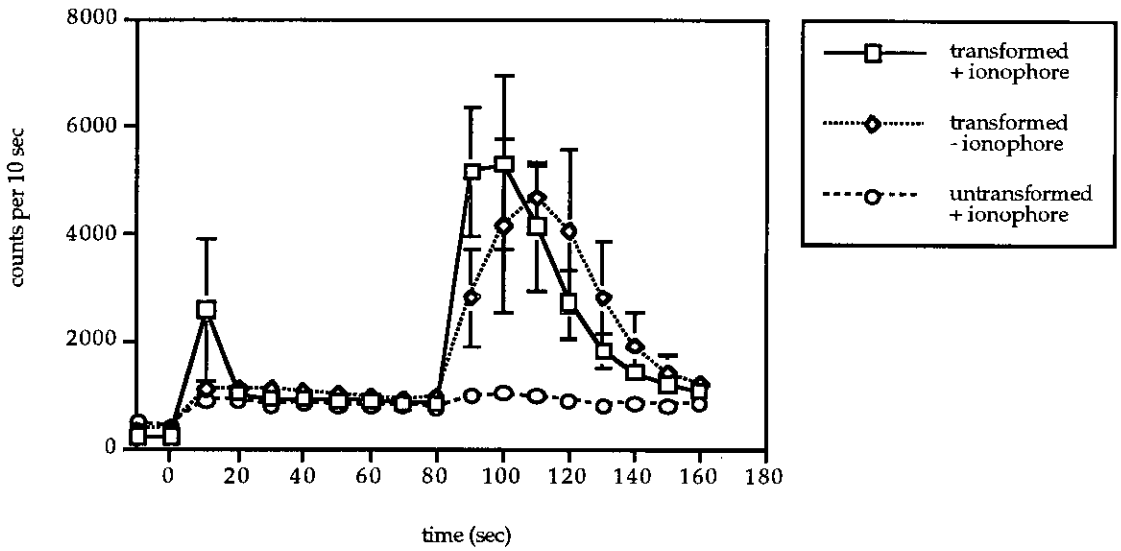
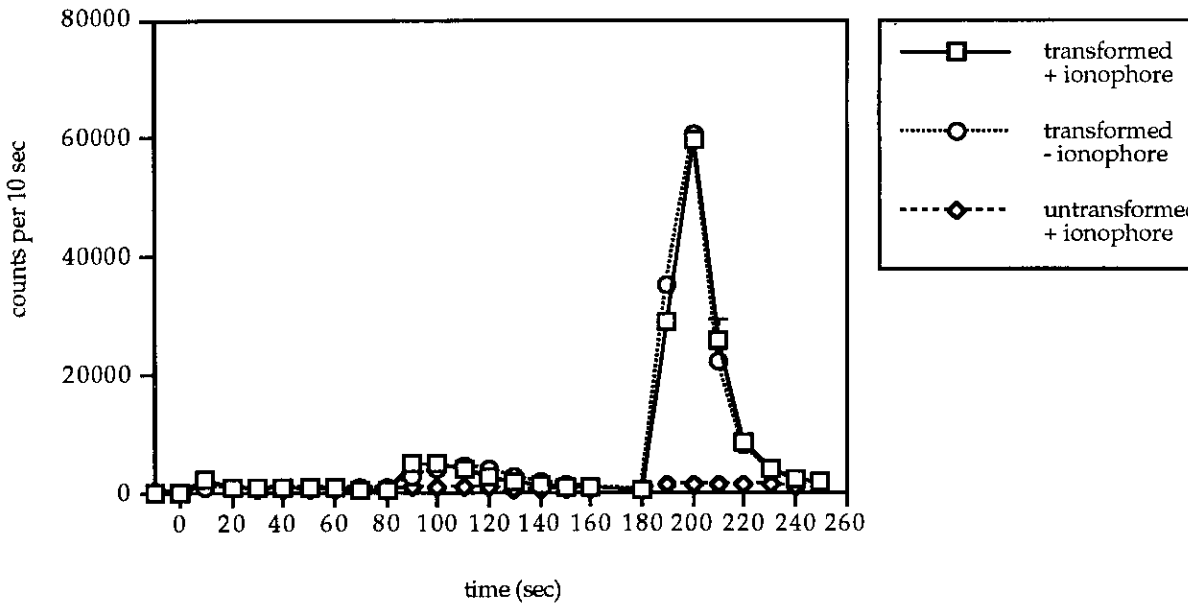


Fig. 5.10 shows that application of the ionophore alone gave a possibly real increase in aequorin luminescence that did not, however, attain statistical significance. Subsequent addition of Ca^{2+} resulted in aequorin discharge to a similar extent whether or not ionophore had been added. The untransformed control gave no light signal, indicating that light emission is due to aequorin and not to background luminescence from other sources.

This experiment was carried out using unbuffered 1 M sorbitol which has a pH of approximately 4.7. As complex formation between Br-A23187 and divalent cations may be reduced below pH 5.0, the experiment was repeated using an osmotic protectant of 1 M sorbitol, 10 mM Tris, pH 7.6.

This experiment did not show a clear ionophore effect, but again the addition of $CaCl_2$ caused a burst of aequorin luminescence.

Figure 5.11: Continuation of the experiment shown in Fig 5.10. After 160 sec, measurement ceased and a 200 µl aliquot of the treated cells was transferred to a clean tube. A single background count was recorded prior to the addition of 1 ml HPLC H_2O at 180 sec. Lysis data are adjusted to represent the entire cell population. Each line represents the mean of three separate cell populations, with the exception of untransformed, + ionophore which was obtained from a single cell population.



By calculating the rate of consumption of aequorin on the addition of Ca^{2+} from the data presented above, $[Ca^{2+}]_c$ was shown to increase to a peak of 4 µM for both the plus and minus ionophore treatment (using the calibration curve from Cobbold *et al.*, 1983).

5.4.2 The effect of exogenous $CaCl_2$ on *N. crassa* containing transgenic aequorin.

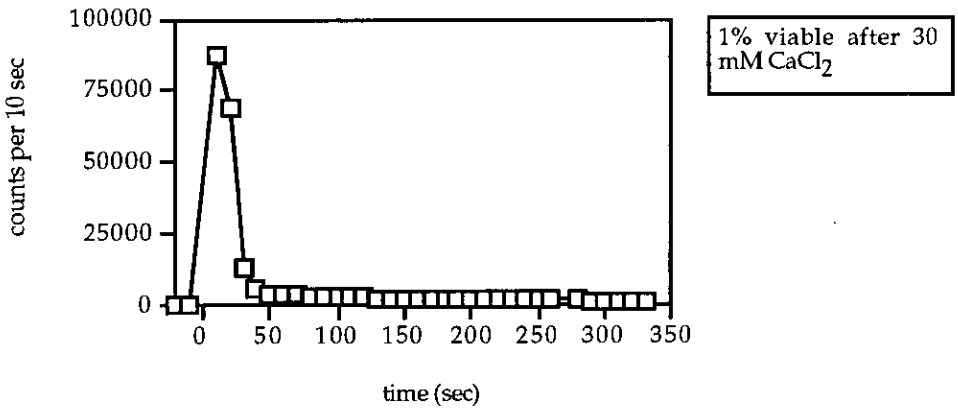
It is possible that the apparent increase in $[Ca^{2+}]_c$, on the addition of exogenous $CaCl_2$ shown in Fig. 5.10 was actually the result of cell damage, resulting in the release of aequorin into the medium where it would be discharged. To test this possibility alive/dead staining of cells exposed to $CaCl_2$ was included in the protocol. In addition, three concentrations of $CaCl_2$ were tested, 30 mM which was hypotonic and caused cell lysis, 333 mM which is isotonic to the osmotic protectant, and 700 mM which is hypertonic.

Spheroplasts ($5x10^6$)(1,13,5 and untransformed *BdA*) were incubated in 100 µl of 1 M sorbitol, 10 mM Tris. Cl, pH 7.6 supplemented with 50 µM coelenterazine. The $CaCl_2$ solution was added to cells while light emission was measured, measurement continued for a further 260 sec. At this point 200 µl of the treated cells were removed to a clean tube, a 10 second background count was recorded and the cells were then lysed with 1 ml of HPLC H_2O. The light emission from the untransformed spheroplasts was subtracted from the light emission from 1,13,5; thus the data shows aequorin luminescence only. Viability was assessed approximately 5 min after the $CaCl_2$ treatment.

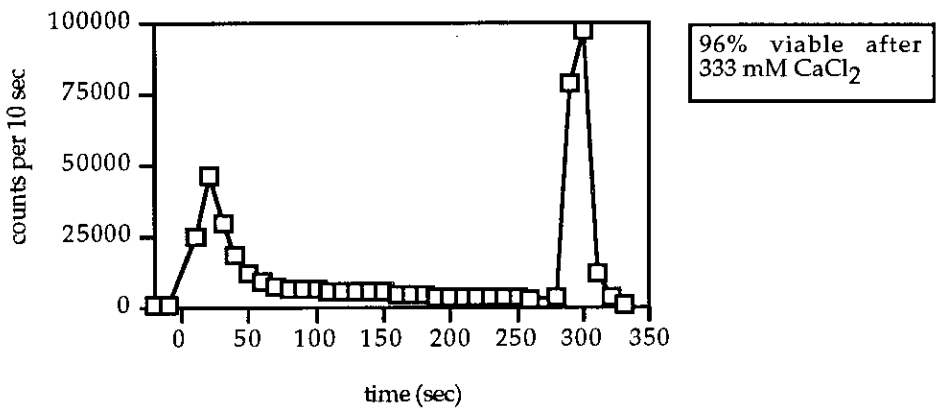
The data presented in Fig. 5.12 shows that the effect of exogenous $CaCl_2$ on spheroplast $[Ca^{2+}]_c$, depended on the concentration applied. The 30 m M $CaCl_2$ treatment caused immediate lysis and release of all aequorin. Using the Ca^{2+}-calibration curve of Cobbold *et al.* (1983) in both the 333 mM $CaCl_2$ and 700 mM $CaCl_2$ treatments, $[Ca^{2+}]_c$ increased to a peak of approximately 5 µM. Spheroplast viability assessment shows that the light signal obtained is not the result of cell damage.

Figure 5.12: The effect of exogenous $CaCl_2$ on *N. crassa* 1,13,5 spheroplasts containing aequorin. The data presents aequorin luminescence only i.e *BdA* luminescence subtracted from 1,13,5.

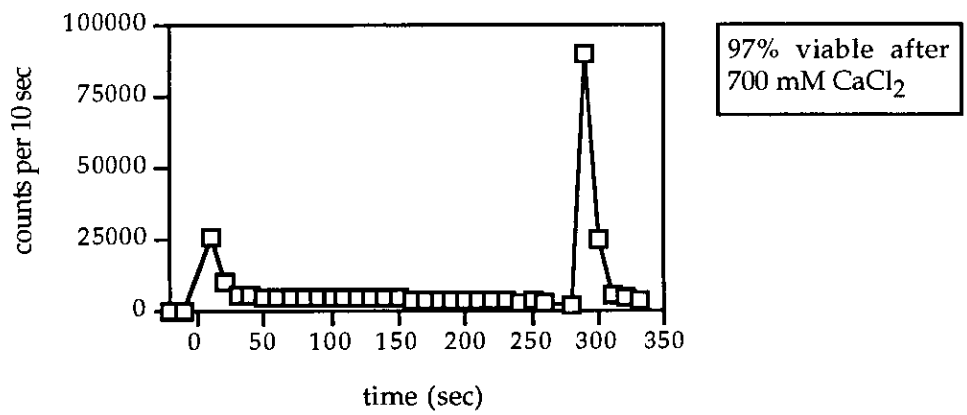
A: 30 mM $CaCl_2$ added at time zero



B: 333 mM $CaCl_2$ added at time zero



C: 700 mM $CaCl_2$ added at time zero



5.4.3 Application of a cold stimulus to *N. crassa* containing transgenic aequorin

In *N. plumbaginifolia* containing aequorin, it has been shown that cooling causes a rapid elevation in $[Ca^{2+}]_c$ (Knight *et al.*, 1991b). To determine if the effects of temperature were mediated through $[Ca^{2+}]_c$ in *N. crassa*, spheroplasts were exposed to a rapid drop in temperature from 25 °C to approximately 3 °C, and the effect on $[Ca^{2+}]_c$ detected by simultaneously measuring light emission from the sample.

Spheroplasts were incubated in 100 µl of 1 M sorbitol, 10 mM Tris.Cl pH 7.6, supplemented with 50 µM coelenterazine for 1 hour at 25°C. The cooling stimulus, 1 ml of ice cooled 1 M sorbitol, 10 mM Tris.Cl pH 7.6, was added to the spheroplasts while simultaneously measuring light emitted from the preparation. Viability of the cells was assessed before and after cooling. After cooling a 200 µl aliquot of the spheroplasts was lysed with 1 ml HPLC H_2O to determine the total amount of aequorin present.

Fig. 5.13 shows an apparent increase in $[Ca^{2+}]_c$ in response to a cooling stimulus. After the cold shock spheroplasts were 68% viable, untreated cells showed 83% viability. The t-test of paired samples on the data in Fig 5.13 gave a probability of the result occurring by chance < 0.1 but > 0.05. Data obtained from the application of room temperature 1 M sorbitol (data not shown) did not differ significantly from background luminescence. It is possible that aequorin discharge resulted from cell damage which was observed in the loss in cell viability following cooling. The experiment would need to be repeated to confirm the significance of the result.

Figure 5.13: The effect of applying a cold shock to *N. crassa* (strain 1,13,5) containing aequorin. Light emission was recorded prior to (20 sec) and during cooling (applied at time zero), and for 120 sec after treatment. Transformed and untransformed samples were paired; the line graph represents the mean of three replica pairs. In each pair the untransformed was subtracted from the transformed light counts, so as to correct for background light emission.

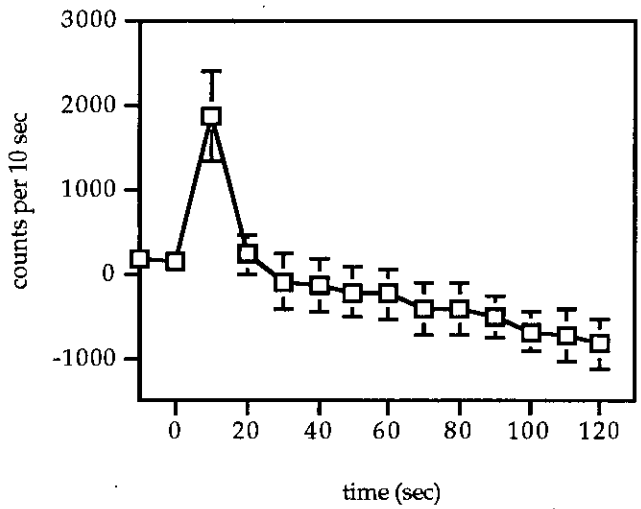
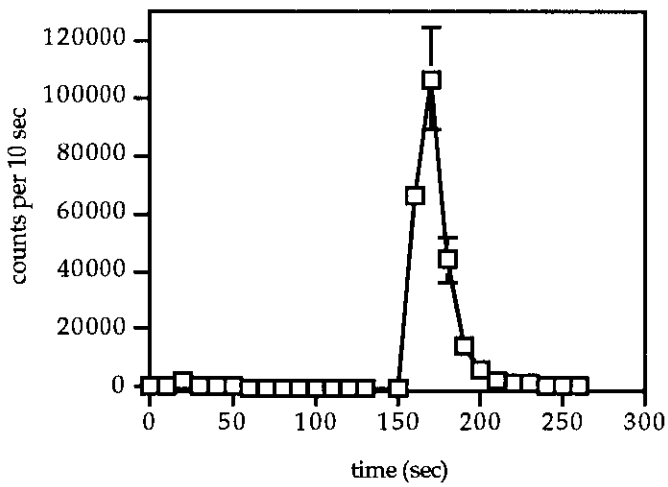


Figure 5.14: Continuation of the experiment shown in Fig 5.13. After 160 sec, measurement ceased and a 200 µl aliquot of the treated cells was transferred to a clean tube. A single background count was recorded prior to the addition of 1 ml HPLC H_2O at 150 sec. Lysis data are adjusted to represent the entire cell population.



5.5 Discussion

In this chapter methods have been developed for monitoring changes in $[Ca^{2+}]_c$ in apoaequorin-expressing strains of *N. crassa*. Aequorin was regenerated *in vivo*, simply by incubating the cells with coelenterazine, as observed in other systems (Nakajima-Shimada *et al.*, 1991b ; Knight *et al.*, 1991a and b). The method was both non-invasive and non-toxic and as such is ideal for monitoring $[Ca^{2+}]_c$ from cell populations.

In *N. crassa*, the possibility of detecting changes in $[Ca^{2+}]_c$ is limited at present by the low level of expression of apoaequorin (see chapter four), and background light emission from other components in the assay. The use of different coelenterazine analogues to form *N. crassa* semi-synthetic aequorin may improve the sensitivity of $[Ca^{2+}]_c$ monitoring at low Ca^{2+} concentrations; *hcp*-aequorin may be particularly useful as it shows a relative intensity of light emission 500 times that of normal coelenterazine at 10 μM Ca^{2+} (Shimomura *et al.*, 1990).

Lysis of the cells made it possible to determine the total light yield of the aequorin present, and this was then used for estimating $[Ca^{2+}]_c$ from light signals from intact cell. This calibration is central to using aequorin as a $[Ca^{2+}]_c$ indicator. In *N. plumbaginifolia* transformed with aequorin, NP40 and EtOH have been used to lyse the cells, but the latter is known to quench aequorin luminescence (N. Wood, pers. comm.). It has been shown here that NP40 is unsuitable, unless all unbound coelenterazine can be removed from the preparation. The excessive perturbation necessary to achieve this may lead to the discharge of aequorin within the cells. It was concluded from tests recorded here that 20% acetone was a possible agent for lysis of germinating conidia, and that spheroplasts were efficiently lysed simply by the addition of water.

An interesting observation from testing different lysis agents in the two *in vivo* systems, was the 47% deficit in aequorin luminescence from germinated conidia, as compared with spheroplasts under the same conditions. This suggests that the cell wall may be impeding coelenterazine entry. If so, higher coelenterazine concentrations or longer incubation periods should reduce this effect.

The estimates of $[Ca^{2+}]_c$ calculated from the fraction of aequorin discharged will be approximate, as it is unlikely that the calibration curve used was constructed under conditions identical to those present in the intracellular milieu. The Ca^{2+} sensitivity of aequorin can change in response to temperature, ionic strength and Mg^{2+} (Cobbold and Rink, 1987). Ideally a Ca^{2+}-calibration curve should be constructed with aequorin isolated from transgenic *N. crassa*, as has been done for recombinant aequorin from *E. coli*, where the level of expression is considerably higher (Watkins *et al.*, 1995).

Addition of the ionophore Br-A23187 did not yield a significant aequorin signal alone, but subsequent addition of Ca^{2+} did lead to a clear increase in $[Ca^{2+}]_c$ both with and without ionophore. Recent work on *S. cerevisiae* and *C. albicans* (T. Perera, pers. comm.) showed a detectable aequorin signal on the addition of 10 μM A23187 and 10 mM $CaCl_2$. The low signal detected from *C. albicans* was at best 11 times less than that obtained from *S. cerevisiae*. In chapter 4 it was shown that transformed *C. albicans* contained 28 times less apoaequorin than *S. cerevisiae* but approximately seven times more than *N. crassa*. It is possible that light emission from *N. crassa* on the application of ionophore was close to the limits of the detection system.

The addition of $CaCl_2$ to spheroplasts did lead to a clear increase in $[Ca^{2+}]_c$, as measured by light emission from aequorin-containing cells, in

spite of the very low levels of aequorin present. Even a fairly modest increase (i.e. 10 fold) in aequorin content in the cell could permit a number of useful experiments to be done. It is of interest to understand the affect of exogenous Ca^{2+} in *N. crassa*. Future experiments could include the application of exogenous $CaCl_2$ in an osmotically stable environment, the replacement of Ca^{2+} with another divalent cation to test Ca^{2+} specificity and the use of Ca^{2+}-channel blockers to determine the type and location of the Ca^{2+}-channels involved. Experiments could be conducted in both spheroplasts and germinated conidia.

Chapter Six

6. Future Work

To measure changes in $[Ca^{2+}]_c$ using aequorin, it is clearly desirable to improve the level of apoaequorin produced by *N. crassa*. As it has been shown that the block in apoaequorin production occurs at translation or beyond; there are two techniques available which may remove or reduce the limiting factors. A synthetic apoaequorin gene in which the codon usage reflects that observed in highly-expressed genes of the host organism could be introduced. Research in this area has produced mixed results in which changes in the codon usage of a gene have resulted in an improvement (Chiu *et al.*, 1996), no change (Demolder *et al.*, 1992) and a drastic reduction (Lammertyn *et al.*, 1996) in protein yield. An alternative approach which has yielded more consistent improvements in yield, is the inclusion of native protein sequences to make a hybrid protein. This may act to improve both mRNA stability (Jeenes *et al.*, 1994; Nyyssönen and Keranen, 1995) and protein stability (Badminton *et al.*, 1995). Fusion of a protein to the N-terminus of apoaequorin is known in at least some cases not to affect aequorin function (Casadei *et al.*, 1990; Badminton *et al.*, 1995). This approach could be combined with the use of the synthetic gene if appropriate.

To maximize light emission from an apoaequorin-transformed organism, selection of a coelenterazine analog which possesses a high relative intensity over that of normal coelenterazine may be useful. Additionally, the level of aequorin present may be increased by growing the organism in the presence of coelenterazine as this is expected to reduce the degradation of apoaequorin by its conversion to a more stable form.

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