Cd-regulated gene expression in Datura innoxia

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ABSTRACT

The effects of Cd on the expression of specific proteins and transcripts have been examined in Cd-resistant (Cd300) and Cd-sensitive cultures of *Datura innoxia*.

2D PAGE analysis of the soluble fraction of *D. innoxia* protein proved problematic. Proteins precipitated on the surface of IEF gels. This was overcome by loading the samples in the gel mixture prior to polymerisation. Polymerisation of gels containing protein extracted from Cd-exposed cells only occurred when PCs, Cd and other components were resolved from the proteins eluting in the void volume following fractionation by gel filtration chromatography (Sephadex G50). Two peptides, designated Cd-1 and Cd-2, were detected in the Cd300 cells only after exposure to Cd. Cd-1 and Cd-2 were also both detected following exposure of the Cd300 cells to 125 μM Cu or Zn, or HS (42 ^{o}C 4 h). Neither Cd-1 nor Cd-2 were observed in protein extracts from WDI cells exposed to 125 μM Cd for 8 h.

Both Cd-1 and Cd-2 proved refractory to Edman degradation while the N-terminal 30 amino acids of a third, constitutively expressed protein, designated Protein-3, were determined using equivalent procedures. This protein showed sequence similarity to PR proteins. Although cleavage of Cd-1 and Cd-2 generated polypeptides which were not terminally blocked, no sequence information was obtained from these polypeptides, even following purification using standard techniques.

Oligonucleotide primers designed from the amino acid sequence of Protein-3 were successfully used to amplify, from cDNA, a fragment which was cloned, sequenced and shown to encode the characterised protein. A longer fragment was also amplified from cDNA by RACE PCR. However, this product was not cloned.

In order to identify cDNA sequences encoding Cd-1 and Cd-2 an expression cDNA library was prepared and antibodies raised against the two peptides. However, no antigenicity could be detected when antisera raised against Cd-1 or Cd-2, or the purified IgG fractions, were used to probe western blots. The λZAP cDNA (Cd-exposed) library was "differentially screened" in order to isolate clones corresponding to Cd-induced genes. This led to the isolation of two Cd-induced clones designated Cd-6 (949 bp) and Cd-8 (659 bp). Both clones hybridised to transcripts of approximately 900 bp. Transcripts were also detected in RNA samples extracted from *D. innoxia* exposed to HS (42 ^{o}C 4 h), however no transcripts were detected in WDI cells exposed to Cd. Southern blots revealed hybridisation to multiple bands, possibly indicating the presence of a gene family. A motif, C-C-X-C-C, found in the α-domain of metallothioneins, was identified in Cd-6. This may represent a putative metal binding site in Cd-6.

DECLARATION

No part of this thesis has previously been submitted for a degree at this or any other university. All the material presented here is my own original work, except where otherwise stated.

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ABBREVIATIONS

1D	one dimensional
2,4D	2,4-dichlorophenoxyacetic acid
2D	two dimensional
ABA	abscisic acid
ADH	alcohol dehydrogenase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
BSO	buthione sulphoxamine
Cys	cysteine
CaMV	cauliflower mosaic virus
CAPS	3-cyclohexoamino-1-propane-sulphonic acid
cDNA	complementary deoxyribonucleic acid
CNBr	cyanogen bromide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanidine triphosphate
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
Glu	glutamine
Gly	glycine

h	hour
His	histidine
HMW	high molecular weight
HPLC	high pressure liquid chromatography
HS	heat shock
HSP	heat shock protein
IEF	isoelectric focusing
IPA	isopropionic acid
Kbp	kilobase
LMW	low molecular weight
Met	methionine
min	minute
Mr	relative mass
mRNA	messenger RNA
MT	metallothionein
NADPH	reduced nicotinamide adenine diphosphate
NBRF	National Biomedic Research Foundation
NBT	nitroblue tetrazolium salt
NMR	nuclear magnetic resonance
OPC	oligonucleotide purification cartridge
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia lyase
PC	phytochelatin
PCR	polymerase chain reaction
PCV	packed cell volume
pI	isoelectric point
PMSF	phenylmethylsulphonyl fluoride
pol	polymerase
PR	pathogenesis related

PTH	phenylthiohydantoin
PVDF	polyvinylidinedifluoride
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
TEMED	N,N,N',N' tetraethylmethylethylenediamine
TFA	trifluoroacetic acid
tRNA	transfer ribonucleic acid
UV	ultra violet
v\v	volume for volume
w\v	weight for volume

CHAPTER 1

INTRODUCTION

The effects of Cd on gene expression were studied in cell suspension cultures of *Datura innoxia*. A long term objective was to isolate a metal regulatory element (MRE) responsive to Cd. MREs have been extensively studied in animal systems (Stuart *et al.*, 1984) but detailed characterisation of plant MREs has still to be performed.

The isolation and characterisation of plant MREs is a prerequisite for more detailed analysis of the signal transduction pathways which lead to metal-induced gene expression. Metal-gene-communication may provide a tractable model for the study of plant gene expression in response to an environmental stimulus. In addition a plant MRE would be a useful molecular tool, allowing the construction of vectors containing promoters which would be switched on following exposure to metal. Such vectors would be valuable in studies requiring controlled expression of particular genes in transgenic plants.

Both Cd-sensitive and Cd-tolerant cell lines of *D. innoxia* were used in this investigation. The analysis of Cd-induced genes in cells with differing metal tolerant phenotypes may lead to a better understanding of metal tolerance mechanisms in plants.

The following introduction describes the known biochemical bases of Cd-toxicity, with emphasis on the effects of Cd on Zn-requiring metalloproteins; the known mechanisms by which plants tolerate this metal, with emphasis on mechanisms involving intracellular sequestration by metal-binding polypeptides and proteins; and reviews the literature relating to environmentally regulated gene expression in plants, with particular emphasis on Cd-regulated gene expression.

The reviews of mechanisms of toxicity, tolerance and gene induction focus on responses to Cd. Information relating to other metals is also mentioned where equivalent data is not available for Cd. In particular the metals of the Cu and Zn triads (Group IB and IIB, Table 1) are discussed when it is considered that the observations may be of relevance to the response to Cd.

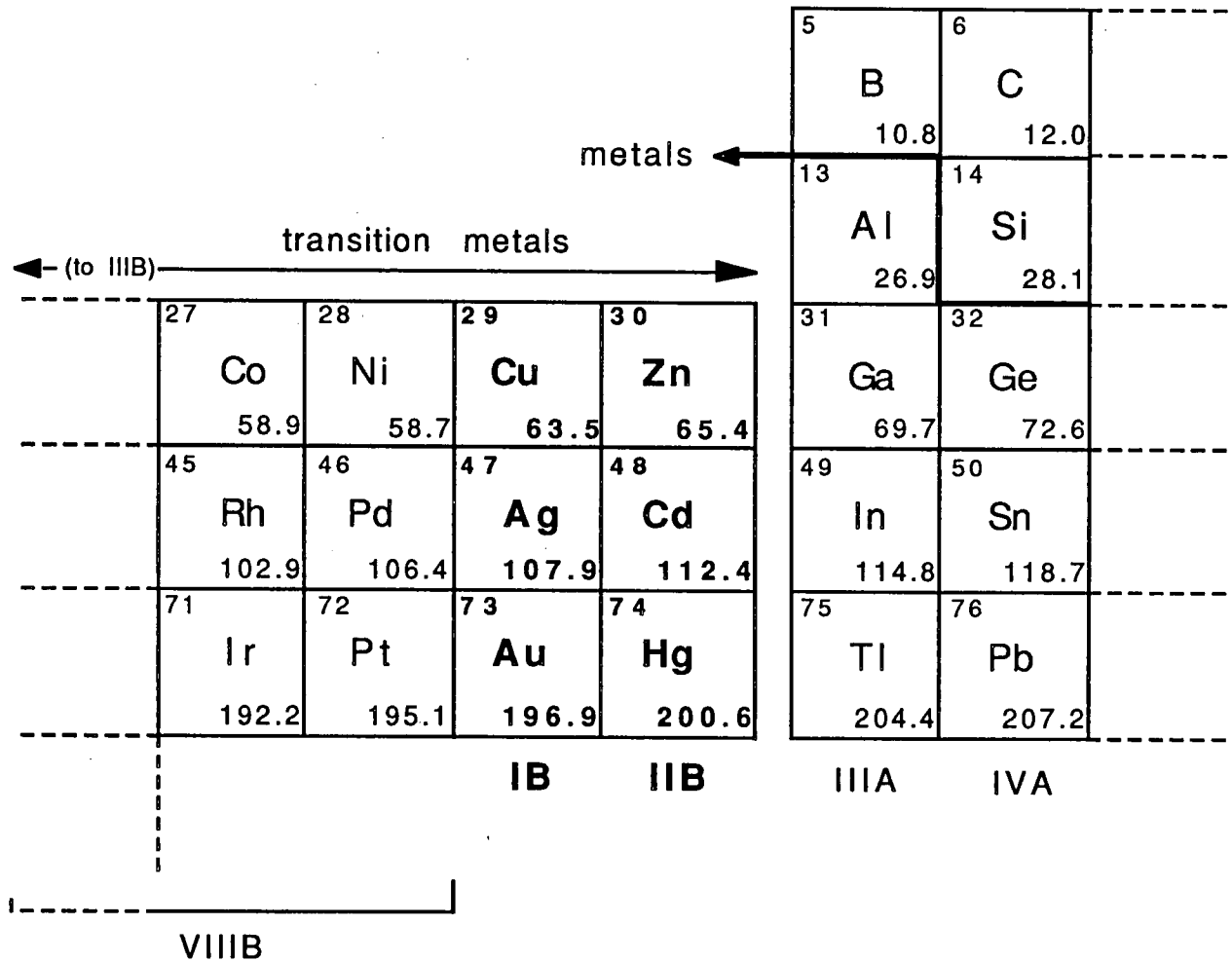
At the end of the introduction is a summary of previous observations on the responses of *D. innoxia* cell suspension cultures to Cd, a more extensive outline of the aims and a flow diagram of the work carried out.

1.1 Cd-TOXICITY

Most toxic effects of Cd are attributable to either the direct interaction of the metal with proteins and other macromolecules or its effect on intracellular redox potential. The propensity for Cd to substitute for Zn in metalloproteins is particularly significant.

TABLE 1

The Cu and Zn triads (groups IB and IIB, respectively) in the periodic table.


metals
transition metals
(to IIIB)
5
B
10.8
6
C
12.0
13
Al
26.9
14
Si
28.1
27
Co
58.9
28
Ni
58.7
29
Cu
63.5
30
Zn
65.4
31
Ga
69.7
32
Ge
72.6
45
Rh
102.9
46
Pd
106.4
47
Ag
107.9
48
Cd
112.4
49
In
114.8
50
Sn
118.7
71
Ir
192.2
72
Pt
195.1
73
Au
196.9
74
Hg
200.6
75
Tl
204.4
76
Pb
207.2
IB
IIB
IIIA
IVA
VIIIB

1.1.1 Direct interaction of metal with proteins

Essential metals are required in biological systems as both cofactors and components of metalloproteins including metalloenzymes. Examples of Zn-requiring metalloenzymes can be found in each of the six classes of enzymes (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases) (Jackson *et al.*, 1990). Excess metal ions (including non-essential metals) are known to directly stimulate or repress the activity of a number of enzymes, many of which would not "normally" associate with these ions. Some of the enzymes whose activities have been demonstrated to be affected in such a manner in plants are summarised in Table 2, along with the metal shown to confer the observed response. Two mechanisms by which enzyme activity is suppressed by Cd predominate; the substitution of Cd for other metal ions in enzymes and the binding of Cd to groups (such as sulphydryl groups) involved in the catalytic action or structural integrity of enzymes (but not mediated by association with metal ions).

1.1.1.1 The binding of Cd to sulphydryl groups involved in the catalytic action or structural integrity of enzymes

Interaction of Cd with SH-groups was proposed as the mechanism of inhibition of photochlorophyllide reductase in *Hordeum vulgare* as the binding of NADPH and protochlorophyllide to the enzyme is coordinated by SH-groups. N-methylmaleimide which inhibits binding at SH-groups elicited a similar response to Cd (Stobart *et al.*, 1985). The inhibition observed after *in vitro* inhibition of Rubisco with Cu and Zn was explained by metal interaction with functional SH-groups (Stiborova *et al.*, 1986). It has also been demonstrated that Cd binds *in vitro* to SH-groups involved in the stabilisation of the Rubisco structure causing dissociation of the enzyme into its subunits and loss of activity (Stiborova, 1988). Chlorophyll biosynthesis is inhibited by certain metal ions. δ-aminolaevulinic acid (ALA) dehydratase and protochlorophyllide reductase are two enzymes involved in this pathway which are known to be highly sensitive to metals. ALA dehydratase activity was significantly inhibited in *Pennisetum typhoideum* treated with Hg. This inhibition was attributed to the interaction of Hg with functional sulphydryl (SH-) groups of the enzyme (Prassad and Prassad, 1978).

1.1.1.2 The substitution of Cd for other metal ions in enzymes

Cd has been shown to inhibit the water splitting enzyme involved in photosystem-2 (PS-2) related electron transfer in the leaves of *Lycopersicon esculentum* (Basinsky *et al.*, 1980). Mn is essential for water splitting activity. Carbonic anhydrase (CA) is a Zn-metalloenzyme which catalyses the reversible hydration of CO_2. Its activity decreases significantly in *Glycine max* following exposure to Cd. It was suggested that the inhibitory effect of Cd resulted from it

TABLE 2

Some enzyme activities known to be sensitive to elevated metal concentrations in plants

ENZYME	SPECIES	EFFECTING METAL	REFERENCE
REPRESSED ACTIVITY			
δ-aminolaevulinic acid	*Pennisetum typhodium*	Hg, Pb	Prassad and Prassad, 1987
Protochlorophyllide reductase	*Hordeum vulgare*	Cd	Stobart *et al.*, 1985
Water splitting enzyme of PS2	*Lycopersicum esculentum*	Cd	Baszinsky *et al.*, 1980
	Phaseolus vulgaris	Zn	Van Assche and Clijsters, 1986
NADPH-oxidoreductase	*Euglena gracilis*	Cd, Hg, Zn	De Filippis *et al.*, 1981
RubisCo	*Hordeum vulgare*	Cd, Cu, Pb, Zn	Stiborava *et al.*, 1986
	Rhodospirillum rubrum	Co, Mn	Robinson *et al.*, 1979
	Hordeum vulgare	Cd	Stiborava, 1988
Cu,Zn-SOD	*Phaseolus vulgaris*	Cd	Cardinaels *et al.*, 1984
Carbonic anhydrase	*Glycine max*	Cd	Lee *et al.*, 1986
Succinic dehydrogenase	*Nicotiana tabacum*	Cd	Reese and Roberts, 1985
Transcription enzymes/factors	*Nicotiana tabaccum*	Cd (100mM)	Hirt *et al.*, 1989
STIMULATED ACTIVITY			
Peroxidase	*Glycine max*	Cd	Lee *et al.*, 1976
	Silene cucubalus	Cu	Mukerji and Das Gupta, 1972
	Lactuca sativa	Zn	Matthys, 1977
Isocitrate dehydrogenase	*Phaseolus vulgaris*	Cd, Zn	Van Assche *et al.*, 1988
Malate dehydrogenase	*Phaseolus vulgaris*	Cd	Van Assche *et al.*, 1988
Glutamate dehydrogenase	*Phaseolus vulgaris*	Cd, Zn	Van Assche *et al.*, 1988
Malic enzyme	*Phaseolus vulgaris*	Cd, Zn	Van Assche *et al.*, 1988
Mn-SOD, Fe-SOD	*Phaseolus vulgaris*	Cd	Cardinaels *et al.*, 1984
Transcription enzymes/factors	*Nicotiana tabaccum*	Cd	Hirt *et al.*, 1989

substituting for Zn in the enzyme (Lee *et al.*, 1976). The enzymic activity of Cu,Zn-superoxide dismutase (SOD) in *Phaseolus vulgaris* has been shown to be inhibited following exposure to Cd. It was proposed that the inhibition was due to competition by Cd for the Zn binding site of the enzyme. However, a significant increase of other SOD enzymes (Fe-SOD and Mg-SOD) was observed following Cd-exposure (Cardinaels *et al.*, 1984). The substitution of intrinsic Zn has been reported in a number of other proteins involved in gene expression. These are reviewed in some detail in sections 1.1.1.3.2 and 1.1.1.4.

1.1.1.3 The role of Zn and the effect of Cd substitution in DNA and RNA polymerase

1.1.1.3.1 DNA polymerase

In higher plants there are three classes of DNA polymerase (Tewari, 1986). DNA polymerase α (pol α) is involved in chromosomal replication (Bollum, 1960). DNA pol β has been associated with DNA repair (Weissbach *et al.*, 1975). The polymerase required for mitochondrial DNA replication, which is encoded in the nucleus, is DNA polymerase γ (Bolden *et al.*, 1977). DNA polymerase has also been isolated from chloroplasts and this appears to represent a fourth class of DNA polymerases in plants (Tewari, 1986).

The requirement for Zn in *E. coli* DNA pol I activity was first reported by Slater *et al.* (1971). It was observed that the chelating agent 1,10-phenanthroline inhibits *E. coli* DNA pol I and sea urchin nuclear polymerase. However, no inhibition of the polymerases was observed in the presence of 1,7-phenanthroline which cannot chelate metals. In these studies Mg^{2+} was present at a 10-fold excess over the chelating agents. These results, together with the measured Zn content of the homogeneous protein, purified in such a way as to retain Zn, suggested a role for Zn in DNA polymerase activity (Slater *et al.*, 1971). Later studies of *E. coli* DNA pol I showed it to contain lg atom of Zn per mole of enzyme. Polymerase activity of the apoenzyme was 7% that of Zn-bound polymerase activity. Incubation of the apoenzyme with Zn^{2+} completely restored activity. Co^{2+} and Mn^{2+} restored activity to approximately 80 % and 45 % respectively, however Mg^{2+}, Ni^{2+}, Hg^{2+}, Fe^{2+} and Cd^{2+} all failed to restore any activity. Dialysis with the chelator 1,10-phenanthroline showed that loss of activity correlated with the loss of ^{65}Zn from the enzyme. It has been suggested, following analysis of enzyme-DNA interactions, that the enzyme-bound Zn facilitates the deprotonation of the 3'-OH group of the terminal dNTP on the growing strand of DNA (Springgate *et al.*, 1973). Cd has been shown to inhibit DNA repair in mammalian cells exposed to UV irradiation. Exposure to Zn at 10x higher concentrations counteracts the inhibitory effects of Cd on DNA synthesis and restores, at least partially, the DNA repair capability of the cells and their survival. It was proposed that Cd and Zn may compete for the same binding site with Cd having the greater afffinity (Nocentini, 1987).

1.1.1.3.2 RNA Polymerases

Higher plants have three distinct RNA polymerases. RNA pol I is located in the nucleolus and synthesises ribosomal RNA (rRNA). RNA pol II is located in the nucleoplasm and synthesises messenger RNA (mRNA). RNA pol III is also found in the nucleoplasm and it synthesises transfer RNA (tRNA) and the 5S rRNA (Mantell *et al.*, 1985). RNA polymerase has also been identified in mitochondria (Kornberg, 1980) and chloroplasts (Bottomley *et al.*, 1971) .

As in DNA polymerases, Zn has been identified in RNA polymerases. Scrutton *et al.* (1971) demonstrated that *E. coli* RNA polymerase contains 2g-atoms of tightly bound Zn per mole of enzyme and that the ratio of Zn to enzyme activity is constant when the purified enzyme is fractionated by gel filtration. Scrutton also showed that addition of 1,10-phenanthroline prior to addition of other reaction components completely inhibited polymerisation. If 1,10-phenanthroline was added after the other reaction components, inhibition of RNA synthesis only occurred after a lag period. 1,7-phenanthroline had no effect on RNA synthesis. This data would support a role for Zn in the initiation stage of RNA synthesis. Zn has also been shown to be an integral part of RNA pol II from *Saccharomyces cerevisiae*. The enzyme activity has been shown to correlate to Zn content during purification of the enzyme. As described for *E. coli* RNA polymerase, 1,10-phenanthroline has been shown to inhibit the yeast RNA pol II, but on addition of excess Zn, polymerase activity was restored (Lattke and Weser, 1976). RNA pol II from wheat germ contains tightly bound Zn as determined by atomic absorption studies. Dialysis of the enzyme with the metal chelator 1,10-phenanthroline results in the loss of enzyme activity and extraction of the bound Zn. Other metals including Cu, Co, Mn, Mg, Cr, Ni and Fe were not detected in significant amounts (Petranyi *et al.*, 1977). A small subunit of yeast RNA pol II, designated RPB9, is unusual among RNA polymerase subunits in that it contains two cysteine repeat motifs, $C\text{-}X_2\text{-}C\text{-}X_{18}\text{-}C\text{-}X_2\text{-}C$ and $C\text{-}X_2\text{-}C\text{-}X_{24}\text{-}C\text{-}X_2\text{-}C$, which may bind some of the Zn that is associated with this enzyme (Young, 1991). The Zn contained in eukaryotic RNA pol III may bind to conserved amino acid sequences of the largest subunit of RNA pol III (Geiduschek and Tocchini-Valentini, 1988).

Studies on the function and binding of Zn have centered on *E. coli* RNA polymerase. The extent to which this information is relevant to plant RNA polymerases, in particular to organellar RNA polymerases, is not, at this time, clear and the literature is therefore only briefly reviewed. The *E. coli* RNA polymerase has been denatured to liberate individual subunits of the core enzyme. After dialysis against 10^{-5} M Zn^{2+} it was established that Zn^{2+} was bound to the β and β' subunits of the core enzyme. No Zn was detected associated with the α-subunit of the holoenzyme (Wu *et al.*, 1977). The isolated β' subunit has been shown to be capable of DNA binding (Fukuda and Ishihama, 1974). Two mutations of the β' subunit have been shown either to have decreased DNA binding ability (Panny *et al.*, 1974) or to be unable to cause the

formation of open promoter complexes (Gross *et al.*, 1976). It has been demonstrated that Zn is essential for not only a catalytic role but also a structural role in *E. coli* RNA polymerase. When RNA polymerase was denatured and the two intrinsic Zn ions removed, subsequent reconstitution of the apoenzyme in the presence of 10^{-5} M Zn^{2+} led to the recovery of the reconstituted RNA polymerase containing one Zn ion with 50 % activity of the original enzyme. The analysis of apoenzyme and reconstituted Zn RNA pol by sucrose density gradient sedimentation showed that the inactive apoenzyme consisted of randomly folded protein indicated by a range of sedimentation values from 5 to 18S. Reconstituted Zn RNA polymerase contained a major active 13S RNA polymerase species and a minor inactive 7.9S species representing the portion of enzyme incapable of reconstitution. Both 13S and 7.9S species contained 1 mol Zn ion and the five subunits (Solaiman and Wu, 1985a).

It has been demonstrated that partial substitution of Zn with divalent metals in *E. coli* RNA polymerase results in the metal hybrid Co-Zn, Mn-Zn, Ni-Zn and Cu-Zn RNA polymerases, which possess 100, 100, 60 and 17 % of the enzyme activity of the reconstituted Zn-Zn polymerase respectively. The substituted metal was found to be located in the β subunit of the polymerase which contains the substrate binding site (Chatterji and Wu, 1982). A method of substituting metal in the enzyme which omitted harsh conditions e.g. low pH, was developed which allowed reconstitution of the enzyme with metal bound to both the β and β' subunits. The enzyme bound all members of the Zn triad, Zn, Cd and Hg, tightly. Only one Hg ion was incorporated, probably due to steric hindrance caused by the large size of the Hg ion. Reconstituted RNA polymerases were obtained which had correct conformation as shown by density gradient sedimentation and fluorescence spectroscopic analysis. It was established that reconstituted *E. coli* RNA polymerase which contained two bound Cd atoms, had 73 % of relative enzyme activity in abortive initiation reactions and 89 % of the relative DNA binding activity compared to the native Zn-containing enzyme (Solaiman and Wu, 1985b).

1.1.1.4 The function of Zn in transcription factors and the effect of its substitution by Cd

Hirt *et al.* (1989) have shown that a Cd concentration of 10 or 50 μM stimulates synthesis of RNA over an initial 6 h exposure in tobacco suspension cultures. Subsequently, RNA synthesis was inhibited. This inhibition was more dramatic in those cells exposed to 10 μM Cd. Incubation of the cells in the presence of 100 μM Cd resulted in the sustained stimulation of RNA synthesis. Exposure to 500 μM and 1 mM Cd resulted in an immediate repression of RNA synthesis with complete inhibition occurring after 72 h exposure to 1 mM Cd. The pattern of protein synthesis observed after exposure of cultures to identical Cd concentrations followed similar trends. As the size of the ribonucleotide pool was not affected by the Cd concentration it was proposed that Cd mainly influenced transcriptional events, but additional changes at the

translational level could not be excluded. A construct comprised of a chloramphenicol-acetyltransferase (CAT) coding region under the control of a nopaline synthase (nos) promoter was transformed into tobacco protoplasts. When such transformed protoplasts were grown on media without Cd, CAT activity was measured and nominally assigned as 100 %. In media containing 50, 100 and 200 μM Cd, CAT activity increased to 160, 180 and 140 % respectively. Of a number of other metals tested, only Zn enhanced the synthesis of RNA and protein synthesis in the same manner as Cd, although at a 10-fold higher concentration. It was proposed that the lower levels of Cd were not sufficient to induce Cd-binding peptides (reviewed in more detail in section 1.2.3.2) but could still exert toxic effects, inhibiting RNA and protein synthesis. At moderate levels of Cd, Cd-binding peptides may be induced, the amount of free Cd reduced and the toxic effects negated. Whilst at high Cd concentrations Cd-binding proteins could not bind all of the free Cd and its toxic effects would still be apparent. This would result in Cd stimulating RNA and protein synthesis *in vivo* at low intracellular levels of free Cd at which level other toxic effects are minimal. Hirt *et al.* (1989) proposed that Cd replaced Zn in DNA binding proteins altering the binding specificity and leading to enhanced transcription.

Transcription factors can, of course, activate or repress their target genes. There are several known types of transcription factors categorised by the structure of their binding domains, including leucine zippers, helix-turn-helix, helix-loop-helix and homeodomain binding proteins. Vallee *et al.* (1991) proposed that Zn-requiring transcription factors could be classified into three groups according to their metal cluster structure. The proposed nomenclature for these categories was Zn-fingers, Zn-twists and Zn-clusters.

1.1.1.4.1 Zn-fingers

A *Xenopus* transcription factor IIIA (TFIIIA) has been shown to contain multiple bound Zn ions which were essential for site-specific DNA binding activity. TFIIIA has been shown to bind specifically to 5S RNA genes. TFIIIA was isolated from *Xenopus* oocytes in association with 5S RNA as a 7S nucleoprotein complex. EDTA treatment of TFIIIA bound to 5S RNA did not release any Zn, however treatment of TFIIIA released from the 5S RNA by nuclease digestion removed Zn ions and resulted in an apoenzyme with no detectable specific DNA binding activity (Hanas *et al.*, 1983). When its amino acid sequence was examined it was observed that with the exception of 90 C-terminal and 10 N-terminal amino acids, TFIIIA consists of 9 repeats with variations of a 27 amino acid motif. Each repeat contains two Cys and two His residues at precisely repeated positions postulated to bind one Zn^{2+} ion to form a structural domain termed a Zn-finger (Miller *et al.*, 1985). The coordination of this Zn^{2+} with 2S and 2N atoms has been confirmed by extended X-ray absorption fine structure analysis (Diakun *et al.*, 1986). It has also been demonstrated that the protein folds into a compact and thermostable structure only in the

presence of Zn^{2+} or Co^{2+}. The Co^{2+} has been shown to complex in a tetrahedral arrangement (Frankel *et al.*, 1987). A single gene encodes *Xenopus laevis* TFIIIA (Taylor *et al.*, 1986) which contains 8 introns and 6 of the 9 exons encode single units of the repeating structure (Vincent, 1986). Numerous eukaryotic genes encoding Zn-fingers have been reported (Berg, 1990). NMR studies of a Zn-finger have led to models of the structural basis of the interaction between DNA and the Zn-finger domains. The model would suggest a two-stranded antiparallel β sheet that includes the two Cys residues, a turn and then a helix that includes two His residues. It is suggested that the Zn-finger wraps around the DNA helix to which it binds (Lee *et al.*, 1989). It has been proposed that the term Zn-finger is only applied to those proteins which have one or multiple repeats of about 30 amino acids each, conserve both two cysteine and two histidine residues and their spacing and have a three dimensional structure that resembles a finger (Vallee *et al.*, 1991).

Transcription factor SpI present in mammalian cells, binds to GC box promoter elements and selectively activates mRNA synthesis. The DNA binding activity has been localised to a C-terminal 168 amino acid region which contains three contiguous Zn-finger motifs. Zn^{2+} has been shown to be essential for DNA binding (Kadonaga *et al.*, 1987). It has also been shown that Cd^{2+} can be exchanged for Zn^{2+} in the Zn-finger of SpI. After such substitution the protein retained its specific DNA binding capacity (Kuwahara *et al.*, 1991).

The majority of transcription factors so far identified in plants appear not to be metal requiring (reviewed by Katagiri and Chua *et al.*, 1992). The metal-requiring factors identified to date are described below and in section 1.1.1.4.2. It is possible that in this review, despite extensive literature searches, other metal requiring transcription factors have been overlooked; in particular in investigations where the use of chelating agents in studies of DNA binding proteins has suggested a role for Zn but where the binding protein has not been extensively characterised, as exemplified by LABF1 (described below).

5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) catalyses an essential step in the shikimate pathway. Nuclear extracts from petunia petal contain a factor that interacts with the 5' upstream region of the gene encoding EPSPS. DNase I footprint analysis of the promoter region showed that four strong binding sites (EP1-EP4) bind the same factor. A cDNA clone (EPF1) encodes a DNA-binding protein which has been shown to have similar binding activity to nuclear factors. Sequence analysis of the full EPF1 revealed two repeats which corresponded to Zn-finger motifs. The two finger motifs in EPF1 are widely separated, which may indicate that EPF1 binds large domains of DNA. Both EDTA and 1,10 phenanthroline inhibit DNA binding activity. Neither of these chelators inhibited the DNA binding capability of the petal nuclear extract. This, together with the results of gel shift assays, led to the suggestion that more than one factor was present in the nuclear extract that interacts with EPSPS upstream sequence and

that EPF1 does not represent a major component among them. In addition, antibodies raised against EPF1 did not recognise the nuclear factor. Northern blot analysis showed that the expression of EPF1 is very high in the petal, low in the floral tube and undetectable in the leaf, stem and root. This pattern of expression was very similar to that of EPSPS. Southern blot analysis indicated that EPF1 belongs to a small gene family. Investigations using a translational fusion of the EPF1 upstream region to the β-glucuronidase reporter gene in transgenic petunia indicate that the cell specific expression pattern of EPF1 in flower and seedling is almost identical to that of EPSPS. This is the only report of a Cys_2/His_2 type Zn-finger from plants (Takatsuji *et al.*, 1991) and EPF1 is one of only two metal requiring transcription factors so far reasonably well characterised and reported in plants, although some others have been identified (see below and Table 3).

A transcription factor, designated LABF1, has been shown to bind to the regions -549 to -316 and -883 to -582 of the *legA* promoter of *P. sativum* (Meakin and Gatehouse, 1991). Binding of LABF1 was not observed using the same two regions of the pea promoter in mobility shift assays carried out in the presence of the chelating agent 1,10-phenanthroline (J. Gatehouse, pers. comm.). This would suggest that the protein LABF1 acts as a metal-dependent-transcription factor.

A cDNA clone, designated SF3, has been isolated by differentially screening a sunflower floral cDNA library. This cDNA encodes a protein of 219 amino acids. Sequence comparison indicates that the encoded protein would contain two potential Zn-finger domains alternating with two basic domains, similar to some other characterised Zn-fingers. It is suggested by the authors that this cDNA encodes a transcription factor required for the expression of late pollen genes (Baltz *et al.*, 1992).

1.1.1.4.2 Zn-twists

The animal glucocorticoid receptor (GR) enhances or represses the transcription of numerous genes which respond to steroid hormones by binding to specific DNA sequences named glucocorticoid responsive elements in the upstream regions of these genes. GRs consist of three functional domains: an N-terminal domain that is involved in transcriptional activation (Evans, 1988), a central Zn-containing one that binds the glucocorticoid responsive element and a C-terminal domain that binds hormones (Evans, 1988). GR is a member of a group of receptors that bind steroid and thyroid hormones, retinoic acid and vitamin D3. The DNA binding domains of members of this group have nine cysteines and one histidine at conserved spaces. The steroid receptors have an additionally conserved tenth cysteine. NMR studies show that the central domain has two tetrahedral Zn binding sites each consisting of four cysteines and one Zn^{2+} ion

(Pan *et al.*, 1990). The distance between the two Zn ions is approximately 1.3 x 10^{-9} m. The two other cysteine and histidine residues are not ligands for Zn. The 15-amino acid spacer that separates the two Zn ions forms the DNA recognition site (Hard *et al.*, 1990). Mineralocorticoid, progesterone and androgen receptors from various species have been shown to contain eight cysteines with conserved spacing identical to that in GR. Amino acid composition around the cysteine residues in these proteins is very similar and the region between the two Zn binding domains (based on rat GR) is invariant. This region forms an α helix in mammalian GR. A Zn-finger based on one Zn atom as described for TFIIIA is not present in these proteins. It has been proposed that these proteins be termed Zn-twists (Vallee *et al.*, 1991).

It has been demonstrated that chelation of Zn in bovine oestrogen receptor by EDTA and 1,10-phenanthroline results in a total loss of DNA binding activity (Sabbath *et al.*, 1987). More recently it has been shown that dialysis of the apoprotein against buffer containing Zn, Cd or Co restored DNA binding activity as measured by mobility shift assays. Dialysis against Cu and Ni containing buffers did not lead to the restoration of DNA binding activity. Competition studies with Zn and Cu or Ni suggested that both Cu and Ni could bind to the protein, which was subsequently unable to bind to DNA (Predki and Sarkar, 1991).

DNase 1 footprinting has been used to characterise nuclear factors that bind to the light-responsive promoter of pea rbcS-3A, a member of the gene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. 3AF1 is a sequence specific binding factor which binds to an AT rich sequence present between -51 and -31 of the rbcS-3A promoter, designated Box IV. Mutations of 3bp in box IV severely reduced DNA-protein interaction. The unmutated Box IV was shown to be active in transgenic tobacco plants when placed under the control of a CaMV 35S promoter. Sequence analysis of a cDNA clone for 3AF1 showed the presence of two repeats in which there is an arrangement of histidines and cysteines which may be related to Zn-finger and Zn-twist motifs. Consistent with the observation of these repeats is the demonstration that 1,10-phenanthroline inhibits the binding of 3AF1 to box IV of rbcS. Northern blot analysis shows that multiple transcripts homologous to this cDNA clone are expressed in different tobacco organs (Lam *et al.*, 1990). This is one of only two Zn-requiring transcription factors whose function has so far been characterised in plants, a third having been described on the basis of sequence comparison only and a fourth transcription factor having been shown to have no binding activity in the presence of the chelator 1,10-phenanthroline (Table 3).

TABLE 3

Known metal requiring transcription factors in plants

NAME	TRANSCRIPTION FACTOR TYPE	SPECIES	REFERENCE
EPF1	Zn-finger	petunia	Takatsuji *et al.*, 1991
SF3	Zn-finger	sunflower	Baltz *et al.*, 1992
3AF1	Zn-finger/Zn-twist	pea	Lam *et al.*, 1990
LABF1	unknown	pea	Gatehouse, pers. comm.

1.1.1.4.3 Zn-clusters

The GAL4 transcription factor from *S. cerevisiae* regulates the expression of the genes encoding the galactose metabolising enzymes (Laughon and Gesteland, 1984). It consists of 881 amino acids of which the N-terminal 62 have been shown to be involved in DNA binding (Pan and Coleman, 1989). The N-terminal 38 amino acids of this domain include six cysteines (Johnson, 1987). NMR studies have demonstrated that these six cysteines interact with two Zn atoms to form a binuclear Zn thiolate cluster, with an interatomic Zn distance of approximately 3.5×10^{-10} m. This Zn-cluster is based on the same principles of coordination as that found in metallothionein (Vallee *et al.*, 1991). The six cysteines and their spacing are conserved in eight other fungal transcription factors. It has been proposed that these proteins be classified as Zn-clusters (Vallee *et al.*, 1991).

To establish the nature of metal binding in GAL4, ^{113}Cd was substituted into the protein for use in NMR studies. The Cd substituted protein was prepared by adding 5 fold excess of ^{113}Cd to the purified Zn containing protein (Gadhavi *et al.*, 1991). No investigations were carried out to establish the effect of this metal substitution on protein function. However this demonstrates that Cd can competitively replace Zn in this transcription factor.

The promoter sequences of the metallothionein (MT) gene of *S. cerevisiae*, CUP1, bind the transcription factor ACE1. It has been shown that ACE1 binds Cu in a cluster, termed a Cu-fist (Dameron *et al.*, 1991). The binding of Cu to ACE1 has been shown to activate the transcription of CUP1 (Furst and Hamer, 1989). The activation and Cu binding of ACE1 is discussed in more detail in section 1.3.5.1. A Zn-cluster remains to be identified in plant tissue.

1.1.2 Direct interaction of Cd with other macromolecules

Possible toxic effects of Cd in relation to binding to macromolecules, other than protein, in the cell have received little attention. Polysaccharides, with anionic properties, are capable of

binding heavy metal cations (Reed and Gadd, 1991). The effects of interaction of Cd with other macromolecules await description.

1.1.3 Alteration of intracellular redox potential

Brodl (1990) has proposed that the heat shock response, which is co-induced by Cd (Chapter 4) is triggered by a change in the cell's redox potential. Inhibitory effects which arise from modulation of redox potential are not extensively documented.

1.2 Cd-TOLERANCE

Reviews examining the broad subject of metal tolerance in plants (Woolhouse, 1983; Thurman and Collins, 1983; Verkleij and Schat, 1990) serve to illustrate the many mechanisms of resistance which have been proposed to occur. As stated by Thurman (1981) these can be described as avoidance mechanisms (exclusion), metabolic adaptation and intracellular compartmentation / complexation.

1.2.1 Exclusion

Metals bind to sites within cell walls. It has been proposed that this may reduce the uptake of metal ions (Woolhouse, 1983). Large quantities of Cd have been localised in the cell walls of *Azolla* (Sela *et al.*, 1988) and *Zea mays* (Klan *et al.*, 1984). Turner and Marshall (1972) recorded a close correlation between the capacity for Zn accumulation in the cell wall and the degree of Zn tolerance in *Agrostis tenuis*. Digestion of the cell walls with trypsin did not release significant amounts of Zn but 66 % of the bound Zn was released by treatment of the cell wall with cellulase. This implies binding to the cellulose, but not the protein, component of the cell wall (Wynn Jones *et al.*, 1971).

Differential uptake of metals from liquid media by tolerant and non-tolerant strains of algae has been observed. The Cu-tolerant, filamentous brown alga, *Ectocarpus siliculosus* has been demonstrated to exclude Cu when compared to non-tolerant strains which accumulate large quanties of the metal (Hall, 1980). Similarly, Cu-tolerance in the green alga *Chlorella vulgaris* has been linked with Cu-exclusion (Foster, 1977). Within higher plants *Becuium homblei*, which is highly Cu-tolerant, when collected from the field has low and similar Cu concentrations in both roots and shoots, indicating restricted uptake of the metal (Reilly, 1969).

1.2.2 Metabolic adaptation

Significant differences have been described in the constants for Zn inhibition of soluble and cell wall bound acid phosphatases of *Anthoxanthum odoratum* plants with differing metal-tolerance phenotypes. These differences were indicative of less stable complexes between Zn and the enzymes in Zn-tolerant rather than Zn-sensitive plants (Cox and Thurman, 1976). Similar differences in inhibition kinetics of enzymes in response to Cd in plants with differing Cd-tolerance characteristics have still to be established.

1.2.3 Intracellular compartmentation / complexation

A number of studies have recorded higher content of organic acids in the metal-tolerant cells compared to non-tolerant cells of the same species (Foy *et al.*, 1978; Thurman and Rankin, 1982). The Zn content of tolerant and non-tolerant ecotypes was shown to be invariant in *Silene cucubalus* (Mathys, 1975). It was later established that there were differences in oxalate and malate content between Zn-tolerant and non-tolerant ecotypes of *S. cucubalus*, *Thlaspe alpestra* and *Agrostis tenuis*. Zn-tolerance was best correlated to malate content. A hypothesis was proposed in which malate would be found primarily in the cytoplasm, capable of binding Zn. This complex would then be transported through the tonoplast into the vacuole where Zn could form a complex with oxalate. The malate could then diffuse back into the cytoplasm to bind Zn again (Mathys, 1977).

Computer modelling has been used to evaluate the possible role of organic and inorganic ligands in tobacco cells exposed to Cd (Wang *et al.*, 1991). The results of the evaluation supported the hypothesis that certain organic acids and sulphydryl containing peptides (the vacuolar compartmentation of phytochelatins (PCs) in the cell vacuole is described in section 1.2.3.2.3) may form soluble Cd complexes in the cell's vacuole. Although complexation of malate and oxalate with Cd was predicted to be less significant, citrate in the concentration encountered in tobacco cell vacuoles had a high potential for forming soluble complexes with Cd particularly over the pH range 4.5 to 7 (Wang *et al.*, 1991).

Cd and other metal ions can be complexed with MTs within cells. MTs are classified as being of three types, class I, II or III. A wealth of information exists concerning the structure and transcriptional regulation of animal and yeast class I and II MTs. In view of this, a brief review of these subject areas is given in sections 1.2.3.1.1 and 1.3.5.1 respectively. The following review focuses on the structure and role of plant MTs and MT-like proteins in the complexation and vacuolar compartmentation of Cd.

1.2.3.1 Metallothioneins

1.2.3.1.1 The animal paradigm for Cd (and other metal ion) detoxification via biochemical sequestration

The name metallothionein (MT) was first given to the Cd-, Zn-, and Cu- containing sulphur rich proteins from equine renal cortex (Kagi and Vallee, 1960). MTs are low molecular weight, cysteine (cys) rich proteins (approximately 30 % cys) that usually lack aromatic amino acid residues. Cys residues in MTs are commonly present in Cys-X-Cys motifs where X is a residue other than cys, or in Cys-Cys clusters. These sequences are involved in binding metal ions in metallo-thiolate clusters (Kagi and Kojima, 1987). Another common feature of all MTs is that their synthesis increases in organisms exposed to elevated concentrations of certain trace metal ions.

MTs can be classified into three groups according to highly conserved primary structures. Class I and Class II MTs are proteins encoded by structural genes. Class I includes mammalian MTs and polypeptides from other phyla which have similar sequences, for example *Neurospora* (Munger *et al.*, 1987) and *Agaricus* (Munger and Lerch, 1985), *Drosophila* (Maroni *et al.*, 1986; Mokdad *et al.*, 1987) and the prokaryote *Synechococcus* (Olafson *et al.*, 1988). Mammalian tissue generally contains two major fractions, MTI and MTII, which at neutral pH usually differ by a single negative charge. In some species, high pressure liquid chromatography (HPLC) has been used to separate these fractions into subforms, which are specified by lower case letters e.g. MT-1_j, MT-1_k. In humans nine MT1 subforms have been identified (Kagi and Kojima, 1987). Class II compromises proteins with locations of cysteine only distantly related to those in equine renal MT. Class II MTs have been identified in *Schizosaccharomyces pombe* (Murasugi *et al.*, 1981) and *Synechococcus TX-20* (Olafson *et al.*, 1988; Olafson, 1984, 1986) and in sea urchin (Nemer *et al.*, 1985).

All class I and II MTs characterised to date are single chain proteins. The number of residues in the molecule varies with species; mammalian forms contain 61-67 amino acids, chicken has 63 and sea urchin has 64 residues. The chain lengths of invertebrate MTs are shorter, the shortest MT being that of the fungus *Neurospora crassa* which has 25 residues (Kagi and Kojima, 1987). As stated earlier, the most notable feature of amino acid content of MT is the abundance of cys residues. In most class I MTs the cys residues occur at positions throughout the chain. In mammals the position of all 20 cys residues is invariant. In addition to this the basic residues lysine and arginine are well conserved in positions adjacent to the cys residues. These residues may play an auxilliary role in the formation of the metal complexes (Pande *et al.*, 1985). Most amino acid substitutions are of conservative type and occur in the N-terminal half of the chain indicating fewer evolutionary constraints in this portion of the chain

(Kagi and Schaffer, 1988). No obvious sequence relationships occur among Class II MTs other than the abundance of cys-rich motifs. In sea urchin MT the order of the amino acids is nearly the reverse of that seen in mammalian MTs (Nemer *et al.*, 1985). The structural similarities of MTs, particularly Class I MTs, have been attributed to convergent evolution imposed by the requirements of metal complexation and other as yet unidentified functional constraints (Kagi and Schaffer, 1988). There has been considerable speculation as to whether or not plants produce MTs or alternatively if the functions of MTs in animal cells are performed by PC molecules in plant cells (Grill *et al.*, 1987).

1.2.3.1.2 The Ec protein from wheat germ

An *in vitro* translation product, designated Ec, was initially isolated from wheat germ as a result of examination of [^{35}S]cysteine incorporation into proteins expressed early in germination (Hanley-Bowdoin and Lane, 1983). Computer analysis of the sequence revealed no significant homology to other proteins, however subsequent visual observation identified several sequences bearing similarity to Class I MT from crab and arrangement of cysteine residues resembling MT (Lane *et al.*, 1987). Isolation of the protein in association with Zn (Lane *et al.*, 1987) resulted in the designation of this protein as a class II MT (Kagi and Schaffer, 1988). The stoichiometry was estimated to be approximately 5 (Zn : protein).

It was noted that although Ec protein could be readily isolated from dry wheat germ, the amount of protein present drops rapidly following germination (Hanley-Bowdoin and Lane, 1983). By analogy with animal systems, for example rats, where the amount of MT present in the livers of neonatal rats is 20-fold greater than in adults (Kern *et al.*, 1981), Lane *et al.* (1987) postulated that the Ec protein may have a role in the deposition of Zn, the requirement for which may alter during proliferative compared to differentiating stages of development.

1.2.3.1.3 MT-like genes from plants

Genes and cDNAs capable of encoding proteins with some sequence similarity to Class I MTs have recently been isolated from several species of higher plant. These include *Mimulus guttatus* (de Miranda *et al.*, 1990), *P. sativum* (Evans *et al.*, 1990), *Zea mays* (de Framond, 1991), *Arabidopsis thaliana* (Takashi, 1991), soybean (Kawashima *et al.*, 1991) and barley (Okumura *et al.*, 1991). Additionally several as yet unpublished cDNAs and genes have been isolated. These include cDNAs from alfalfa (A.M. Tommey, unpublished results) and genes from *P. vulgaris* (J. Bryden, unpublished results), *Nicotiana tabacum* (A.M. Tommey, unpublished results), *D. innoxia* and a citrus species (P.J. Jackson, pers. comm.) and a further *Arabidopsis* homologue (Goldsborough, pers. comm.). All of these cDNAs and genes, for which

sequence information is available, exhibit significant sequence similarity to each other. The deduced amino acid sequences of the putative protein products of these genes are extremely well conserved, especially with regard to the cysteine residues which occur in the N- and C-terminal portions of the predicted sequences. For each of the sequences reported, excepting the soybean and *Arabidopsis* sequences, the cysteine residues can be aligned perfectly. The soybean and *Arabidopsis* sequences have two additional cysteine residues at the N-terminus. The aligned predicted protein sequences of the known MT-like DNA sequences are presented in Figure 1.

All of these MT-like sequences were isolated as a result of screening cDNA libraries. In the case of the genes from *P. sativum* and *Z. mays*, cDNA clones encoding MT-like proteins were isolated as a result of differential screening of cDNA libraries for organ abundant clones. In each case the MT-like cDNAs were isolated as a result of being highly expressed in root relative to other tissues. *M. guttatus* cDNA clones, which were isolated from libraries prepared from mRNA isolated from root tissue on the basis of repression by Cu shock, were subsequently shown to be highly abundant in root tissues relative to leaf tissue.

Okumura *et al.* (1991) isolated the barley gene, *ids*1, as a result of screening for Fe-deficiency-specific cDNAs. These authors speculate that the protein may have a role relating to Fe metabolism, potentially at the level of regulation of the genes involved with synthesis of Fe-binding substances such as mugeneic acid or that the MT-like protein may have a direct role in the transport of iron. Mugeneic acid is a member of a family of phytosiderophores which solubilise sparingly soluble Fe^{3+} in the rhizosphere and allow reabsorption of Fe(III)-phytosiderophore complexes (Takagi, 1976). No evidence was presented for these roles other than an increase in abundance of these cDNAs in response to Fe-deficiency. Recently reported data has shown a link between Fe metabolism and MT gene expression in *S. cerevisiae* (cited in Lindsay, 1992). Cells selected for increased Cu-requirement were found to be mutated in a membrane-associated Fe reductase system (FRE1). This system was shown to be active in Cu uptake and reduction (CuII to CuI). It was subsequently shown that in normal *S. cerevisiae* cells FRE1 is expressed in low Cu concentrations to improve Cu uptake and is repressed in high Cu concentrations. Root systems of a *P. sativum* mutant, designated E107, grown under Fe-sufficient or Fe-deficient conditions showed 4 to 7-fold greater reduction of Fe(III) compared to the roots of wild type *P. sativum* grown under Fe-sufficient conditions. Specific staining techniques showed reduction occurred over the entire length of the roots, excepting the root apices, in E107 grown under both Fe-conditions and wild type plants grown under Fe-deficient conditions. Wild type plants grown under Fe-sufficient conditions showed no or very limited Fe(III) reduction. It was concluded that E107 mutants acted functionally as Fe-deficient plants which use an Fe(III) reductase system to elevate Fe^{2+} transport capacity (Grusak *et al.*, 1990). It has most recently been demonstrated that an Fe/Cu reductase is active in *P. sativum* roots grown in Fe deficient conditions (A.M. Tommey, unpublished). This is discussed in greater

FIGURE 1

Amino acid alignment of the predicted protein sequences of the MT-like genes and cDNAs isolated from plants.

The sequences are presented with breaks to allow maximal alignment of conserved residues. References are given in the text.

TYPE 1

```
Tobacco          ..........................................................AAEGGNGCKCGSNCTCDPCNC.
Alfalfa          ....................................TVILGVGPAKIHFEG...AEMGVAAEDGGCKCGDSCTC.PCNCK
PsMT_A           MS.GCGCGSSCNCGDSCKCNKRSSGLSYSEMETTE..TVILGVGPAKIQFEG...AEMSAASEDGGCKCGDNCTCDPCNCK
PsMT_B           MS.GCGCGSSCNCGDSCKCNKRSSGLSYSEMETTE..TVILGVGPAKIQFNG...AEMSVAAEDGGCKCGDSCTCDPCNCK
PsMT_C           MS.GCGCGSSCNCGDSCKCSKRSSGLSYSEMETTE..TVILGVGPAKIQFNG...AEMSVAAEDGGCKCGDSCTCDPCNCK
Barley-Ids1      MS..CSCGSSCGCGSNCNCGKMYPDLEEKSGATMQVTVIVLGVGSAKVQFEEAA...EFGEAAH.GCSCGANCKCNPCNC.
M.Gutattus       MSSGCSCGSGCKCGDNCSCSM......YPDMETNTTVTMIEGVAPLKMYSEG..SEKSFGAEGGNGCKCGSNCKCDPCNC.
Maize            MS..CSCGSSCGCGSSCKCGKKYPDLEETSTAAQP..TVVLGVAPEKKAAPEFVEAAAESGEAAHGCSCGSGCKCDPCNC.
Consensus        MS--C-CGS-C-CG--C-C----------------------GV---K------------------GC-CG--C-C-PC-C-
Cys-signature    ----CXC---CXC---CXC-----------------------------------------------CXC---CXC--CXC-
```

TYPE 2

```
Arabidopsis      MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKNQYEASGESNNAESDACKCGSDCKCDPCTCK
Soybean          MSCCGGNCGCGSSCKCGNGCGGCKMYPDLSYTES.TTTETLVMGVAPVK.AQFEGAEMGVPAENDGCKCGPNCSCNPCTCK
Consensus        MSCCGGNCGCGSSCKCGNGCGGCKMYPDLSY----TTTET-V-GVAP----Q-E-------AE-D-CKCG--C-C-PCTCK
Cys-signature    --CC---CXC---CXC---C--C-----------------------------------------CXC---CXC--CXC-
```

detail below.

As discussed above, the MT-like gene from pea, $PsMT_A$, was isolated by screening a genomic library using a cDNA clone which had been isolated on the basis of high transcript abundance in pea root. The comparison of the cDNA and genomic clones of $PsMT_A$ revealed the presence of a large intron in the coding sequence (Evans *et al.*, 1990). This feature is conserved in the genomic clone from *Z. mays* (de Framond, 1991). Southern analysis of several restriction enzyme digests of *P. sativum* DNA revealed the existence of a small multi-gene family comprising up to 5 members. Several of these have recently been cloned (J. Bryden, unpublished results) (Figure 1, $PsMT_B$ and $PsMT_C$). The predicted amino acid sequence of $PsMT_A$ has two regions which are rich in cysteine residues. These two regions are located at the ends of the predicted protein and the N-terminal domain has extensive homology to the Cu-MT from *N. crassa*. The central portion has no homology to other MTs and contains several aromatic and hydrophobic residues. Northern analysis of expression of the $PsMT_A$ gene shows that expression is most abundant in root relative to other tissues (Evans *et al.*, 1990). Etiolated leaf tissue exhibited increased abundance relative to leaves grown under normal light. In root tissue, $PsMT_A$ transcripts could not be detected in pea 14 days after germination (Robinson *et al.*, 1992). The phenotypic effect of constitutive $PsMT_A$ expression has been examined in *E. coli* and *A. thaliana* (Evans *et al.*, 1992). This data showed that *E. coli* cells expressing recombinant $PsMT_A$ have an 8-fold greater accumulation of Cu than the control cells. No significant increase in the accumulation of Zn or Cd was detected. In transgenic *A. thaliana* expressing $PsMT_A$ under the control of the CaMV 35S promoter, 75 % of the plants derived from the F1 parents showed increased Cu accumulation compared to untransformed control plants. These observations support the suggestion that $PsMT_A$ can bind Cu *in planta* and plays a role in Cu homeostasis (Evans *et al.*, 1992).

Estimations of the pH at which 50 % of the metal is dissociated from $PsMT_A$-GST (glutathione), isolated from *E. coli* transformed with the gene encoding this fusion protein, have been made. The pH of half-dissociation of Zn, Cd and Cu was determined to be approximately 5.25, 3.95 and 1.45 respectively (Tommey *et al.*, 1991). The same study determined the pH of half-dissociation of equine MT, for the metals Zn, Cd and Cu, to be 4.5, 3.0 and 1.8 respectively. These figures agreed with previously published results. The pH of half-dissociation of Cd from PC complexes in *S. pombe* cells has been determined as 4.0 or 5.4, depending on the S^{2-} content of the PC complex (see section 1.2.3.2.1) and that of Cu to be 1.3 (Reese *et al.*, 1988). In tobacco, values of Cd displacement from PC complexes ranged from 5.0 to 5.8 (Reese and Wagner, 1987). The comparison of these pH of half-dissociation constants would indicate that $PsMT_A$ has high affinities for Cu and Cd in particular. Kille *et al.* (1991) reported that the expression of $PsMT_A$ in *E. coli* led to an increased accumulation of Cd in cells grown on medium containing the metal compared to non-transformed cells.

Kille *et al.* (1991) expressed a *PsMT*$_A$ cDNA in the heat-inducible expression vector pPW1, in *E. coli.* Upon purification of the expressed product three pools of Cd-containing material were generated. Sequence analysis confirmed that these components were derived from PsMT$_A$. It appeared that PsMT$_A$ had been proteolysed within the extended region between the two cysteine rich regions. It was proposed by the authors that the middle sequence of PsMT$_A$ is involved in the correct folding of the putative metal binding regions into the correct juxtaposition or in targeting the product to specific subcellular locations. Similar results were obtained by Tommey *et al.* (1991). In their studies PsMT$_A$ was expressed as a glutathione fusion protein. Attempts to purify the components of the fusion protein by cleavage with factor Xa released PsMT$_A$ and an additional second sequence which corresponded to the internal region of PsMT$_A$.

Analysis of pea plants grown hydroponically in medium containing high and low concentrations of available Fe indicated that the activity of a root surface Fe-reductase, a marker for the Fe-efficiency mechanisms in *P. sativum,* was greater in plants grown under low Fe conditions and that roots from plants grown under low Fe conditions accumulated twice as much Cu as roots grown under conditions of high available Fe (Evans *et al.*, 1992). More recently it has been demonstrated that when root Fe-reductase activity increases a concomitant Cu-reductase activity is observed (A. M. Tommey, unpublished). These results have led to the proposal that under low Fe conditions in pea roots PsMT$_A$ acts to detoxify Cu which accumulates as a result of the increased activity of root surface Fe/Cu reductase (a schematic diagram to describe such a model is shown in Figure 2). The findings of Evans *et al.* (1992) (above) support such a hypothesis and this would be consistent with the observations in yeast.

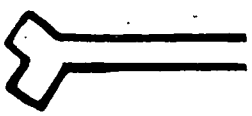
The cDNA clone containing the MT-like sequence from soybean (*Glycine max* L), reported by Kawashima *et al.* (1991), was isolated as a result of heterologous screening of a cDNA library using a synthetic oligonucleotide probe corresponding to the N-terminal part of mammalian MT. Analysis of expression of the gene by northern hybridisation of the cDNA to mRNA from soybean revealed slightly higher levels of expression of the corresponding gene in leaves relative to root. Exposure of plants to 6 mM Cd resulted in a slight decrease in expression of the gene in root tissue, which is consistent with the response to Cu shock observed in *M. guttatus.* It is unclear whether this response is specific to the MT-like gene, or is a general effect of reduced transcription in response to Cu toxicity.

Additional work in soybean involving immunohistochemistry studies using an anti-rat MT antibody revealed an antigenic response in soybean seedlings. Positively immunostained regions were observed in the ground meristem, protoderm and cap, especially the columella of the root tip but not in the procambium. The authors regard these immunostaining results as evidence for the localisation of MT in soybean seedlings (Chongpraditnum *et al.*, 1991). However the soybean root antigen was not characterised.

Figure 2

Schematic diagram showing the proposed role of $PsMT_A$ and its involvement with Fe-reductase

It has been proposed that $PsMT_A$ can bind Cu and plays a role in Cu homeostasis (Evans *et al.*, 1992). When root Fe-reductase activity increases a concomitant Cu-reductase activity is observed (A.M. Tommey, unpublished) and so under low Fe conditions $PsMT_A$ may act to detoxify Cu which accumulates as a result of the increased activity of root surface Fe/Cu reductase.

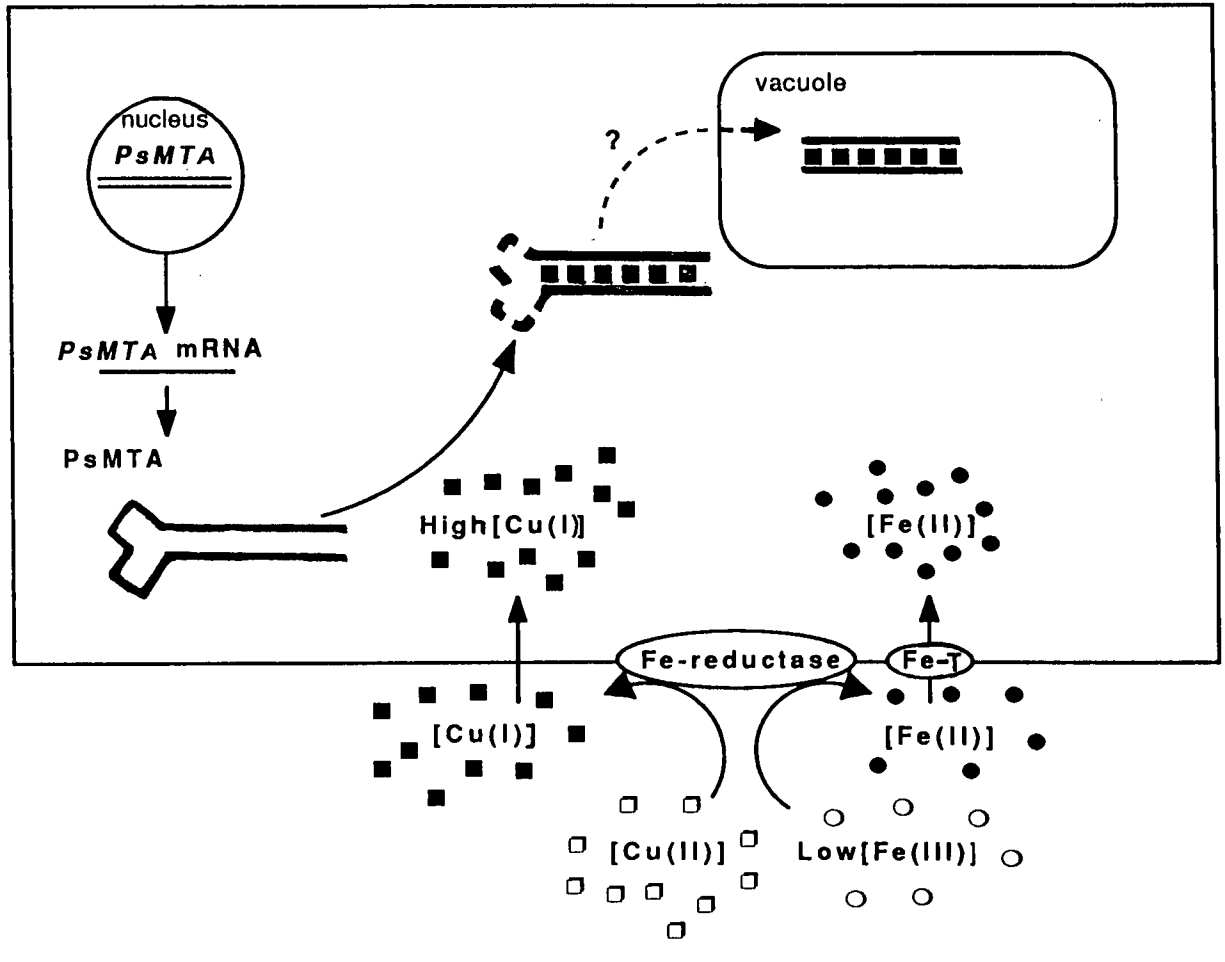


= $PsMT_A$ - represented according to the proposed structure of Kille *et al.* (1991). The arms of the molecule are the cysteine-rich regions which bind metal.

= $PsMT_A$ bound to copper. The part of the protein indicated by the broken line may serve to target the molecule to the vacuole (Kille *et al.*, 1991).

Fe-T indicates a putative Fe^{2+} transporter identified by Kaplan (pers. comm.).

The mechanism of Cu uptake into the cell is unknown.


nucleus
PsMTA
PsMTA mRNA
PsMTA
?
vacuole
High[Cu(I)]
[Fe(II)]
Fe-reductase
Fe-T
[Cu(I)]
[Fe(II)]
[Cu(II)]
Low[Fe(III)]

The role of products of MT-like genes is clearly unknown, but observed *in vitro* metal affinities and *in vivo* effects of expression suggest a role in the metabolism / detoxification of Cu is likely and a role in Cd detoxification is a formal possibility.

In addition to MT-like proteins plants also synthesis phytochelatins (PCs). The involvement of PCs in the response to exposure to elevated concentrations of Cd and other metals is discussed below. The response of PCs to Cd exposure contrasts to that of MTs, the role of which is the subject of debate (discussed by Karin, 1985).

1.2.3.2 Phytochelatins

In this report the term phytochelatin (PC) will be used. Different nomenclature exists for PCs; cadystins (Murasugi *et al.*, 1981), gamma-glutamyl metal binding peptide (Reese *et al.*, 1988), phytometallothionein (Rauser, 1987) and poly(gamma-glutamylcysteinyl)glycine (Robinson and Jackson, 1986; Jackson *et al.*, 1987). Cd binding peptides which form aggregates in the presence of Cd were first isolated from extracts of *S. pombe* (Murasugi *et al.*, 1981) and subsequently from higher plants (Bernhard and Kagi, 1985; Grill *et al.*, 1985; Robinson *et al.*, 1985). They have been identified in a wide range of plant species (reviewed by Rauser, 1990), the unicellular flagellate *Euglena gracilis* (Shaw *et al.*, 1989), eukaryotic algae (Gekeler *et al.*, 1988) and in *Candida glabrata* in response to Cd-salts (exposure of *C. glabrata* to Cu-salts leads to the induction of two MT polypeptides, MT-1 and MT-2) (Mehra *et al.*, 1988).

1.2.3.2.1 Structure and metal binding

PCs have the general structure γ(Glu-Cys)nGly, where n can range from 1 to 11. The repeating unit γ(Glu-Cys) is terminated with an α-linked glycine residue. PCs have been identified in the order Fabales in which the C-terminal glycine is replaced by β-alanine (Grill *et al.*, 1986). In *Agrostis gigantea* two Cd-binding peptides have been isolated which contain serine. In one form the amount of serine was equivalent to the amount of glycine but in the other the amount of glycine was twice that of serine (Rauser *et al.*, 1986). There have been reports of desglycyl forms in which the carboxy terminal amino acid is cys, because of the lack of a glycine or β-alanine residue (Steffens, 1986).

PC molecules have been isolated from plant cells in association with Cd and Cu, although Zn was a minor constituent of a complex isolated from tomato root which was subsequently shown to have a PC component (Leu-Kim and Rauser, 1986). Zn was also a major component of the Cd-PC complex isolated from Cd exposed *E. gracilis* (Shaw *et al.*, 1989). Wagner (1984) demonstrated Hg and Cu (but not Zn) binding to PC *in vitro*, as monitored by changes in

absorbance at 250nm brought about by addition of these metals to a solution containing Cd-PC. Thumann *et al.* (1991) demonstrated that Zn-PC complexes could be formed *in vitro* by addition of Zn to apo-PC.

Reese and Winge (1988) reported that PC-Cd complexes isolated from Cd-exposed *S. pombe* cells were isolated as a cluster composed of several PC molecules. Two distinct forms were present one of which contained acid labile sulphide (S^{2-}). These authors reported that the presence of S^{2-} resulted in an increase in the affinity of the complexes for Cd as demonstrated by a decrease in the pH required to displace Cd from complexes reconstituted with S^{2-}, relative to complexes without S^{2-}. The presence of S^{2-} in reconstituted complexes formed *in vitro* resulted in an alteration of the UV absorption characteristics of the complexes which was consistent with increasing size of Cd-S^{2-} crystalites (Reese and Winge, 1988). It is postulated from the results of these experiments that the Cd-S^{2-}-peptide complexes consist of a Cd-S^{2-} crystalite core, surrounded by peptide molecules. Reese *et al.* (1988) demonstrated that the S^{2-} containing complexes isolated from *S. pombe* had increased capacity for Cd, having a higher stoichiometric ratio of Cd : peptide relative to complexes that did not contain S^{2-}. S^{2-} has also been shown to be present in the Cd complexes isolated from *D. innoxia* (Robinson *et al.*, 1990a), *E. gracilis* (Shaw *et al.*, 1989) and *C. glabrata* (Dameron and Winge, 1989). There are no reports of S^{2-} in the Cu complexes from any source. A comparison of the published data for the pH of half dissociation of metal from various PC complexes shows, however, that the affinity of PCs for Cu is much higher than for Cd (Robinson, 1990). It has been proposed that the S^{2-} containing PC-Cd complex of *S. pombe* is involved with Cd-storage in the cellular vacuole and that the non-S^{2-} complex is involved in the sequestration of cytoplasmic Cd (Ortiz *et al.*, 1991) (This is reviewed in more detail in section 1.2.3.2.3). It has been shown that *D. innoxia* cell cultures selected for resistance to Cd produce more of the longer chain PCs than do non-selected cultures (Delhaize *et al.*, 1989a). The finding that longer chain PCs have a higher affinity for Cd and Cu than do shorter forms (Hayashi *et al.*, 1988; Mehra and Winge, 1988) indicates that the length of the molecule may also be of importance in metal sequestration in metal-resistant cells.

1.2.3.2.2 Biosynthesis of PCs

PCs are not encoded by structural genes but are the product of a biosynthetic pathway (Robinson and Jackson, 1988). Glutamyl γ-carboxamide bonds have been confirmed by numerous methods. Incubation of the peptides with γ-glutamyltransferase liberated glutamic acid (Grill, 1987). Peptides also proved refractory to Edman degradation and insensitive to digestion with *Staphylococcus aureus* V8 protease (Robinson and Jackson, 1986). Both treatments involve cleavage across glutamyl α-carboxamide bonds whereas the PCs contain glutamyl γ-carboxamide bonds. Oligonucleotide sequences were designed to hybridise to any mRNA molecules encoding

such proteins. No such hybridisation was observed in cells grown in the presence or absence of Cd (Robinson *et al.*, 1988). The structure of PCs and the observation that PC synthesis is accompanied by a concomitant depletion in cellular pools of glutathione (Scheller *et al.*, 1987) indicated that glutathione is a precursor in the synthesis of PC molecules. Inhibition of the production of PCs in cells exposed to BSO, a potent inhibitor of γ-glutamyl-cysteine synthetase (Griffith and Meister, 1979), provided further evidence for a biosynthetic pathway which involved glutathione consumption (Reese and Wagner, 1987). Additionally, mutants of *S. pombe* which were unable to synthesise cadystin (PC) were also unable to synthesise glutathione (Mutoh and Hayashi, 1988). Pulse chase experiments using ^{35}S-labelled cysteine have indicated that longer chain forms of PC are synthesised from shorter chain forms. Gradual migration of ^{35}S-labelled cysteine was observed from glutathione to elongating species of PC molecule (Robinson *et al.*, 1988).

Grill *et al.* (1989) reported the partial purification of an enzyme from *Silene cucubalus* which acts as a PC synthase, catalysing the formation of $\gamma(Glu\text{-}Cys)_{n+1}Gly$ from $\gamma(Glu\text{-}Cys)_nGly$ via transfer of the γ(Glu-Cys) from glutathione, thereby acting as a γ(Glu-Cys) dipeptidyl transpeptidase. In a cell free reaction system the enzyme was activated by Cd and the reaction was stopped by the sequestration of the activating metal ion by the newly formed PC molecules. Hayashi *et al.* (1990) reported the existence of two pathways of cadystin synthesis in *S. pombe*. As well as the reaction described above they report that synthesis proceeds by the addition of γGlu-Cys units from glutathione to elongating chains of $(\gamma Glu\text{-}Cys)_n$ units, to which glycine is added as the final step of the reaction. The amino acid sequence of the putative PC synthase has not yet been reported.

1.2.3.2.3 The role of PC in metal detoxification

The synthesis of PC molecules in response to elevated concentrations of metal ions and their association with the inducing metal ion, strongly suggests that they play a role in metal detoxification via sequestration. PCs isolated from *D. innoxia* cultures which had been selected for tolerance to Cd were found to be associated with up to 80 % of the cellular Cd (Jackson *et al.*, 1984). The observations that mutants of *S. pombe* which are unable to synthesise these molecules are hypersensitive to Cd toxicity (Mutoh and Hayashi, 1988) and that tobacco cells exposed to BSO are Cd-hypersensitive (Reese and Wagner, 1987), also indicate a role in the detoxification of metal ions.

It has been shown that a Cu ligand (subsequently shown to include PC (Salt *et al.*, 1989) was induced in both Cu-tolerant and non-tolerant ecotypes of *M. guttatus* in response to metal administration (Robinson and Thurman, 1986). Similarly Delhaize *et al.* (1989) reported that a

D. innoxia cell line which had been selected for Cd-tolerance synthesised PCs at a similar rate to a none Cd-selected line when exposed to Cd. It was noted however that in the Cd-tolerant cell line, Cd-PC complexes were formed more rapidly than in the non-tolerant cell line. In addition, the complexes formed by the non-tolerant cell line were of lower molecular weight than those of the tolerant cells and did not bind all the cellular Cd. Following studies in Cu-tolerant *Silene vulgaris* Schatt and Kalff (1992) reported that variation in PC production appeared to be a consequence of variation in tolerance. They proposed that PCs are not decisively involved in differential Cu-tolerance in *S. vulgaris*. PCs have been reported to be localised in the cell vacuole. The synthesis of Cd-binding peptides was induced upon addition of 20 μM $CdCl_2$ to the medium of hydroponically grown *Nicotiana rustica* var. Pavonii. Amino acid analysis showed that the main components were γ(Glu-Cys)$_3$-Gly and γ(Glu-Cys)$_4$-Gly. To directly determine the location of these PCs, protoplasts and vacuoles were isolated from leaves of Cd-exposed seedlings. HPLC was used to detect the presence of LMW thiols and atomic absorption was used to determine the presence of Cd. Both PCs and Cd were found localised in the vacuole (Vogeli-Lange and Wagner, 1990). Evidence from Ortiz *et al.* (1992) indicates the presence of a specific transporter, designated *hmt1*, of Cd-PC complexes into the vacuole in *S. pombe* cells. *S. pombe* produce LMW-PC-Cd and HMW-PC-Cd-S^{2-} complexes. The presence of S^{2-} imparts a higher binding capacity (reviewed in section 1.2.3.2.1). The *hmt1* gene has been identified as an *in vivo* requirement for the accumulation of HMW-PC-Cd-S^{2-} complex. A Cd-sensitive mutant of *S. pombe*, designated LK100, was isolated which accumulated less HMW-PC-Cd-S^{2-} than the wild type cells. LK100 cells transformed with *hmt1* showed increased accumulation of HWM-PC-Cd-S^{2-}. The amino acid sequence deduced from *hmt1* cDNA suggests that the translation product is similar to ABC (ATP-binding casette)-type membrane transport proteins. ABC-type transporters mobilise proteins and other compounds across membrane barriers and are involved in mediating cellular resistance to a number of toxins (refs cited in Ortiz *et al.*, 1992). Analysis of subcellular fractions derived from *S. pombe* containing an *hmt1-lacZ* fusion indicated that the encoded fusion protein was localised in the vacuolar membrane. Transformed cells over-expressing *hmt1* showed increased Cd-tolerance, suggesting that the hybrid protein was sorted correctly. In addition these cells accumulated more Cd ions when exposed to the metal, further supporting a vacuolar location for the HMT1 protein. Sequences which would act to direct the HMT1 protein to the vacuole were not clearly apparent, but limited similarity of the amino acid terminus of HMT1 to other signal sequences was observed. The authors hypothesise that the LMW-PC Cd complex is transported across the vacuolar membrane where sulphide would be incorporated to form the HMW complex, the LMW complex acting as a Cd-scavenger in the cytoplasm and the HMW complex being involved with Cd storage. The authors suggest that as *S. pombe* strains which over-expressed *hmt1* were more tolerant to Cd than the wild type strain, that HMT1-mediated transport may be the rate limiting step in the formation of the HMW-PC-Cd-S^{2-} complex. Thus it is likely that although synthesis of PCs is essential for detoxification of free Cu

and Cd ions, other factors such as the sequestration of the complexes in the vacuole have a role in the cellular response to supraoptimal concentrations of these metal ions. It was observed that decreased peptide formation in tobacco cells exposed to BSO, while increasing Cd toxicity, did not affect Zn uptake or toxicity (Reese and Wagner, 1987). Thus it is likely that PCs do not have a role in Zn detoxification.

1.2.3.2.4 Possible alternative functions for PCs

Several alternative functions, not directly relating to metal ion detoxification, have been proposed for PCs. This is suggested by the observation that the enzymes involved in their synthesis are constitutively present (Steffens *et al.*, 1986). Thumann *et al.* (1991) reactivated Cu- and Zn-requiring enzymes using *in vitro* reconstituted Cu-PC and Zn-PC complexes and suggested a role in the storage and mobilisation of these ions *in vivo*. The presence of inorganic sulphide in one of the two types of Cd-PC complexes isolated has led to the suggestion that PCs may play a role in sulphur metabolism (Steffens *et al.*, 1986). Structural similarity to glutathione has also led to suggestions that PCs may have analogous roles to glutathione (e.g. detoxification of hydrogen peroxide). Rieger *et al.* (1990) demonstrated that *Vicia faba* root cells could be protected from the effect of both triethylenemelamine and maleic hydrazine (which cause chromatin aberrations in dividing cells) by pre treatment with $NiCl_2$. BSO treatment of the roots prior to treatment with $NiCl_2$ nullified the effect, leading to the hypothesis that PC molecules have a role in the protection of the plant from these compounds. As is the case for MTs, the evidence for a non-detoxification role is indirect. Further research is required to demonstrate whether or not these molecules have a constitutive function in non metal-stressed tissue.

1.3 ENVIRONMENTALLY REGULATED GENE EXPRESSION

The following section aims to review very briefly the literature relating to the known responses of certain plant genes to changes in environmental factors such as extreme temperature, anoxia, water stress, biotic stress and discuss responses to elevated metal concentrations in greater detail. In reviewing metal-related gene expression a discussion relating to the animal and yeast systems is also reviewed as these are, at present, better characterised than plant systems. Specific mention is made of Cd-gene-regulation in the section discussing general metal-gene-regulation in plants. Some cellular responses are co-inducible by different environmental factors, in which case appropriate cross references are used. In particular, aspects of heat shock are co-induced by Cd and these are described in section 1.3.5.3. Table 4 gives a list of known *cis*-acting consensus sequences which are the target sites for environmentally regulated *trans*-acting factors.

TABLE 4

Consensus sequences which act as binding sites for environmentally responsive *trans*-acting factors in plants.

a) n denotes a less strongly conserved nucleotide. These 5bp sequences are arranged contiguously in alternating orientation.

b) obtained from consensus sequence

Abbreviations: SOD, superoxide dismutase; ABA, abscisic acid.

ENVIRONMENTAL STRESS RESPONSE	SEQUENCE	PROMOTER IDENTIFIED IN	TERMED AS	REFERENCE
Annoxia	CCGTGGTTTGCTTGCC CTGCAGCCCCGGTTTC	*Adh*1	Anaeroic regulatory element (ARE)	Walker *et al.*, 1987
Water stress	ACAC..TACGTGGCGG.GC CCGCCGCGCTG	ABA induced genes[b]	Abscisic acid responsive element (ABRE)	Mundy *et al.*, 1990
	ACGTGCGCC CGAGCA	ABA induced genes[b]	Abscisic acid responsive element (ABRE)	Marcotte *et al.*, 1985
Heat shock	nGAAn[a]	Heat shock genes[b]	Heat shock element (HSE)	Xiao and Lis, 1988
Biotic stress	AATATTTTAATT	pea PR1		Datta and Cashmore, 1989
	TCACGTGATGT	parsley PR1		Schulze-Lefert *et al.*, 1989

1.3.1 Response to extreme temperature

The control of the heat shock (HS) response is one of the better categorised environmental responses. In contrast to HS very little information is available on the control of gene expression following exposure to low temperatures.

1.3.1.1 Heat shock

The HS response is typified by the suppression of normal protein synthesis and the induction of new proteins termed heat shock proteins (HSPs). These proteins are classified according to their molecular weight. The major classes of HSP are HSP110, HSP90s, HSP70s, HSP60s and LMW HSPs. The dramatic thermoinducibility of HS genes has been attributed to the presence of specific *cis*-acting elements located in the 5' upstream regulatory regions of the genes. The elements have been designated heat-shock consensus elements (HSEs) (Schoffl *et al.*, 1984). The HSE was originally identified in *Drosophila* HSP70 as 5' CTnGAAnnTTCnAG 3' (Pelham *et al.*, 1982). This has more recently been described as arrays of variable numbers of the 5bp sequence nGAAn arranged in alternating orientation (Xiao and Lis, 1988). The n denotes less strongly conserved nucleotides that may nevertheless play an important role in protein binding interactions.

The HSE was shown to be a binding site for heat-shock transcription factor (HSF) which has a central role in activation of transcription (Goldenberg *et al.*, 1988). The first demonstration that plants also use the same mode of transcriptional activation came when the *Drosophila* HSP70 promoter was shown to direct heat inducible transcription in transgenic tobacco (Spena *et al.*, 1985). It appears that a single HSE is sufficient to confer HS activation on plant genes (Wing *et al.*, 1989). Soybean has since been shown to have HSP promoter sequences which confer thermoinducibility in transgenic tobacco (Strittmatter and Chua, 1987).

A gene encoding a heat shock factor was first isolated from *S. cerevisiae* (Wiederrecht *et al.*, 1988). S. *cerevisiae* heat shock factor (Sc-HSF) and *Drosophila* HSF (D-HSF) associate to form protein trimers in solution and when bound to DNA (Perisic *et al.*, 1989; Sorger and Nelson, 1989). Each subunit of D-HSF multimer is thought to bind to a single nGAAn unit. The binding to successive units (Xiao *et al.*, 1991) and the binding of trimers to adjacent sites is highly cooperative (Shuey and Parker, 1986). Despite the strong conservation of the heat shock element sequence across species boundaries, heat shock factor proteins from different species have only limited sequence similarity. This similarity is confined to the DNA binding domain and the region that is involved in trimerisation (Sorger, 1991). Tomato cells contain two proteins shown to have similarity to the originally isolated HSF from *S. cerevisiae* (Sorger, 1991).

In *Drosophila* and humans, DNA binding of HSF and transcriptional activation is enhanced by HS (Czarnecka *et al.*, 1990; Goldenberg *et al.*, 1988). In animal systems there is evidence that phosphorylation of HSF creates a complex with high transcriptional activity (Laron *et al.*, 1988). There is no evidence available to suggest a similar role for phosphorylation of plant HSF. It has been speculated that following activation of the genes, HSPs act in an autoregulatory manner. When *Drosophila* cells are gradually raised to high temperatures, allowing them to accumulate HSPs, HSP synthesis during recovery is repressed far more strongly than in the recovery period of cells exposed rapidly to the same high temperatures (Didomenico *et al.*, 1982). Treatment with various agents which inhibit the synthesis and accumulation of HSPs in soybeans also delays their repression during recovery (Gurley and Key, 1991). HSP70 is currently thought to be the most important HSP involved with autoregulation. For a given heat shock a specific quantity of HSP70 is always produced before repression begins (Didomenico *et al.*, 1982).

The HSF has been shown to activate the transcription of a yeast metallothionein (MT) gene. The ACE1 gene product is the Cu-dependent activator of CUP1. In attempts to identify other genes involved in transcriptional CUP1 activation a mutant (ADS) was isolated which was found to have a single-base change in the HSF gene. The mutation changed a valine codon to an alanine codon in the DNA-binding domain of HSF. This mutation suppressed the requirement for ACE1 in the activation of CUP1 transcription and reduced transcription of the SSA 3 gene, a member of the yeast HSP70 gene family. These results were taken to indicate that MT transcription is under HSF control and that MT synthesis is important in response to heat shock (Silar *et al.*, 1991).

1.3.1.2 Exposure to low temperatures

It has been shown that synthesis of novel mRNA species can be induced within hours to a day after exposure to low temperatures (Cattivelli and Bartels, 1989). Some mRNAs decline following exposure to low temperatures (Hahn and Walbot, 1989).

It has been established that cold acclimation leads to the accumulation of ABA (Chen *et al.*, 1983) as does water stress (Greelman and Zeevart, 1985). In addition, a subset of proteins accumulated in response to cold acclimation are also induced by ABA (Guy, 1990). Rice seedlings exposed to low temperatures accumulate large quantities of the *Rab* transcript (Hahn and Walbot, 1989), a gene induced by ABA or desiccation (Mundy and Chua, 1988). The induction of this gene is discussed in the following section (1.3.2).

1.3.2 Water stress

Abscisic acid (ABA) has long been known to accumulate in leaves as a result of water stress (Wright, 1969). Water stress and ABA have been shown to induce a protein of Mr 26,000 designated osmotin (Singh *et al.*, 1989) and a glycine rich protein of Mr 16,000, designated Rab 21 (Mundy and Chua, 1988). The precise role of these two proteins has not yet been established.

An abscisic acid responsive element (ABRE) has been identified in the Em gene of wheat. Em is a major protein of the mature wheat embryo that begins to accumulate in the embryo by 21 days post-anthesis (Williamson *et al.*, 1985). Using hybrid gene constructs two functional fragments of the Em 5' region; an ABRE from -152 to -103 and an element between +6 and +86, have been identified that quantitatively increase the ABA response. Comparison of the two regions to other ABA-regulated genes led to the identification of two motifs which may be ABRE core sequences (Marcotte *et al.*, 1989). These two motifs are ACGTGCGCC and CGAGCA (Table 4).

An ABRE has also been identified in the upstream region of the *Rab* gene. Transcriptional elements between -294 and -52 of the *Rab* gene are sufficient to confer ABA-dependent expression on the chloramphenicol acetyl transferase (CAT) reporter gene in rice protoplasts. Gel retardation and DNase 1 experiments show nuclear factors binding to regions of this sequence. The motifs ACAC..TACGTGGCGG.GC and CCGCCGCGCTG present in this sequence have been shown to be present in other *rab* genes and cotton genes induced by ABA, and these may act as ABREs (Mundy *et al.*, 1990) (Table 4).

1.3.3 Anaerobic response

Exposure of maize seedlings to anoxia leads to the synthesis of approximately 20 new proteins (Sachs *et al.*, 1980). In the regulation of gene expression the most compelling evidence for the identity of anaerobic elements comes from investigations using transformed maize protoplasts. Studies using deletion constructs of the alcohol dehydrogenase (*adh1*) promoter indicate that a 40-base-pair (bp) DNA sequence within the *Adh*1 promoter is required for anaerobically regulated expression of the hybrid gene. Clustered point mutations in the sequence show that it is composed of two essential regions each 16bp separated by a 10bp sequence. Attachment of this 40bp element to an unrelated promoter is sufficient to confer inducibility in an anaerobic environment. The sequence of this *Adh*1 40bp element designated anaerobic response element (ARE) is:

CTGCagCCCcGGTTTCgcAagcCGcgCCGtGgtTTgCTTgCC

Underlined sequence denotes the two 16bp motifs. In the above sequence upper case denotes identical nucleotides, lower case denotes nonhomologous nucleotides between ARE and the *Adh*2 gene (Walker *et al.*, 1987). Sequences homologous to ARE have been identified in the *Adh*1-like gene from pea (Llewellyn *et al.*, 1987).

1.3.4 Biotic stress

Pathogenesis related proteins (PRs) have been shown to be induced by both biotic (Lotan and Fluhr, 1990; Casacubetra *et al.*, 1991) and abiotic (Antoniw and White, 1981; Brederode *et al.*, 1991) elicitors.

The basic PR1 gene upstream region contains several consensus sequences which may participate in protein binding. The AT-1 binding site identified in several light regulated genes has a similar (11 out of 12 matches) motif 750 bp upstream in the PR1 gene, AATATTTTAATT (Datta and Cashmore, 1989). There is also an element, TCACGTGATGT, 177bp upstream which displays high homology to a light responsive chalcone synthase gene region shown to be protected in DNA footprinting studies (Schulze-Le Fert *et al.*, 1989). The internal motif CACGTG is conserved in a further two specifically protected motifs. The same core motif is identical to the core of the G-box sequence, a protein binding motif. This core motif is also found in other plant genes involved in defence against pathogens, including the phenylalanine ammonia lyase (PAL) genes, central in the biosynthesis of some phytoalexins (Dron *et al.*, 1988) and the wound induced proteinase inhibitor II gene from potato (Keil *et al.*, 1986).

1.3.5 Metal-regulated gene expression

Metallo-regulation of yeast and higher eukaryotic metallothionein genes has been extensively studied. The available information is reviewed here as an archetype for eukaryotic metal-gene-communication.

1.3.5.1 Metal-gene communication: Metallothionein as the animal and yeast model system

Metal regulatory elements (MREs) have been identified in the 5' regulatory regions of animal MT genes. The mouse MT-1 promoter contains six MREs designated MREa to MREf. As stated in section 1.2.3.1.1, a notable feature of all MTs is their inducibility by metals. The metals Cd, Zn, Cu, Hg, Au, Co, Ni and Bi have all been shown to induce MTs (Palmiter, 1987). In addition, induction of MTs can be achieved by hormones, cytotoxic chemicals and physiological conditions associated with physical or chemical stress (Palmiter, 1987).

Comparison of the sequences of the metal regulatory elements (MREs) of animal MT genes and examination by point mutation of their function has identified the core MRE to be 5' TGCRCNCX 3' (where R represents G or A, X represents G or C and N can be any base except A) (Palmiter, 1987) (Table 5). These MREs are thought to be acted upon by positive transcription factors when activated by the appropriate metal ion (Seguin and Hamer, 1987).

In *S. cerevisiae* the MT gene is CUP1. Expression of this gene confers Cu-resistance on the cells (Butt and Ecker, 1987). It has been demonstrated that the CUP1 protein binds 8 Cu atoms per polypeptide in a trigonal arrangement through cysteine thiolates in a Cu-S polynuclear cluster (Winge *et al.*, 1985). Both Cu and Ag have been shown to induce CUP1 transcription (Karin *et al.*, 1984; Furst *et al.*, 1988). The -105 to -230 region of the gene has been demonstrated to be important in Cu-inducibility and has been designated the CUP1 Upstream Activation Sequence (UAS_{CUP1}) (Thiele and Hamer, 1986). Two Cu-sensitive strains have been isolated, these strains were designated *ace1* and *cup2* (Thiele, 1988; Welsh *et al.*, 1989). The two recessive mutations were shown to be allelic (Buchman *et al.*, 1989). Further investigations have shown that cells which have defective ACE1\CUP2 genes lack UAS_{CUP1} binding activity (Welsh *et al.*, 1989). ACE1 protein has 12 cysteine residues present within the N-terminal 105 amino acids (Szczypka and Thiele, 1991) which are arranged in Cys-X-Cys or Cys-X_2-Cys configurations (Winge *et al.*, 1985). This region has been shown to be important in Cu-activated DNA binding capacity (Furst and Hamer, 1989). Investigations have shown that Cu ions activate ACE1 by altering the conformation of the protein and hence its DNA binding activity. It was demonstrated that this conformational switch occurs in an all-or-none highly cooperative fashion and that although Ag(I) also activates ACE1 it acts in a less cooperative manner than Cu (Furst and Hamer, 1989). Characterisation of the DNA binding region of ACE1 has shown that the ACE1 DNA binding structure is based on a polynuclear Cu(I)-cysteine thiolate cluster, termed a Cu-fist. This Cu-fist consists of 6 or 7 Cu(I) ions coordinated to cysteine thiolates in a triagonal geometry. It is this Cu-fist that organises and stabilises the conformation of the N-terminal domain of the transcription factor for specific DNA binding (Dameron *et al.*, 1991). The binding site for ACE1 bears no resemblance to the core sequence of higher eukaryotic MREs (Table 5).

Table 5

Consensus MRE sequence and the ACE1 binding region

	SEQUENCE	REFERENCE
MRE	TGCRCNCX[a]	Palmiter, 1987
ACE1	TCY(4-6)GCTG[b]	Theile, 1992

a) R can be G or an A, X can be C or G b) Y can be a C or T

Candida glabrata contains a family of MT genes which are only activated by Cu and Ag (Mehra *et al.*, 1989, 1990). Investigations have revealed that *C. glabrata* contains a gene (AMT1) that encodes a 265 amino acid protein which may activate MT-1 and MT-2 gene transcription *in vivo*. AMT1, similar to ACE1, has 11 cys residues within the 105 amino acid N-terminal region (Zhou and Thiele, 1991).

In the mouse MT-1 gene promoter an MRE, designated MREd, is both a functional Sp1 binding site and a binding site for a Zn-activated factor from HeLa cells designated MTF-1 (Westin and Schaffer, 1989). A specific nuclear protein which binds to MREd of the mouse gene encoding MT-1 has also been shown to require Zn^{2+}. The chelating agents EDTA and 1,10-phenanthroline inactivated DNA binding activity. This activity was restored by Zn^{2+} but not by Cd^{2+} (Seguin, 1991). Searle (1990) has reported a Zn-dependent factor found in rat liver cells which binds to the mouse MREs, MREa and MREd, designated Zn-activated protein (ZAP) which, it is suggested, is equivalent to MTF-1. A mouse nuclear factor of Mr 108,000, designated metal element protein 1 (MEP-1), has been shown to bind to the MREd of the mouse MT-1 gene (Seguin and Prevost, 1988). The binding affinity of MEP-1 to MREd has been shown to be greater than to other mouse MREs, MRE4 of the human MT-IIA gene and MREa of the trout MT-B gene (Labbe *et al.*, 1991). A mouse nuclear factor of Mr 74,000, purified to near homogeneity and designated metal response element binding factor 1 (MBF-1), can bind to the MREa element of the trout MT-B gene in addition to MREe and MREd of the mouse MT-1 gene (Imbert *et al.*, 1989).

1.3.5.1.1 MT localisation at S-phase and its interaction with Zn-fingers

Zeng *et al.* (1991a; 1991b) were able to inhibit the DNA binding of the Zn-dependent transcription factors Sp1 and TFIIIA *in vitro* by the addition of thionein (apo-metallothionein), thus abolishing transcription activation in an *in vitro* assay. By contrast, transcription factor Oct-1 which binds DNA via a homeo-domain i.e. a helix-turn-helix motif not involving Zn ions, was refractory to thionein action. These authors postulate that similar activity *in vivo* could potentially act as a control mechanism for modulation of a large subset of genes whose transcription is activated by Zn-dependent transcription factors. The involvement of metallothionein (MT) in gene regulation is inferred by recent studies showing a change in the subcellular localisation of MT in cultured rat hepatocytes. A clear shift from cytoplasm to the nucleus was observed by immunofluorescence using anti-rabbit MT antisera. This change in localisation was shown to occur in early S-phase.

1.3.5.2. Metal-regulated gene expression in plants

Regulation of the metalloproteins plastocyanin and ferritin and the metalloenzyme superoxide dismutase are discussed below. In addition, the induction of pathogenesis related proteins (PRs) by mercuric chloride is reviewed. These compounds are usually associated with responses to biotic stress. The regulation of the induction of these two protein classes is described in section 1.3.5.2.4. The effect of Cd on gene-regulation is discussed in section (1.3.5.3).

1.3.5.2.1 Plastocyanin

Plastocyanin is a Cu-protein of Mr 10,500 (Katoh, 1960) whose expression is regulated by the availablity of Cu in the medium (Wood, 1978).

Some cyanobacteria and some green algae are capable of producing both plastocyanin and a c-type cytochrome (c552, c553) depending on the Cu concentration of the medium. Plastocyanin is expressed under the Cu-sufficient condition and cytochrome c under the Cu-deficient condition. In the green alga *Scenedesmus actus,* synthesis of plastocyanin appears to be regulated at the post-translational level (Bohner *et al.*, 1981). In *Chlamydomonas reinhardtii,* it has been demonstrated that c552 expression is repressed by Cu at the transcriptional level in Cu-sufficient cells (Merchant and Bogorad, 1987; Merchant *et al.*, 1991) while the level of plastocyanin is controlled at the post-translational level (Merchant and Bogorad, 1986a, 1986b).

1.3.5.2.2 Superoxide dismutase

Superoxide dismutases (SODs) are metalloproteins that catalyse the conversion of O^{2-} to H_2O_2 and O_2. Three SOD isoenzymes have been discovered, containing either Cu and Zn, Mn or Fe (Malstrom *et al.*, 1989).

In *S. cerevisiae* the transcription of both Cu,Zn-SOD and MT genes was induced by Cu and Ag in wild type cells, but not in mutants lacking ACE1. Both metals have been shown to activate ACE1 mediated MT transcription (Karin *et al.*, 1984, Furst *et al.*, 1988). It was concluded that the transcriptional level of Cu,Zn-SOD is co-regulated with MT (Carri *et al.*, 1991). Gralla *et al.* (1991) have demonstrated that Cu activated ACE1 binds to a single site in the promoter of the *S. cerevisiae* gene encoding Cu,Zn-SOD. A major Cu-induced protein which cross-reacted with antibodies against cytosolic Cu,Zn-SOD from spinach has been isolated from soybean. The N-terminal 40 amino acids of this protein were found to be 70 % and 62.5 % homologous to sequences of Cu,Zn-SOD from rice and tomato respectively. It was concluded from these studies

that the induction of SOD by such treatment may be the result either of a direct effect of Cu on the gene for SOD or of an indirect effect via an increase in levels of O^{2-} (Chongpraditnum *et al.*, 1992).

1.3.5.2.3 Ferritin

Ferritin is a protein found in eukaryotes which sequesters Fe for subsequent metabolic utilisation (Thiel, 1987). Regulation of ferritin gene expression by Fe was first observed by Drysdale and Munro (1966). The mechanism in animals is mainly post transcriptional, involving ferritin mRNA storage and translational competition. Analysis of ferritin mRNA untranslated regions (UTR) has led to the identification of a conserved 28-nucleotide region in animal ferritin mRNAs, termed the iron responsive element (IRE) (Thiel, 1990). IREs have also been identified in the 3' UTR of transferrin receptor (TfR) involved with Fe uptake (Casey *et al.*, 1989). The IRE is known to bind a regulatory protein (Walden *et al.*, 1988; Brown, *et al.*, 1989; Dix *et al.*, 1992).

In plants, transcriptional control seems to play a more important role in regulation of ferritin expression. In bean leaves excess iron increased the concentration of both mRNA and protein (Vandermark *et al.*, 1983). A 50-60 fold increase of ferritin protein is accompanied by an equivalent accumulation of hybridisable ferritin mRNA and by increased transcription of ferritin genes in soybean (Lescure *et al.*, 1991).

1.3.5.2.4 Pathogenesis related proteins

Pathogenesis related (PR) proteins have been shown to be induced following exposure to mercuric chloride. These molecules are normally associated with the biotic response (section 1.3.4). Bean leaves treated with mercuric chloride showed the novel synthesis of proteins designated PR1-4. Three of these appear to be serologically identical to PR proteins induced by alfalfa mosaic virus (AMV) (de Tapia *et al.*, 1988).

1.3.5.3 Cd-regulated gene expression

As decribed by Hirt *et al.* (1988) exposure of *N. tabacum* cultures to Cd led to an increase in transcription. The authors proposed that this may be due to the alteration of DNA binding by specific transcription factors (section 1.1.1.4). It has been established that Cd can substitute for Zn in the three classes of metal-requiring transcription factor proposed by Vallee *et al.* (1991) (sections 1.1.1.4.1 to 1.1.1.4.3). Such alteration of binding activity effected by Cd substitution

could cause a change in the pattern of expression of numerous genes.

De novo PC synthesis can be detected in metal tolerant *D. innoxia* cultures within 5 min of Cd exposure. This rapid synthesis occurs even in cultures which have been treated with the protein synthesis inhibitor cyloheximide. When protein synthesis was inhibited 99.5 %, synthesis of PC was only reduced 2-fold (Robinson *et al.*, 1988). Enzymes required for the synthesis of PCs and its biosynthetic precursors must be present constitutively, at least at low levels, in the absence of toxic metal ions.

At present there is no evidence of Cd-induced expression of MT-like genes in plants, indeed no change in transcript abundance was observed following exposure of *M. guttatus* to either Zn or Cd (de Miranda *et al.*, 1990).

Cd co-induces the HS response. Czarnecka *et al.* (1984) demonstrated that exposure of soybean seedlings to 500 μM Cd induced a set of polyA$^+$ RNAs to a similar level as HS. The induction of the HS polyA$^+$ RNAs was far more pronounced following exposure to Cd than exposure to other metals, water stress, anaerobiosis and high concentrations of hormones. Only exposure to arsenate resulted in induction of transcripts to as great a degree as HS. The transcriptional control of the HS response is detailed in section (1.3.1.1).

1.4 KNOWN EFFECTS OF Cd ON *D. innoxia* CELLS

D. innoxia cells from suspension cultures have been selected for their ability to grow and divide rapidly in normally lethal concentrations of Cd. A concentration of 12.5 μM Cd which is toxic to sensitive *D. innoxia* cells (survival frequency 1 in 10^{-2} cells) was used to select Cd-resistant callus. Callus resistant to this concentration was replated at the same Cd concentration. Callus from this second plating was used to establish suspension cultures. The Cd concentration was increased in a stepwise manner and resulted in cultures capable of rapid growth in medium containing 12.5, 25, 50, 100, 120, 160 and 250 μM Cd. *D. innoxia* cells grow in clumps in suspension culture, therefore to ensure a suspension culture was not derived from a genetically mixed population of cells, the selected Cd-resistant cultures were converted to protoplasts and individual protoplasts used to initiate new suspension cultures (Jackson *et al.*, 1984). The *D. innoxia* cells which showed sustained resistance to the highest Cd concentration, 250 μM Cd, were selected by growing for a number of cell generations at 300 μM Cd (Personal communication). This cell line was designated Cd300 and the wild type cell line from which it had been derived was designated WDI.

Several of the biochemical responses of *D. innoxia* cells to Cd are well characterised. Resistance of isolated cultures to Cd was correlated with the synthesis of low molecular weight,

cysteine rich, Cd-complexes, shown to be PCs (Jackson *et al.*, 1987). Induction of these compounds was detected within five minutes of exposure to Cd (Robinson *et al.*, 1988). There was direct correlation between the maximum accumulation of the γ(EC)nGs and the concentration of Cd to which the cells were resistant. The γ(EC)nGs form multimeric aggregates in the presence of metal ions. Cd binds to both the high and low molecular weight aggregates. In contrast, Cu binds preferentially to the higher molecular weight forms (Jackson *et al.*, 1987). The effect of Cd on γ(EC)nG synthesis and formation of γ(EC)nG-Cd complexes has been compared in the Cd300 cell line and the WDI cell line. WDI cells are sensitive to exposure to 250 μM Cd, however the synthesis of γ(EC)nG during the initial 24 h exposure was the same as that in Cd300 cells. However, the WDI cells formed γ(EC)nG-Cd complexes later than the Cd300 cells and the peptides forming the complexes in the sensitive line appeared to be smaller than those of the tolerant cell line and did not bind all the cellular Cd (Delhaize *et al.*, 1989a).

In vivo labelling experiments have shown that Cd induces the synthesis of a similar range of proteins in both cell lines even at concentrations toxic to the WDI cells. *In vitro* translation of polyA$^+$ RNA identified two abundant mRNAs in both Cd-exposed and non-exposed Cd300 cells which encode proteins of Mr approximately 11,000. These two mRNAs are absent, or present at low levels, in the WDI cell line. Some mRNAs are Cd300 or WDI specific. A subset of the Cd-induced transcripts (and hence transcription products) is co-induced by exposure to an elevated temperature of 12^{o}C (42^{o}C incubation) for 4 h, a number are heat shock specific and some transcripts are specifically induced by Cd and not heat shock. No difference was detected in the ability of the two cell lines to tolerate heat shock (Delhaize *et al.*, 1989b). These observations indicate that it should be possible to isolate genes which respond exclusively to Cd and are differentially regulated in Cd300 compared to WDI cells.

1.5 AIMS

The initial aim of the research was to identify proteins which were induced by Cd in *D. innoxia* using two dimensional polyacrylamide gel electrophoresis (2D PAGE). It was to be established if Cd-induced peptides were solely induced by Cd or whether such proteins were co-induced by other stimuli, including other metals and HS. The differential synthesis of proteins in the two cell lines of *D. innoxia*, Cd-tolerant Cd300 and non-tolerant WDI, following Cd-exposure was to be examined.

A second aim was then to sequence Cd-induced proteins and construct degenerate oligonucleotide primers from this sequence. The use of 2D PAGE to identify individual polypeptides has now been coupled with protein microsequencing to allow amino acid sequence to be determined from small quantities of individual peptides. Peptides often prove refractory to

Edman degradation due to the N-terminus being blocked. To overcome this the peptides can be cleaved and the cleavage products further purified using either electrophoretic methods developed to separate small peptides, such as tricine gels (Shagger and Von Jagow, 1987) or high pressure liquid chromatography (HPLC).

Primers designed from the protein sequence would then be used to amplify the corresponding DNA sequences using the polymerase chain reaction (PCR). These DNA sequences could be used to probe genomic libraries to isolate the regulatory regions of the genes.

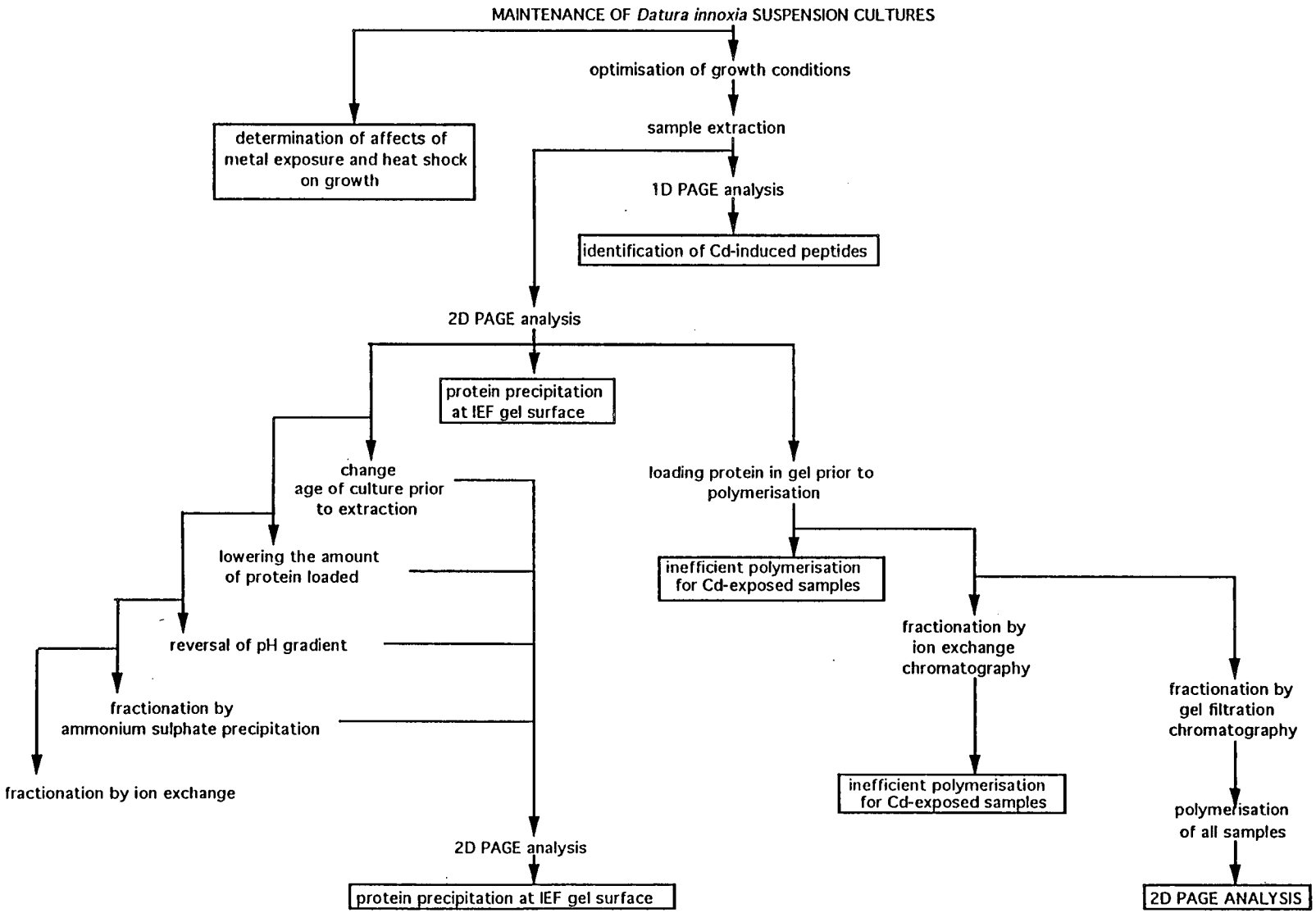
If the procedure outlined above proved unsuccessful then it was planned to use an alternative approach of screening a cDNA library. Constructing a cDNA library in λZAP II would allow screening with either antibodies or DNA probes since levels of gene expression and vector copy number are sufficiently high for the detection of either the insert DNA or its encoded protein using this vector. If antibodies could not be used then a differential screen could be carried out using probes constructed from RNA extracted from Cd-exposed cells and non-exposed cells.

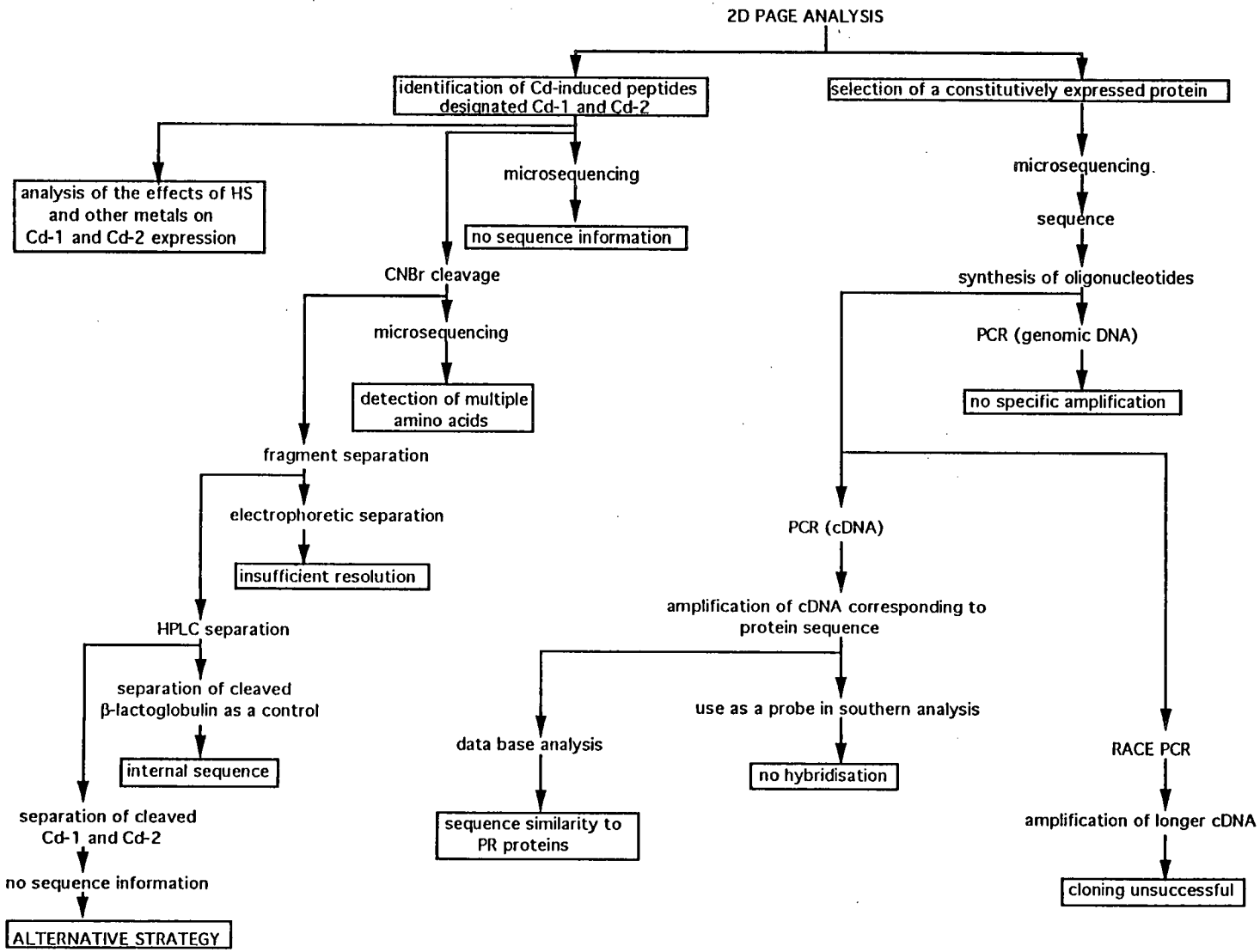
cDNA isolated from a library would be characterised and used to screen a genomic library to isolate the corresponding gene. An isolated gene could be sequenced, searched for an open reading frame (ORF) and the predicted product compared to databases in an attempt to find similar proteins. Upstream regions could be searched for any putative consensus *cis*-acting regulatory sequences with a longer term aim of using such sequences in deletion construct studies.

The following flow diagram represents the progress made towards these objectives and hence summarises the work presented and discussed in the following chapters (Figure 3).

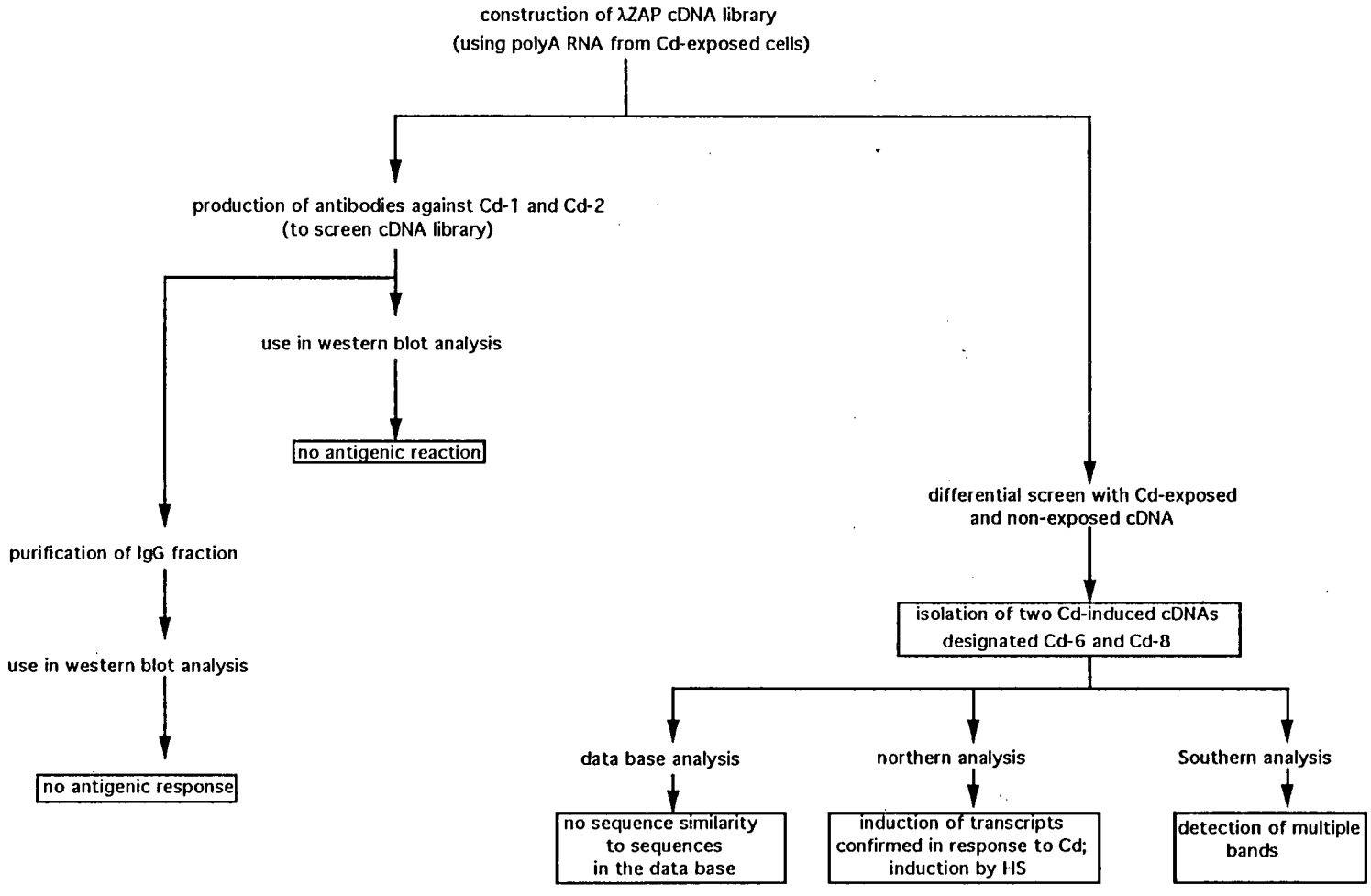
FIGURE 3

A flow diagram summarising the work carried out


MAINTENANCE OF *Datura innoxia* SUSPENSION CULTURES
optimisation of growth conditions
determination of affects of metal exposure and heat shock on growth
sample extraction
1D PAGE analysis
identification of Cd-induced peptides
2D PAGE analysis
protein precipitation at IEF gel surface
change age of culture prior to extraction
loading protein in gel prior to polymerisation
lowering the amount of protein loaded
inefficient polymerisation for Cd-exposed samples
reversal of pH gradient
fractionation by ion exchange chromatography
fractionation by gel filtration chromatography
fractionation by ammonium sulphate precipitation
fractionation by ion exchange
inefficient polymerisation for Cd-exposed samples
polymerisation of all samples
2D PAGE analysis
protein precipitation at IEF gel surface
2D PAGE ANALYSIS


2D PAGE ANALYSIS
identification of Cd-induced peptides designated Cd-1 and Cd-2
selection of a constitutively expressed protein
analysis of the effects of HS and other metals on Cd-1 and Cd-2 expression
microsequencing
no sequence information
CNBr cleavage
microsequencing
detection of multiple amino acids
fragment separation
electrophoretic separation
insufficient resolution
HPLC separation
separation of cleaved β-lactoglobulin as a control
internal sequence
separation of cleaved Cd-1 and Cd-2
no sequence information
ALTERNATIVE STRATEGY
microsequencing
sequence
synthesis of oligonucleotides
PCR (genomic DNA)
no specific amplification
PCR (cDNA)
amplification of cDNA corresponding to protein sequence
data base analysis
sequence similarity to PR proteins
use as a probe in southern analysis
no hybridisation
RACE PCR
amplification of longer cDNA
cloning unsuccessful

ALTERNATIVE STRATEGY



CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

All chemicals and biological reagents were from Sigma Chemical Company Ltd or BDH Ltd, both Poole, Dorset, UK with the exception of those listed below.

Restriction endonucleases, DNA modifying enzymes, X-gal and IPTG were from either Northumbria Biologicals Ltd or Boehringer Mannheim.

Agarose was from Bethesda Research Laboratories (UK) Ltd.

Bacteriological agar and yeast extract were from Oxoid Ltd.

Trypticase peptone (tryptone) was from Becton Dickinson Microbiology Systems, Cockeysville, USA.

Sodium chloride was from Reidel de Haen.

Phenol (redistilled) was from International Biotechnologies, Inc., Newhaven, Connecticut, USA.

The reagent for the protein micro-assay was from Bio-Rad Laboratories, Herts., UK.

Nitrocellulose and nylon filters were from either Schleicher and Schuell or Amersham International plc.

3MM chromatography paper was from Whatman, Maidstone, Kent, UK.

All reagents used in HPLC procedures were supplied by Rathburns, Walkerburn, UK.

Taq polymerase was supplied by Promega, Madison, USA.

[α-^{35}S]dATP (1000 Ci m mol^{-1}), [γ-^{32}P]ATP (3000Ci m mol^{-1}), [α-^{32}P]dCTP (400Ci m mol^{-1}) and nylon membrane (Hybond N) were obtained from Amersham International, Aylesbury, UK.

dNTPs, oligo dT primer$_{15}$, RNase inhibitor and AMV reverse transcriptase were obtained from Boehringer Mannheim, Sussex, UK.

β-lactoglobulin (sequencing grade) was supplied by Applied Biosystems, Warrington, UK.

Water used in all procedures was distilled, double deionised (MilliQ) water.

2.1.1 *Escherichia coli* strains

The *E. coli* strains used during the course of this work were, JM101 Δ(*lac-proAB*), *supE, thi-1*, F'(*traD36, proAB*$^+$, *lacI*^{q}, *lacZ*Δ*M15*) (Yanisch-Peron *et al.*, 1985); SURE, *mcrA,* Δ*(mcrBC-hsdRMS-mrr)171, supE44m thi-1,* λ-, *gyrA96, relA1, lac, recB, recJ, sbcC,*

umuC::Tn5(kan^r), uvrC, [F', *proAB, lacI^q, lacZΔM15*, Tn*10, (tet^r)*] (Stratagene) and XL1-Blue, *gyrA96, endA1, recA1, thi-1, hsdR17, supE44, relA1, lac*, [F', *proAB, lacI^q, lacZΔM15*, Tn*10, (tet^r)*] (Stratagene).

2.1.2 Plasmids

The plasmids used during the course of this work were, pUC19 (amp^R) (Vieira and Messing, 1982) and pBluescript (amp^R, with T7 promoter) (Stratagene).

2.2 METHODS

2.2.1 Growth conditions

2.2.1.1 Growth conditions for *E. coli*

Bacterial strains were routinely grown in L-broth or L-agar (1 % (w/v) Bactotryptone, 5 % (w/v) Tryptone, 85.6 mM NaCl (for L-agar 1 % of agar was added)) at 37 ^oC. For selection of *E. coli* harbouring plasmids which encoded antibiotic resistance, the appropriate antibiotic was added to the media as described by Sambrook *et al.* (1989).

Strains of *E. coli* harbouring F' plasmids were maintained on minimal agar plates lacking proline in order to select for the presence of the plasmid:-

Agar, 1.3 % (w/v), total volume of 768 ml, was autoclaved and cooled and made to 1x M9 salts, 2 mM $MgSO_4$, 100 μM $CaCl_2$, 1 mM thiamine and 0.2 % (w/v) glucose.
(5x M9 salts are 451 mM $Na_2HPO_4.7H_2O$, 110 mM KH_2PO_4, 44 mM NaCl, 93mM NH_4Cl),

2.2.1.2 Growth conditions for *Datura innoxia*

Suspension cultures of *D. innoxia* were maintained in the dark as 40 ml batch suspensions in 250 ml baffled Duran flasks. Cells were grown on a gyratory incubator (Infors-AG) at 120 rpm and 30 ^oC in medium based on Gamborg's 1B5 medium (Gamborg *et al.*, 1968). The *D. innoxia* suspension cultures were maintained on a 96 h cycle. After that time the cells were subcultured; 10 ml of culture were added to 30 ml of fresh media. The composition of the medium was:

Macronutrients	mM
KNO_3	247
$CaCl_2.2H_2O$	1.0
$MgSO_4.7H_2O$	1.0
$(NH_4)_2SO_4$	1.0
NaH_2PO_4	1.2

Vitamins and hormones	μM	Micronutrients	μM
Nicotinic acid	12.2	KI	452
Pyridoxine HCl	486	H_3BO_3	48.5
Thiamine HCl	889	$ZnSO_4.7H_2O$	6.96
Riboflavin	266	$Na_2MoO_4.2H_2O$	103
Ascorbic acid	255	$CuSO_4.5H_2O$	0.10
Calcium panthothenate	419	$CoCl_2.6H_2O$	0.10
Biotin	409	Na_2EDTA	100
Hypoxanthine	1.84	$FeSO_4.7H_2O$	96.7
Myo-Inositol	768		
Choline Chloride	71.6		

Other constituents

Casamino acids	0.1 % (w/v)
Sucrose	5.84 mM
2,4-D	0.45 μM

All solutions were made to a final volume with freshly drawn deionised distilled water. The final media was autoclaved.

2.2.2 *D. innoxia* growth analysis

The growth of *D. innoxia* suspension cultures was monitored by measuring packed cell volumes (PCVs). Every twelve hours 2.5 ml of suspension culture was removed and put into graduated centrifuge tubes, cells pelleted at 1600 g for 1 min (MSE Centaur 2 centrifuge) and the PCV recorded. At 96 h the cells were subcultured and one more PCV measurement taken from the original culture at 120 h. No more measurements were then taken as the remaining volume of culture was too small to allow further sampling. The growth of the new culture was monitored in the same manner.

2.2.2.1 Growth analysis of *D. innoxia* exposed to metals and heat shock

Cultures were exposed to a particular treatment, exposure to Cd, Cu, Zn or heat shock (HS), at 48 h. Following a treatment the cultures were replaced in their normal growth conditions. Those cultures which had been exposed to elevated metal concentrations were transferred to sterile tubes (Falcon, 2098) and the cells pelleted by centrifugation at 800 g for 5 min (MSE Centaur 2) after which the supernatant was discarded and the cells resuspended in 40 ml of medium. This was repeated and the resuspended cells returned to new sterile flasks. All manipulations were carried out in a sterile environment. Cultures exposed to HS were immediately returned to an incubator set to the correct temperature. All control cultures were treated in an identical manner to their sister culture which had been exposed to a particular stress. At 96 h four individual PCV measurements were taken as described in section 2.2.2 and mean values were calculated.

2.2.3 Isolation of nucleic acids

2.2.3.1 DNA purification from *D. innoxia* cells

Two 40 ml *D. innoxia* suspension cultures at 48 h in their growth cycle were placed in sterile tubes (Falcon, 2098) and the cells pelleted by centrifugation at 800 g for 5 min (MSE Centaur 2). The cells were then transferred to a sterile mortar and pestle and ground under liquid N_2. This was transferred back to two fresh sterile tubes (Falcon, 2098) with 10 µl of β-mercaptoethanol, 10 ml of boiling extraction buffer (1.4M NaCl, 100 mM Tris pH 8.0, 20 mM EDTA) and 20 ml of PCA (10 % (v/v) extraction buffer, 2 % (v/v) isoamylalcohol, 48 % (v/v) chloroform, 40 % (v/v) phenol). The tubes were inverted to mix the contents prior to centrifugation at 800 g for 10 min. The upper phase was collected and PCA extraction repeated. The aqueous phase was then extracted three times with an equal volume of chloroform/isoamylalcohol (24:1) which was mixed with the sample by inversion. The aqueous phase was separated by centrifugation at 800 g for 10 min. Total nucleic acids were precipitated by addition of 0.2 volumes of 5 M ammonium acetate and 2.5 volumes of ethanol and incubation at -80 ^{o}C for 1 h. The nucleic acids were pelleted at 14,000 g for 30 min (MSE high speed 18). The pellet was washed with 100 % ethanol and then dried under vacuum. The nucleic acid pellet was resuspended in 5 ml of H_2O. The sample was weighed and 0.94 x weight of caesium chloride was dissolved in the sample. 100 µl of ethidium bromide (10 mg ml^{-1}) was added to every 3 ml of sample. The sample was placed in two 5 ml "quick seal" ultracentrifuge tubes (Beckman) and balanced with 1 mg ml^{-1} ethidium bromide, 5.7 M CsCl. Tubes were sealed, placed in a rotor (Beckman VTI65) and centrifuged at 230,000 g (r_{ave}) for approximately 17 h (Sorval OTD65B). After centrifugation, tubes were observed under UV and the DNA band

removed. Ethidium bromide was removed from the sample by extraction with CsCl saturated isopropanol until the sample was clear. The CsCl was removed by dialysis (Sambrook *et al.*, 1989) and the DNA sample was ethanol precipitated as described in section 2.2.5.4. The DNA was resuspended in sterile H_2O.

2.2.3.2 RNA purification from *D. innoxia* cells

Total nucleic acids were extracted from *D. inoxia* cells as described in section 2.2.3.1. All solutions were treated with diethylpyrocarbonate (DEPC) as described by Sambrook *et al.* (1989). Solutions containing Tris were made in DEPC treated water and filter sterilized. All bottles used to store solutions were baked at 180 °C overnight. Following precipitation of total nucleic acids the pellet was resuspended in 9.25 ml 4 M guanidine thiocyanate. 3.5 ml of 7.5 M CsCl was layered into the bottom of an ultracentrifuge tube and the sample carefully overlayed. The tube was placed in a swing-out rotor (Sorval Ti70) and centrifuged at 112,000 g (r_{ave}) and 20 °C for 20 h (Sorvall OTD65B) balanced against an identical tube. Following centrifugation the supernatant was removed and the RNA pellet resuspended in 1ml sterile H_2O. Purity of RNA was estimated by measuring absorbance at 230, 260 and 280 nm and calculating $^{260}/_{230}$ and $^{260}/_{280}$ ratios. RNA yield was estimated from the absorbance reading at 260 nm.

2.2.3.3 Isolation of polyA$^+$ RNA

PolyA$^+$ RNA was isolated from the total RNA sample. 100 µg of oligo-dT cellulose (Sigma) was placed into a 10 ml syringe and equilibrated with the binding buffer (0.5 M NaCl, 20 mM Tris pH 7.5, 1 % (w/v) SDS) until the pH of the eluate was pH 7.5. An equal volume of 2x binding buffer was added to the total RNA sample and heated at 65 °C for 5 min then quenched immediately on ice. This was loaded onto the column. The eluant was collected and applied to the column a second time. The column was washed with 4 x 5 ml of 1x binding buffer and polyA$^+$ RNA was then eluted with 4 x 1 ml of elution buffer (20 mM Tris pH 7.5, 0.5 % (w/v) SDS). An equal volume of 2x binding buffer was added to the polyA$^+$ RNA and heated to 65 °C for 5 min then quenched on ice immediately. The above procedure was repeated from the loading step.

The polyA$^+$ RNA sample was precipitated by adding 0.2 volumes of 5 M ammonium acetate and 1 volume of isopropanol and was stored at -20 °C overnight. The poly A$^+$ RNA was pelleted by centrifugation at 14,000 g (r_{ave}), 30 min at 4 °C (MSE high speed 18) and dissolved in a small volume of sterile H_2O.

The purity of the polyA$^+$ RNA was determined by measuring the absorption at 230, 260

and 280 nm and calculating the $^{260}/_{230}$ and $^{260}/_{280}$ ratios. PolyA$^+$ RNA yields were determined from the absorbance at 260 nm.

2.2.3.4 Plasmid minipreps

Small amounts of plasmid DNA were prepared essentially according to Sambrook *et al.*, (1989).

A single bacterial colony was grown overnight in 5 ml of L-broth with antibiotic selection, then a 1.5 ml aliquot of the culture was transferred to a sterile eppendorf tube and centrifuged at 13,000 g for 1-2 min in a microfuge (MSE microcentaur). The supernatant was removed and the bacterial pellet resuspended in 100 µl of ice-cold Solution 1 (1 % (w/v) glucose, 10 mM EDTA, 25 mM Tris.HCl pH 8.0). The bacterial suspension was allowed to stand at R.T. for 5 min, then 200 µl of freshly made Solution 2 (0.2 M NaOH, 1 % (w/v) SDS) was added, the tubes mixed gently by inversion and placed on ice for 5 min. 150 µl of ice-cold Solution 3 (11.5 % (v/v) of glacial acetic acid, 3 M potassium acetate pH 4.8) was added, the contents of the tube mixed by inversion then vortexed briefly and stored on ice for a further 5 min. Cell debris was pelleted by centrifugation at 13,000 g for 5 min. The supernatant was removed to a fresh tube and extracted with an equal volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was transferred to a clean tube and the DNA precipitated by the addition of 2 volumes of ethanol. After 5 min at R.T., the plasmid DNA was pelleted by 5 min centrifugation at 12,000 g, then washed with 70 % (v/v) ethanol, dried under vacuum and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) plus RNase at a concentration of 20 µg ml^{-1}.

2.2.4 Transformation of bacteria

2.2.4.1 Calcium chloride method for preparing competent cells

This was based on the method of Mandel and Higa (1970).

50 ml of L-broth was inoculated with 1 ml of an overnight culture of *E. coli* and incubated with shaking at 37 ^{o}C until the absorbance at 550 nm was 0.3-0.4 (2-3 h). The culture was placed on ice for 10 min before transfer to a chilled, sterile Sorvall tube and centrifuged at 12,000 g (r_{ave}) for 5 min at 4 ^{o}C in an MSE 18 centrifuge. The supernatant was discarded and the cell pellet gently resuspended in 25 ml of ice-cold 50 mM $CaCl_2$, 10 mM Tris.HCl (pH 8.0). The cell suspension was maintained on ice for 15 min and then recentrifuged as before. The supernatant was removed and the pellet now resuspended in 2.5 ml of the same solution. For storage 0.7 ml of 80 % (v/v) glycerol was added and the cells dispensed into 200 µl aliquots prior

to flash freezing in liquid nitrogen and storage at -80 ^{o}C.

2.2.4.2 Transformation of *E. coli* competent cells

Competent cells prepared by the above method were transformed as follows.

Pure DNA, or a ligation mixture was added to freshly made competent cells, or frozen cells which had been thawed on ice. The tube of cells was mixed gently and kept on ice for 30-60 min, heat-shocked at either 42 ^{o}C for 2 min, or 55 ^{o}C for 30 sec and placed back on ice for 5 min. 1 ml of L-broth was added and the cells incubated at 37 ^{o}C for 1 h before appropriate aliquots, usually 100 µl, were spread onto selective agar plates.

2.2.5 DNA manipulations

2.2.5.1 Quantitation of DNA solutions

The DNA solution was diluted with an appropriate volume of TE buffer, usually a 1:50 or 1:100 dilution. The absorbance of the solution at 260 nm and 280 nm was measured. A pure DNA sample has $^{260}/_{280}$ ratio of 2.0. An absorbance at 260 nm of 1.0 is equivalent to a double-stranded DNA concentration of 50 µg ml^{-1}, or a single-stranded DNA concentration of 40 µg ml^{-1}.

2.2.5.2 Restriction enzyme digests

Plasmid DNA was generally digested in a volume of 10-30 µl, with 5 units of the desired restriction enzyme and 0.1 volumes of the appropriate 10x concentrated restriction enzyme buffer (supplied with the enzyme). The volume was made up with H_2O and the reaction incubated at the recommended temperature (usually 37 ^{o}C) for 1-2 h. Digestions which were to be analysed by gel electrophoresis were terminated by the addition of 0.1 volumes of stop-dye (1 % (w/v) SDS, 50 mM EDTA pH 8.0, 20mM Tris. HCl pH8.0, 50 % (v/v) glycerol, 1.49 mM bromophenol blue).

If DNA was being digested for use in subcloning procedures, then the reaction was terminated by phenol/chloroform extraction (see section 2.2.5.3).

2.2.5.2.1 Restriction of genomic DNA

10 μg of genomic DNA was incubated with 20 units of restriction enzyme in a final volume of 50 μl made to a concentration of 1x buffer (10x is supplied with the enzyme). The reaction was incubated for 4 h at 37 ^{o}C and stopped by addition of 5 μl of stop dye.

2.2.5.3 Phenol:chloroform extraction of DNA

DNA samples were deproteinised by the addition of an equal volume of redistilled, water-saturated phenol equilibrated with 0.1 M Tris.HCl pH 8.0. The phases were mixed by vortexing and then separated by microcentrifugation at 13,000 g for 3-4 min. The upper, aqueous layer was carefully removed and re-extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) saturated with the same buffer. The sample was extracted in the same manner with an equal volume of chloroform:isoamyl alcohol (24:1), again the upper aqueous phase was collected.

2.2.5.4 Ethanol precipitation of DNA

DNA was precipitated from solution by the addition of 0.1 volumes of 3 M sodium acetate pH 4.8 and 2 volumes of ethanol, unless otherwise stated. For fragments under 1 Kb in size, 1 mM glycogen was added to assist precipitation. The sample was vortexed and stored at -80 ^{o}C for at least 30 min before centrifugation at 12,000 g for 10 min in a microfuge. The DNA pellet was washed with 70 % (v/v) ethanol, dried under vacuum and resuspended in either TE buffer or sterile distilled water.

2.2.5.5 Filling in recessed ends of DNA fragments

If it was necessary to convert a DNA fragment with recessed ends, to one with blunt ends the following method was used.

The DNA fragment was resuspended in 13 μl of sterile distilled water following isolation from an agarose gel (section 2.2.7). To this was added 2 μl of 10x reaction buffer (supplied with the enzyme), 1 μl each of 0.5mM dATP, dCTP, dGTP and dTTP and 1 μl (1 unit) of T4 polymerase. The reaction was incubated at room temperature for 15 min and was then phenol/chloroform extracted (section 2.2.5.3), ethanol precipitated (2.2.5.4) and resuspended in sterile water.

2.2.5.6 Ligation of DNA

DNA fragments with compatible cohesive or blunt termini were ligated by the action of T4 DNA ligase. The fragments to be ligated were mixed together in the approximate ratio of 3:1 insert:vector moles of termini, with 0.1 volumes of 10X concentrated ligation buffer (supplied with the enzyme) and 1-2 units of T4 DNA ligase. For blunt-ended ligations the amount of enzyme was increased to 5 units. The ligation reaction was incubated at either 15 ^{o}C overnight, or at R.T. for 2-3 h before it was used to directly transform competent *E. coli* cells.

2.2.6 Agarose gel electrophoresis

The correct amount of agarose required to give the desired concentration (usually 0.7-1.0 % (w/v) depending upon the sizes of the DNA fragments to be separated) was added to 200 ml of TBE buffer (0.77 M Tris-HCl, 0.83 M boric acid, 12.6 mM EDTA , for 10x) for a large gel, or 70 ml for a minigel then heated in a microwave oven for 2-3 min and the agarose cooled before being poured into the gel mould and allowed to set. The gel was placed in a tank containing TBE buffer, the DNA samples containing stop-dye were loaded into the wells and electrophoresis was carried out at an appropriate voltage for the desired length of time. Following electrophoresis the gel was stained by incubation in 1 μg ml^{-1} of ethidium bromide, 5 min for a minigel and 15 min for a large gel. The gel was destained for an equal period in H_2O prior to being viewed on a UV transilluminator (UVP Inc.) and, if necessary, photographed using a red filter and Polaroid 667 film.

Electrophoresis of RNA was carried out in formaldehyde-agarose gels. Formaldehyde-agarose gels were prepared and electrophoresed as decribed by Sambrook *et al.* (1989).

2.2.7 DNA fragment isolation

Gel slices containing fragments of interest were cut from agarose gels using a clean scalpel blade and released from the block by electroelution (Sambrook *et al.*, 1989). The DNA was purified by phenol extraction (section 2.2.5.3) and precipitated by ethanol precipitation (section 2.2.5.4).

2.2.8 Radioactive labelling of DNA

DNA was radioactively labelled using one of two methods, depending on the source of the DNA. Chemically synthesised oligonucleotides were end-labelled with [γ-^{32}P]ATP with the enzyme T4 polynucleotide kinase, as described by Sambrook *et al.* (1989). Double stranded

DNA sequences were labelled by random priming using [α-^{32}P]dCTP with klenow polymerase (Feinberg and Vogelstein, 1983). Following the labelling reaction, unincorporated radioactivity was separated from the DNA fragments by Sephadex G50 gel permeation chromatography using 10 ml (total volume) columns.

2.2.9 Transfer of nucleic acids to nylon membranes

The method used was a modification of that of Southern (1975).

The DNA-containing agarose gel was photographed and then soaked for 45 min in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) with occasional shaking. The gel was rinsed twice with distilled water and soaked for a further 45 min in neutralisation buffer (3 M NaCl, 0.5 M Tris-HCl pH 7.0), then rinsed with 20x SSC (3 M NaCl, 0.3 M sodium citrate). The gel was placed on a sheet of Whatman 3MM paper soaked in 10x SSC which was positioned over a glass plate "bridge", so that the ends dipped into a reservoir of 10x SSC and acted as a wick. A piece of nylon was cut to the same size as the gel, wetted with distilled water, immersed in 10x SSC and placed on top of the gel, taking care to remove any air bubbles. 3 pieces of Whatman 3MM paper, cut to the same size as the gel, were soaked in 10x SSC and laid on top of the nylon filter, again removing any air bubbles. Finally, 3 layers of absorbent "nappy pads" were positioned on top followed by a glass plate and the whole apparatus was compressed with a lead weight. DNA transfer was then allowed to proceed overnight.

The blotting apparatus was dismantled and the nylon filter removed, once the positions of the wells had been marked. The filter was baked between two pieces of 3MM paper in a vacuum oven at 80 ^{o}C for 2 h.

RNA was transferred to nylon filters as decribed by Sambrook *et al.* (1989).

2.2.10 Hybridisation of radioactive probes to nylon-bound nucleic acids

All hybridisations were carried out in heat sealed polythene bags contained in plastic boxes. Hybridisation of probe to northern blots was carried out at 42 ^{o}C and Southern blots at 65 ^{o}C. In both cases filters were prehybridised for 1 h prior to addition of probe. Solutions for both prehybridisation and hybridisation were as described by Sambrook *et al.* (1989). Hybridisations were carried out for 16 h after which the filters were washed to a particular stringency using SSC and 3.5 mM SDS. Filters were exposed to X-ray film (Fuji RX) at -80 ^{o}C with intensifying screens (Feinberg and Vogelstein, 1983). After exposure to film the filters could be stripped of radioactivity by incubation at 90 ^{o}C in 0.1 % (w/v) SDS. The filter could then be reprobed.

2.2.11 *In situ* hybridisation of bacterial colonies

The method used was based on that described by Sambrook *et al.* (1989).

A gridded nitrocellulose filter was placed onto a selective agar plate and individual colonies to be screened were transferred to it using sterile cocktail sticks. Each colony was also transferred to an identical position on a master plate. The plates were inverted and incubated at 37 ^{o}C overnight and both the filter and the master plate were marked in three identical positions in order to identify positive colonies. Using forceps, the filter was peeled from the plate, placed colony side up on a piece of 3MM paper saturated with 10 % (w/v) SDS and left for 3 min. The filter was transferred to 3MM paper soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 5 min, followed by 5 min on 3MM paper saturated with neutralising solution (1.5 M NaCl, 0.5 M Tris.HCl pH 8.0). The filter was laid on a sheet of dry 3MM paper and allowed to dry at room temperature for 30-60 min before being baked for 2 h at 80 ^{o}C in a vacuum oven.

To prepare the filter for hybridisation, it was first floated on the surface of a tray of 6x SSC until thoroughly wetted from beneath and then submerged for 5 min. A prewashing step followed, to remove any fragments of agar or loose bacterial debris. The filter was incubated at 65 ^{o}C for 1-2 h with shaking in 100 ml of washing solution (1 M NaCl, 1 mM EDTA, 0.1 % (w/v) SDS, 50 mM Tris.HCl pH 8.0). Prehybridisation, hybridisation and detection of positive colonies were carried out as described in section 2.2.10.

2.2.12 Chemical synthesis of oligonucleotides

Oligonucleotides were designed and synthesised using an 381A DNA Synthesiser, Applied Biosystems, operated using standard protocols. The primers were dissolved in H_2O to a concentration of 1 $\mu g\ \mu l^{-1}$.

2.2.13 PCR amplification

2.2 13.1 Amplification of DNA corresponding to known protein sequence

Amplification was performed using the polymerase chain reaction (PCR) based on the methods described by Fordham-Skelton (1990).

A reaction mixture was set up containing 5 µl of 10x Taq buffer (Promega), 1.25 mM dATP, dCTP, dGTP and dTTP, 1.25 µg of template DNA, 5 µl of both 5' and 3' primers and 1 µl of Taq polymerase. The volume of the reaction mix was brought to 50 µl with H_2O. A control reaction was prepared in which no template DNA was added. The reaction mixes were

overlayed with 100 µl of mineral oil. The PCR reaction was carried out in an "Intelligent heating-block" (Hybaid) according to the programme of a denaturing step of 92 ^{o}C for 1.5 min, a primer annealing step of 45 ^{o}C for 1 min followed by an extension step of 72 ^{o}C for 1.5 min. Thirty two of these cycles were performed and these followed by a single cycle comprising a 92 ^{o}C for 1.5 min denaturing step, a 40 ^{o}C for 1.5 min annealing step and 72 ^{o}C for 4 min extension time.

2.2.13.2 Amplification of cDNA corresponding to known protein sequence

The following procedure was based on selected procedures of Gillard *et al.* (1990) and the protocol for cDNA synthesis (Boehringer Mannheim).

cDNA was synthesised using a reaction mixture containing 100 ng of total RNA, 2 µl of oligo dT primer$_{15}$, 2 µl of 10x Taq polymerase buffer, 2 µl of DTT (100 mM), 1 µl of RNase inhibitor and 1 µl of AMV reverse transcriptase. The reaction mixture was made to 20 µl with sterile H_2O. The reaction was carried out at 42 ^{o}C for 1 h. Half of the cDNA (10 µl) was used in the PCR amplification. Degenerate oligonucleotide primers were designed and amplification carried out as described in section 2.2.13.1 using 10 µl of synthesised cDNA instead of 2.5 µl of genomic DNA. Inosines were included in the oligonucleotide primers at positions of 4-fold redundancy.

2.2.14 DNA sequence analysis

Plasmid sequencing was performed by the dideoxy-sequencing method of Sanger *et al.* (1977) using fluorescent dye-linked universal M13 primers and analysed by using an Applied Biosystems 370A DNA sequencer. Plasmids were sequenced in both directions using forward and reverse primers as described in the suppliers protocol (1988) and Sequenase enzyme (USB).

2.2.15 Construction of a cDNA λZAP library

cDNA was prepared from the polyA^{+} RNA (section 2.2.3.3) using a ZAP-cDNA synthesis kit (Stratagene). cDNA was ligated into the Uni ZAP II XR vector (Stratagene) and the recombinant phage were packaged with Gigapack gold packaging extract (Stratagene). The protocols supplied by Stratagene were followed throughout. *E. coli* SURE cells (Stratagene) were used throughout the construction of the library.

2.2.15.1 Phagemid recovery

cDNA inserts were recovered from the recombinant phage by *in vivo* excision of the plagemid pBluescript SK$^-$. *E. coli* XL1-Blue cells (Stratagene) were used during the recovery. The protocol supplied by Stratagene was followed throughout. Plasmid recoveries were performed as described in section 2.2.3.4.

2.2.15.2 Differential screen of λZAP cDNA library

Differential screening of a λZAP cDNA library was performed as described by Olszewski *et al.* (1989).

This is an unusual method which uses a dual-labelling for identifying differentially expressed genes. The RNA population in which the RNAs of interest were more abundant was used as a template for the synthesis of [α-^{35}S]dATP-labelled cDNAs and the second RNA population, in which the RNAs of interest were less abundant, was used as a template for the synthesis of [α-^{32}P]dCTP-labelled cDNAs. The labelled cDNAs were pooled and hybridised to plaque lifts on nitrocellulose filters (Schleicher and Schuell) obtained according to the Stratagene λZAP protocol. 80,000 plaques from the amplified cDNA library were screened. Prehybridisation, hybridisation and washing procedures were the same as those described earlier (section 2.2.10). When exposing the filters to X-ray film (Fuji RX) one piece of film was placed directly next to the filter and a piece of card, (Studland mounting board, 1275 micron, white) was placed on top of it. A second film was placed behind the card. Recombinant phage derived from constitutively expressed mRNA hybridised to both the ^{35}S- and ^{32}P-labelled probes. These phage were represented on both developed films. Recombinant phage derived from Cd-induced mRNA hybridised to only ^{35}S-labelled probe. This probe only fogged the film directly adjacent to the filter, radioactive emissions from this probe being too weak to pass through the card.

Plaques which hybridised solely to Cd-induced RNA were taken from the plate using the back end of a pastette. This was put into 1 ml of SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 0.01 % (w/v) gelatin, 50 mM Tris pH 7.5) and vortexed for 2 min. The phage released from the plug were plated at increasing dilutions. A suitable dilution was chosen to rescreen the plaques obtained from the primary screen in an identical manner. Any recombinant phages which still appeared to carry inserts corresponding to Cd-induced cDNA after the secondary screen were isolated and then screened a third time using identical procedures.

2.2.16 Polyacrylamide gel electrophoresis (PAGE)

2.2.16.1 Sample preparation for 1D SDS PAGE

D. innoxia cells were incubated for 48 h after initiation of a fresh culture. Metal solutions, if added, were filter sterilised through a disposable microfilter, 0.2 μm pore size (Sartorius) prior to inoculation. Cultures were transferred to 50 ml (Falcon, 2098) tubes and centrifuged at 800 g for 5 min (MSE centaur 2). The supernatant medium was removed and the cells washed with homogenisation buffer (0.0625 M Tris pH 6.8, 0.5 mM phenylmethylsulphonylfluoride). The cells were spun at 800 g for 5 min, the pellet was collected and stored on ice. The cells were placed in a precooled hand-held homogeniser, together with 1 ml of homogenisation buffer. After 20 passes on ice the homogenate was collected into 10 ml sterile plastic tubes. The homogeniser was rinsed with 1 ml of homogenisation buffer and this was added to the tubes. The homogenate was centrifuged at 800 g for 5 min and the supernatant was collected.

2.2.16.2 1D SDS PAGE

The following procedure is based on that described by Hames and Rickwood (1981).

All protein electrophoresis was carried out using a Protean II xi. (Biorad). Acrylamide : bisacrylamide was mixed with a mixed ionic resin (Biorad) prior to use. The consituents of the SDS-PAGE resolving gels were:

	7.5 %	15 %
Acrylamide: bisacrylamide (30 %:0.8 % (both w/v))	4 ml	8 ml
Resolving buffer (3.0 M Tris, pH 8.8)	2 ml	2 ml
SDS (10 % (w/v))	0.16 ml	0.16 ml
Ammonium persulphate (2 % (w/v) made fresh)	0.8 ml	0.8 ml
H_2O	9 ml	5 ml
TEMED	2 μl	2 μl

A linear gradient resolving gel between 7.5-15 % acrylamide was poured using a Gilson minipulse 3 pump. After the gel was poured overlay buffer (0.375 M Tris, 0.15 % (w/v) SDS) was layered onto the surface of the resolving gel. The tops of the plates were sealed and the gel left to polymerise overnight. The overlay buffer was removed and the stacking gel was poured on top of the resolving gel prior to use.

The constituents of the stacking gel were:

	4.7 %
Acrylamide: Bisacrylamide (30 %:0.8 % (both w/v))	1.25 ml
Stacking buffer (0.5 M Tris, pH 6.8)	2.5 ml
SDS (10 % (w/v))	0.1 ml
Ammonium persulphate (2 % (w/v) made fresh)	0.5 ml
H_2O	6.65 ml
TEMED	7.5 µl

Protein samples (60-80 µg) were mixed with an equal volume of sample buffer (0.0625 M Tris pH 6.8, 65.7 mM SDS, 5 % (v/v) β-mercaptoethanol, 0.29 M sucrose, 597 mM Bromophenol blue), boiled for 4 min and microfuged for 2 min. Samples were loaded after reservoir buffer (0.025 M Tris, 0.192 M glycine pH 8.3) had been poured into the apparatus. Gels were run at 100 V until the tracker dye reached the bottom of the gel (overnight).

Specific electrophoretic methods designed to separate small peptides were also used. Urea 1D PAGE was carried out using the exact procedures of Hames and Rickwood (1981) and tricine-SDS PAGE was carried out precisely as described by Shagger and Von Jagow (1987).

2.2.16.3 Visualisation of protein separated by PAGE

Following electrophoresis gels were stained with Coomassie blue R250 (0.1 % (w/v) Coomassie blue R250, 25 % (v/v) methanol, 10 % (v/v) acetic acid) for a minimum of 4 h with gentle agitation. The gel was washed with H_20 prior to incubation in destaining solution (25 % (v/v) methanol, 10 % (v/v) acetic acid), which was renewed every 2-3 h.

Gels were silver stained by incubating the gel in 40 % (v/v) ethanol, 5 % (v/v) formaldehyde for 30 min. The gel was then washed with H_2O for 30 min prior to incubating in 50 % (v/v) methanol for 3-4 h or overnight. The gel was washed in 1 mM DTT for 30-60 min prior to being soaked in 0.1 % (w/v) silver nitrate for 30-60 min. Excess silver nitrate was washed off the gel with H_2O and the gel was developed (0.28 M sodium carbonate, 5×10^{-5} % (v/v) formaldehyde). Development was stopped by addition of 2.4 M citric acid. Stained gels were then dried. The gels were first soaked for 4 h in soak solution (25 % (v/v) methanol, 10 % (v/v) acetic acid, 3 % (v/v) glycerol), a sheet of soaked cellophane was placed on a glass plate and the gel placed on top. Another piece of cellophane was placed on top of the gel. Any air trapped between the layers was excluded. An agarose "gel frame" was placed on top and clamped with bulldog clips.

The gels were allowed to dry under an incandescent lamp.

2.2.16.4 Sample preparation for 2D PAGE

Protein extracts were taken from *D. innoxia* as described in section 2.2.16.1. At that point samples were concentrated by adding 4 volumes of acetone and storing at -20 ^{o}C overnight. The protein was spun down at 800 g for 10 min and resuspended in 0.5 ml lysis buffer (9.5 M urea, 0.02 % (v/v) Non-idet NP40, 10 mM DTT) based on the sample preparation of Delhaize (pers. comm.). After protein estimation (section 2.2.18) Pharmalytes were added to a final concentration of 0.5 % (v/v).

2.2.16.5 2D PAGE

The first dimension of isoelectricfocusing (IEF) was run in tube gels, the IEF gels were composed of (for 16):

Urea	8.25 g
H_2O	6 ml
Acrylamide: Bisacrylamide (30 %:0.08 % (both w/v))	2 ml
Pharmalytes 3-10	0.75 ml
Non-idet NP40	0.3 ml
Ammonium persulphate (10 % (w/v) made fresh)	25 µl
TEMED	5 µl

The gels were cast in disposable 1 ml pipettes sealed with parafilm at one end. The gel was poured, overlayed with 10 µl of isoamyl alcohol and allowed to polymerise. The gels were stored at approximately 28 ^{o}C to retain the urea in solution. Once set the isoamyl alcohol and parafilm were removed and the tube gels were loaded into the apparatus, the upper cathodic chamber contained 400 ml of 20 mM NaOH and the lower anodic chamber 4 l of 10 mM phosphoric acid. 300 µg of prepared sample was loaded on top of each gel. Electrophoresis was carried out at 200 V for 0.5 h, 400 V for 16 h and 800 V for 2 h. Upon completion of electrophoresis the tube gels were removed and stored immediately at -20 ^{o}C until needed for the second dimension.

The IEF gel was extruded from the tube when frozen using water pressure supplied by a syringe. The IEF gel was then incubated in sample buffer for SDS-PAGE for a minimum of 3 h. The IEF gel was then placed on the SDS-PAGE gel (section 2.2.16.2) and overlayed with 1 % (w/v) agarose (in reservoir buffer). Electrophoresis was carried out at 100 V until the tracker dye reached the end of the gel (overnight). Protein was visualised by Coomassie staining as described

2.2.17 Western blotting

2D PAGE gels were run as described in section 2.2.16.5 and the unstained gels were electroblotted using a semidry apparatus (Sartoblot, Sartorius). Proteins were blotted onto polyvinylidene difluoride (PVDF) membrane, Problot (Applied Biosystems), which was first washed in 70 % (v/v) methanol to ensure no air was trapped in the membrane. Excess methanol was rinsed off with water and the gels were equilibrated in transfer buffer (10 mM CAPs, 10 % (v/v) methanol, pH 11) for 15 min. Glycine containing transfer buffer was not used as glycine would act as a contaminant in protein microsequencing. The gel and 3MM paper used in the sandwich were both soaked for 5 min in transfer buffer and the blotting cassette was assembled as described by Sartorius.

In blotting the 2D PAGE gels, the area of gel containing Cd-1 and Cd-2 was excised from the gel. This region measured 7.5 x 5 cm. Electro-blotting was carried out on this gel piece at 0.8 mA cm^{-2} (30 mA) for 47.5 min.

2.2.17.1 Detection of electroblotted proteins

The protein transferred to the membrane was visualised by staining with Coomassie blue (0.1 % (w/v) Coomassie brilliant blue R250, 50 % (v/v) methanol) for 1 min. The membrane was destained (50 % (v/v) methanol 10 % (v/v) acetic acid). Proteins were identified and destained with fresh destain solution.

2.2.17.2 CNBr cleavage of electroblotted proteins

Proteins were cleaved using cyanogen bromide (CNBr). An electroblotted protein spot was cut into small pieces (2 x 4 mm) and suspended in an 0.5 ml eppendorf tube containing 100 µl of 0.15 M CNBr in 70 % (v/v) TFA. Tubes were incubated overnight in the dark at room temperature. Excess reagents were removed via vacuum centrifugation in a vacuum centrifuge (Jouan RC10.22). When dry, 50 µl H_2O was added, vortexed briefly and the sample redried in the vacuum centrifuge. This last washing step was repeated. 50 µl of 0.1 % (w/v) ammonium hydroxide was then added and incubated at room temperature for 15 min to convert all the peptides to the homo-serine form.

2.2.17.3 Release of protein from Problot

Protein was eluted from Problot (Applied Biosystems) by soaking the membrane in H_2O for 2 h followed by 24 h incubation in 5 % (v/v) TFA, 75 % (v/v) IPA at 60 ^{o}C (P. Jackson (Applied Biosystems) pers. comm.). The protein was dried in a vacuum centrifuge (Jouan RC 10.22) and resuspended in 2 % (w/v) SDS, 10 μM DTT.

2.2.18 Protein concentration

2.2.18.1 Ammonium sulphate precipitation

Ammonium sulphate ($(NH_4)_2SO_4$) was added to the protein sample prepared as described in section 2.2.16.1 to yield a final specific percentage saturation. Samples were precipitated at 10, 20, 30, 40, 50, 60, 70 and 80 % (w/v) saturation and pelleted by centrifugation at 800 g for 5 min. The supernatant of each sample was removed and precipitated at 90 % (w/v) saturation. All precipitates were resuspended in 200 μl of homogenisation buffer and added to an equal volume of IEF lysis buffer. Up to 300 μg of protein was loaded onto the IEF gels.

2.2.18.2 Lyophilisation

Samples were concentrated by freeze drying in an Edwards Modulo freeze drier. The samples were shell frozen at -80 ^{o}C and lyophilised overnight. The protein was resuspended in 300 μl of homogenisation buffer.

2.2.19 Determination of protein concentration

The protein concentration of cell extracts was determined by a Bradford assay using the Bio-Rad micro assay reagent (Bradford, 1976). The dye reagent concentrate was diluted 1 in 5 with distilled water and 200 μl of this was mixed with 100 μl of sample, diluted if necessary. This was carried out in a microtitre plate (Falcon). The absorbance at 595 nm was measured using a Titertek Multiscan MCC plate reader. The protein concentration was calculated from the absorbance readings using a calibration curve constructed using known quantities of BSA.

2.2.20 Chromatography

2.2.20.1 Ion exchange chromatography

A DE52 pre-swollen cellulose anion exchange matrix (Whatman) was packed into an Econo column, height 10 cm, diameter 1 cm (Biorad). The column was equilibrated with homogenisation buffer. *D. innoxia* protein samples (as described in section 2.2.16.1) were loaded onto the column. The column was eluted in a step-wise manner with 100, 150, 200, 250, 300, 400, 500 mM and 1 M NaCl made in 10 mM Tris pH 7.5. A volume of 25 ml elution buffer was gravity fed at each step and 2.5 ml fractions were collected. The protein concentration of each sample was estimated (section 2.2.19).

2.2.20.2 Gel filtration chromatography

A Pharmacia C-column (height 50 cm, diameter 2 cm) was packed with Sephadex G50. The matrix was swollen by boiling in homogenisation buffer for 2 h. Calibration of the column using dextran blue showed the void volume to be approximately 70 ml. Samples as prepared in section 2.2.16.1. were loaded onto the column. The column was eluted at a flow rate of 15 ml h^{-1} (Gilson minipulse 3) with homogenisation buffer. Fractions were collected every 15 min (3.8 ml). The protein concentration was calculated for each fraction. All chromatography was carried out at 4 ^{o}C. An atomic absorption spectrophotometer (Perkin Elmer 5000) was used to construct a calibration curve from which the Cd concentration of each sample was estimated.

2.2.20.2.1 Desalting of cell extracts

D. innoxia samples collected as described in section 2.2.16.1, were desalted using prepacked Sephadex G25 columns, PDP10s (Pharmacia). The PDP10 columns were equilibrated with 25 ml of homogenisation buffer. 2.5 ml of sample was then loaded onto the column and eluted off in 3 ml of homogenisation buffer.

2.2.20.3 Narrow Bore HPLC

Reverse phase HPLC was performed using an Applied Biosystems 140A solvent delivery system, Applied Biosystems 1000s diode array detector and an Applied Biosystems (Brownlee) RP300 Aquapore octyl # Co3032 column. Water used was freshly drawn MilliQ. Prior to loading samples onto the column, particulates were removed by centrifugation and the supernatant transferred to a new eppendorf tube. A flow rate of 100 µl min^{-1} was used in all investigations. A 100 µl injection loop was used and following injection the sample loop was

closed to prevent peak trailing.

Two protocols were used. The first was employed to elute intact proteins and used elution buffers of 0.1 % (v/v) TFA (A) and 2:1 isopropanol:acetonitrile, 0.1 % (v/v) TFA (B). A gradient elution was used with a gradient of 5 % (v/v) B to 100 % (v/v) B. The second protocol was used to resolve mixtures of small polypeptides. It used elution buffers of 0.1 % (v/v) TFA (A) and 80 % (v/v) acetonitrile, 0.1 % (v/v) TFA and a gradient elution was used with a gradient of 5% (v/v) B to 100 % (v/v) B. Elution buffers were filtered through a 0.2 μm pore size filter (Sartorius) under vacuum which served to degas the buffers.

2.2.21 Protein microsequencing

An Applied Biosystems model 447A equipped with a 120 PTH-amino acid analyser (Applied Biosystems) was used to obtain protein sequence information from electroblotted proteins.

2.2.22 Production of antibodies

D. innoxia protein samples separated by 2D PAGE (section 2.2.16.5) were electroblotted onto nitrocellulose (section 2.2.17). Individual "protein spots" were isolated from ten blots (500 pmoles). The sample was ground to a powder in a mortar and pestle under liquid N_2. The ground nitrocellulose was taken up in 1.5 ml of PBS (2.7 mM KCl, 137mM NaCl, 1.8 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) The nitrocellulose acts as an adjuvant to prolong the half life of the antigen in the animal. 0.5 ml of this suspension was mixed with an equal volume of Freunds complete adjuvant (Freund and McDermot, 1942; Freund, 1956). 0.5 ml of the resulting water in oil suspension was injected subcutaneously and 0.5 ml was injected into the thigh muscle of a rabbit. The rabbits were unfed for 24 h prior to the first injection. One rabbit was used for each of the proteins. Two further injections were given to boost the immune response. For both of these booster injections 0.5 ml of suspension was mixed with an equal volume of incomplete Freunds adjuvant. 0.5 ml was injected into the thigh muscle and 0.5 ml was injected subcutaneously. The first of these was 3 weeks after the initial injection and the second was 2 weeks following the first boost. The rabbits were terminally bled 2 weeks after the final injection.

Blood was collected in 50 ml sterile tubes (Falcon 2098) and centrifuged at 1000 rpm for 10 min (MSE Centaur 2). The supernatant was collected into 1.5 ml Eppendorf tubes and microcentrifuged for 10 min at 4 ^{o}C. 200 μl aliquots of supernatant were stored in 0.5 ml Eppendorf tubes at -20 ^{o}C.

Prior to injecting the rabbits with protein a pre-immune bleed was taken from each animal. These pre-immune bleeds were treated in exactly the same manner as the immune bleeds.

2.2.23 Purification of the immune fraction from serum

Protein-A attached to glass beads, Prosep-A (Bioprocessing), was used to separate the immunoglobulins from the serum. 0.75 ml of Prosep-A was equilibrated with 10 ml of PBS for 10 min. An OPC cartridge (Applied Biosystems) was emptied and washed with PBS prior to being filled with Prosep-A. An adaptor (usually used to join two OPC cartridges together) was attached to one end of the OPC cartridge. A syringe was attached to the opposite end and used to draw PBS over the Prosep-A. The OPC cartridge was inverted and PBS washed over the Prosep A to exclude any air. 4ml of serum was dialysed overnight against 25 mM sodium acetate, 10 mM NaCl pH 7.2. The dialysed serum was centrifuged and diluted with an equal volume of PBS. The prepared serum was drawn over the Prosep-A and the IgG fraction allowed to adsorb for 10 min. More serum was then passed over the Prosep-A until 2 ml had been processed in this manner. The column was washed with 5 x 0.5 ml of PBS and each wash eluted from the column was collected. The serum fraction bound to the column was eluted off the Prosep-A with 0.1 M glycine pH 3. A hypodermic needle was attached to the end of the adaptor to enable drops to be collected. Four drops were collected as one fraction in 0.5 ml Eppendorf tubes (approximately 40 µl) containing 1.5 µl of 1 M Tris pH 9.6, to neutralise the elution buffer. The washes and each fraction were analysed by absorption at 230 nm and 280 nm.

2.2.24 Western blots screened with an antibody

1 and 2-dimensional western blots of *D. innoxia* protein were collected as described in sections 2.2.16.2 and 2.2.16.5 with the exception that the transfer buffer used was 25 mM Tris, 192 mM glycine, 20 % (v/v) methanol. The proteins were stained with Ponceau S (0.2 % (w/v) Ponceau S in 3 % (v/v) TCA) for 5 min and the membrane destained in PBS for 2 min. After noting the position of the size markers the membrane and proteins were completely destained.

The membrane was blocked in TBS (20 mM Tris pH 7.8, 0.15 M NaCl, 3 % (w/v) gelatin) overnight. The membrane was then washed in TBS, 0.5 % (v/v) Tween three times and then incubated with the antibody diluted 1 in 1000 in TBS, 1 % (w/v) gelatin, 0.5 % (v/v) Tween for 2 h at R.T. The membrane was washed five times in TBS, 0.5 % (v/v) Tween before being incubated with a secondary antibody (Anti-rabbit IgG from goat (Sigma)) diluted 1 in 9000 in TBS, 1 % (w/v) gelatin, 0.5 % (v/v) Tween. The membrane was washed three times in TBS, 0.5 % (v/v) Tween and then colour developed by incubating in alkaline phosphatase (AP) buffer (100 µM NaCl, 5 µM $MgCl_2$, 100 mM Tris pH 9.5). BCIP was made up at 50 mg ml^{-1} in 100 %

(v/v) DMF and added to the AP buffer (1 in 200 dilution). A 35 mg ml^{-1} in 70 % (v/v) DMF solution of NBT was made and added to the AP buffer (1 in 100 dilution). Development was carried out for approximately 15 min or to optimal contrast. The reaction was stopped in 1 % (v/v) acetic acid.

2.2.25 Atomic absorption spectrophotometry (AAS)

Atomic absorption analysis for the measurement of the concentration of metal ions in solution was performed using a Perkin Elmer HGA spectrophotometer. Analyses were performed according to the manufacturer's protocols. Three replicate readings were taken for each sample and the mean value calculated. Metal was quantified via the construction of a calibration curve using Spectrosol standards (BDH).

CHAPTER 3

RESULTS

3.1 IDENTIFICATION AND MICROSEQUENCING OF Cd-INDUCED PEPTIDES

3.1.1 Growth of *D. innoxia* suspension cultures

Three independent original cultures were monitored (section 2.2.2). The original culture was designated n.1, it gave rise to a daughter culture n.2 and that, in turn, to n.3. The growth curves are shown in Figure 4. A lag phase following initial transfer of cells to fresh media was only observed in two of the nine cultures. Up to 96 h, at the point of subculturing, the cells showed no signs of entering a stationary phase. Between 96 and 120 h the rate of growth of most of the cultures had decreased.

3.1.1.1 Effect of Cd and heat shock on *D. innoxia* suspension cultures

Exposure to elevated temperatures resulted in a very small but significant difference in the packed cell volume (PCV) measurements; cells exposed to elevated temperatures had a PCV of 1.8 ml (S.D. 0.06) and the non-exposed cells had a PCV of 2.0 ml (S.D. 0.06), (Figure 5). Exposure of Cd300 cells to Cu and Zn had no effect on cell survival as PCV measurements of cultures exposed to either metal are not significantly different from those of non-exposed cells. The WDI cells did show a small but again significant difference between Cd-exposed cells (PCV of 1.65, S.D 0.05) and non-exposed cells (PCV of 1.9, S.D. 0.05) (Figure 6).

3.1.2 1D SDS PAGE

The soluble protein fraction was collected as described in section 2.2.16.1, desalted (section 2.2.20.2.1) and the sample was lyophilised (section 2.2.18.2) to increase the protein concentration. After lyophilisation, problems were encountered in rehydrating the protein, which were surmounted by desalting to 10 mM sodium borate ($Na_2B_4O_7$) buffer. This lessened protein aggregation and aided protein solubilisation by complexing with the protein and integrating with the glycoproteins while maintaining only a very low ionic strength. Borate also maintains its pH over a wide range of temperatures, i.e. it has a low temperature co-efficient.

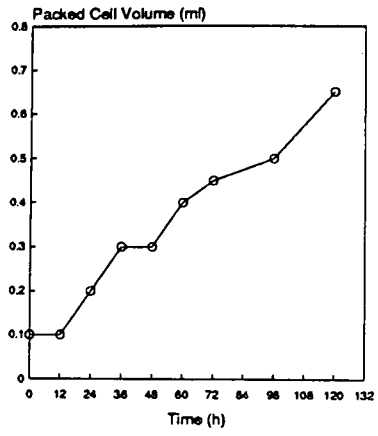
1D-SDS PAGE was used to identify differences in protein expression in *D. innoxia* due to exposure to Cd (section 2.2.16.2). At 48 h in the growth cycle (section 3.1.1) Cd300 cultures were exposed to 125 μM Cd for 2, 4 and 8 h. This is half the Cd concentration used by Delhaize *et al.* (1989b) to examine the effect of Cd on gene expression in *D. innoxia* The cultures used by Delhaize *et al.* (1989b) were growing at twice the rate of cultures used here. As a control, a culture was inoculated with H_2O and harvested immediately (0 h). The results of such

FIGURE 4

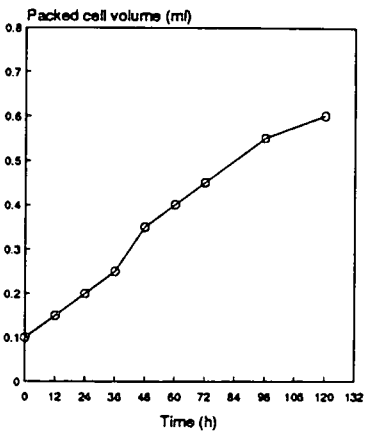
***D. innoxia* suspension culture growth analysis**

Three independent original cultures were monitored, numbered 1, 2 and 3. The original culture was designated n.1. It gave rise to a daughter culture n.2, and that in turn to n.3. Each culture was subcultured at 96 h, 10 ml of culture was added to 30 ml of fresh media. PCVs were taken from 2.5 ml of culture.

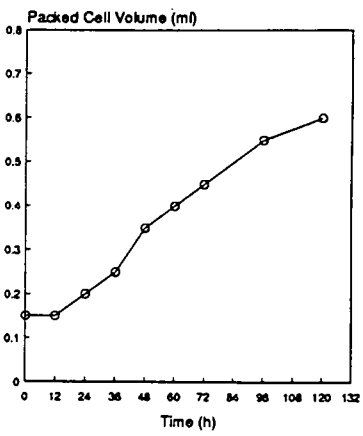
Culture 1.1



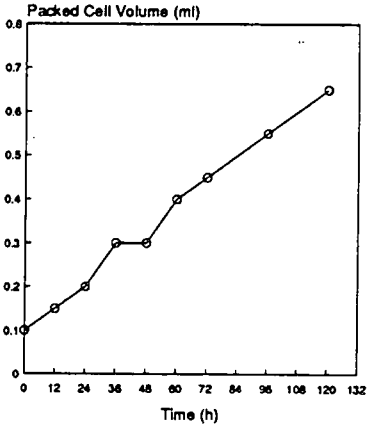
Culture 1.2



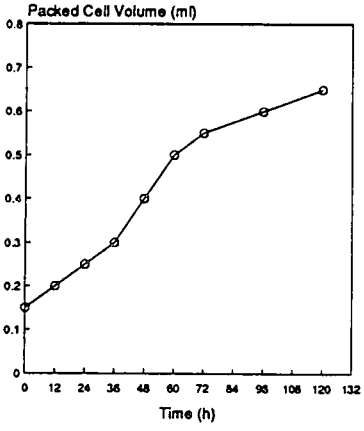
Culture 1.3



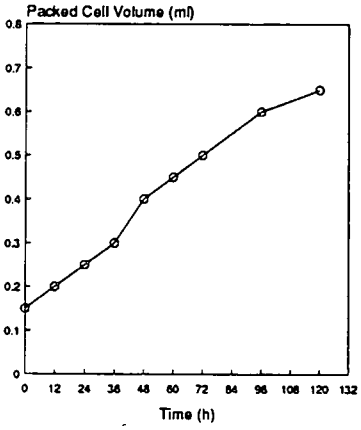
Culture 2.1



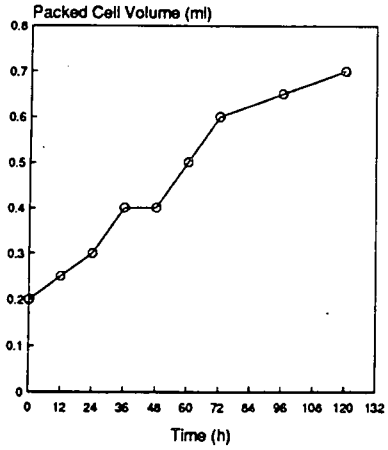
Culture 2.2



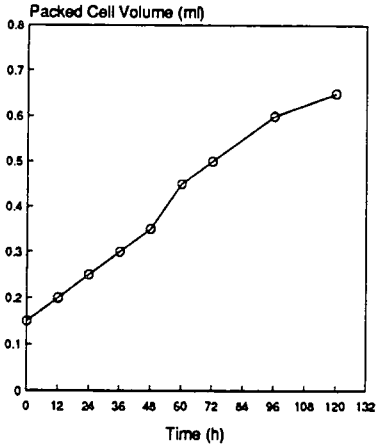
Culture 2.3



Culture 3.1



Culture 3.2



Culture 3.3

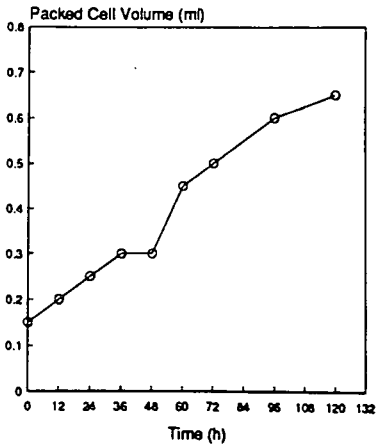


FIGURE 5

Effect on PCVs of Cd and HS on Cd-tolerant (Cd300) *D. innoxia* cells.

After exposure to the particular Cd treatment cultures were returned to normal growth conditions. At 96 h PCVs were measured from four 10 ml aliquots of cultures. Error bars show the standard deviations from the means of the four measurements.

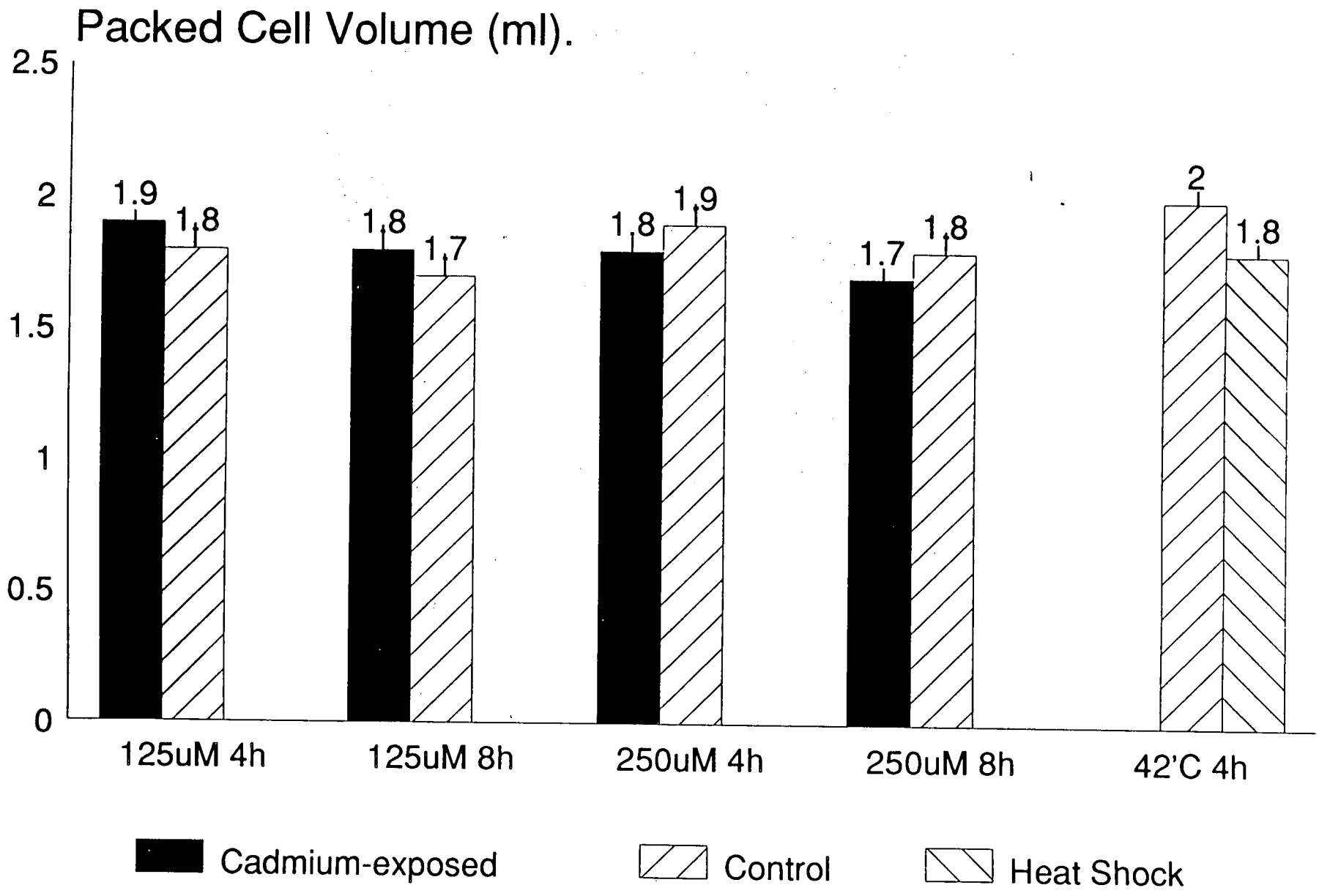
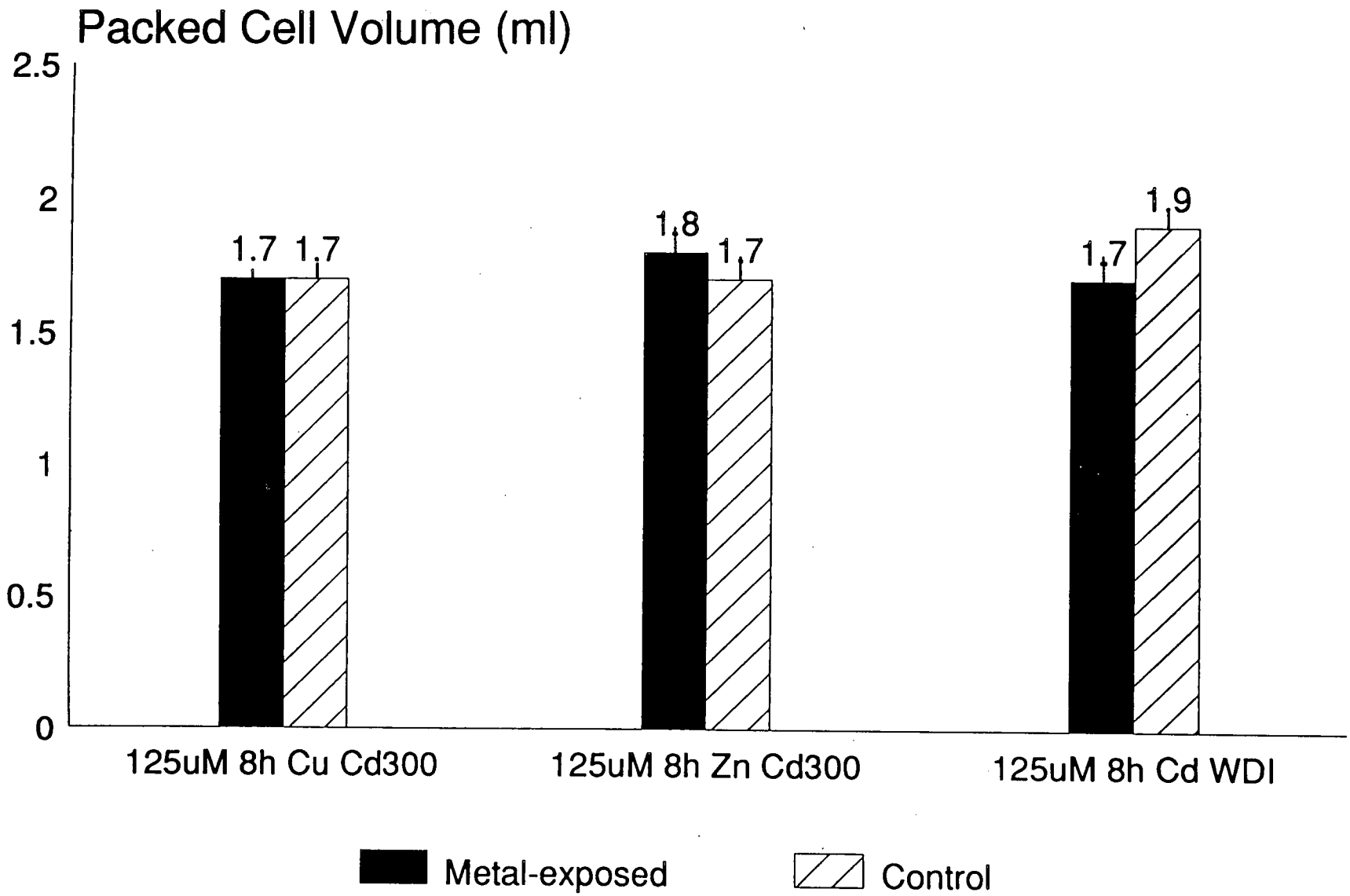

Packed Cell Volume (ml).
2.5
2
1.5
1
0.5
0
1.9
1.8
1.8
1.7
1.8
1.9
1.7
1.8
2
1.8
125uM 4h
125uM 8h
250uM 4h
250uM 8h
42'C 4h
Cadmium-exposed
Control
Heat Shock

FIGURE 6

Effect of Cu and Zn on the growth on Cd-tolerant (Cd300) and Cd on non-tolerant (WDI) *D. innoxia*

After exposure to a particular metal treatment cultures were returned to normal growth conditions. At 96 h PCVs


Packed Cell Volume (ml)
2.5
2
1.5
1
0.5
0
1.7
1.7
1.8
1.7
1.7
1.9
125uM 8h Cu Cd300
125uM 8h Zn Cd300
125uM 8h Cd WDI
Metal-exposed
Control

comparative analysis are shown in Figure 7.

The gel shows clearly that a number of novel proteins were induced, or existing proteins enhanced in all the samples exposed to Cd. Any inductions that did occur were observable after this time exposure to Cd. After 2 h no further inductions could be observed. The proteins induced display a wide range of molecular weights from approximately 12 to 60,000. The repression of individual protein species, following exposure of Cd300 cells to Cd, was not observed.

3.1.3 2D PAGE

To increase resolution of polypeptide separation, 2D PAGE was used (section 2.2.16.5). At 48 h in the growth cycle cultures were exposed to 125 μM Cd for 8 h and control sister cultures were treated in an identical manner with an equal volume of H_2O. Soluble protein was recovered (section 2.2.16.4), loaded onto the IEF gels and electrophoresed. The protein precipitated on the surface of the IEF gel, in samples prepared from both Cd300 and WDI cultures.

To investigate if precipitation was due to treating the cultures specifically at 48 h, due to a particular cellular component accumulating at that specific stage, the experiment was repeated, treating the cultures at either 24 h or 72 h. However, precipitation again occurred on electrofocusing these samples.

In order to establish whether the problem of protein precipitating at the IEF gel surface was specific to *D. innoxia* samples, protein was collected from salt grass (*Distichlis spicata*) suspension cultures (as described for *D. innoxia* in section 2.2.16.4) and electrofocused (section 2.2.16.5). No precipitation occurred at the surface of the IEF gel. On running an SDS PAGE second dimension (section 2.2.16.2) and staining the gel with Coomassie blue (section 2.2.16.3) individual polypeptides were observed. This indicated that the problem of protein precipitation on the IEF gel surface was a consequence of some inherent quality of *D. innoxia* cell extracts.

To establish if too much protein had been loaded on to the IEF varying amounts of protein were loaded and electrofocused, the results are shown in Figure 8. However, even at the lower loadings (30 μg) the protein precipitated at the gel surface. On loading greater quantity of protein some of the protein migrated into the gel and precipitated as a smear without focusing at particular isoelectric points (pIs).

No individual polypeptides were observable on any of the gels generated following SDS PAGE resolution of the IEF gels (data not shown). Streaking was seen on silver stained SDS PAGE gels. The streaking seen in the final SDS PAGE gel corresponded to the cathodic end of

FIGURE 7

Identification of Cd-induced proteins in *D. innoxia* using 1D PAGE

Markers (Mr): 110,000 84,000 47,000 33,000 24,000 16,000

1= Protein extract from Cd300 cells incubated with H_2O for 0 h
2= Protein extract from Cd300 cells incubated with 125 μM Cd for 2 h
3= Protein extract from Cd300 cells incubated with 125 μM Cd for 4 h
4= Protein extract from Cd300 cells incubated with 125 μM Cd for 8 h.

The arrows identify differences in protein bands between the various treatments, indicating Cd-inductions and Cd-enhancements.

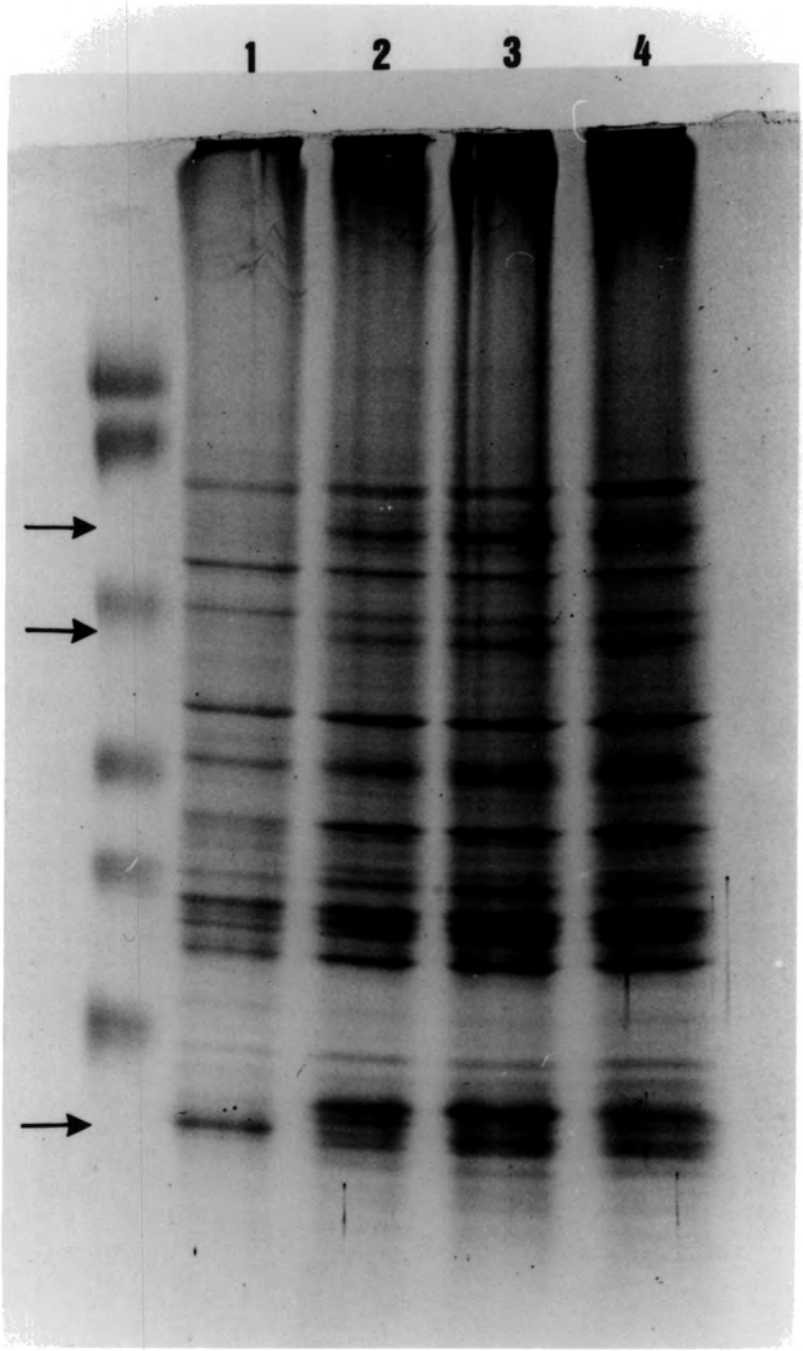

1
2
3
4

FIGURE 8

Precipitation of protein on the IEF gel surface after electrofocusing

Tube	1	2	3	4	5	6	7
Protein loading (μg)	900	600	300	225	150	75	30


1
2
3
4
5
6
7
BIO-RAD
MODEL 225 TUBE GEL CASTING STAND

the IEF indicating proteins had not focused to their pIs in the IEF dimension.

3.1.3.1 pH dependence

To establish if the precipitation was pH dependent the running buffers used in IEF were reversed. The upper, cathodic, chamber was filled with 400 ml of 10 mM phosphoric acid and the lower, anodic, chamber with 4 l of 20 mM NaOH. This maintained an acidic environment at the IEF gel surface at which the protein was loaded. Electrofocusing (section 2.2.16.5) was repeated with protein extracted from Cd300 cells exposed to Cd (125 μM, 8 h) and from non-exposed cells (section 2.2.16.4). Precipitation of both protein samples occurred at the IEF gel surface.

3.1.3.2 Ammonium sulphate precipitations

Ammonium sulphate ($(NH_4)_2SO_4$) precipitation is often carried out as a preparative step to remove contaminants, such as carbohydrates and nucleic acid, from protein samples. $(NH_4)_2SO_4$ precipitations were carried out (section 2.2.18.1) on protein extracted from Cd300, Cd-exposed (125 μM, 8 h) cultures (section 2.2.16.1). Protein samples from each $(NH_4)_2SO_4$ "cut" were recovered and prepared for IEF (section 2.2.16.4). 100 μg of protein or the total sample, which ever was the least, was loaded onto IEF and the gels electrophoresed (section 2.2.16.5). Regardless of the percentage $(NH_4)_2SO_4$ saturation used, protein precipitated on the surface of the IEF gel.

3.1.3.3 Fractionation by ion exchange chromatography

Abundant protein species can cause precipitation of protein at a particular pI (An der Lan and Chrombach, 1981). Proteins extracted from a Cd-exposed (125 μM, 8 h) Cd300 culture (section 2.2.16.1) were fractionated by stepped elution from an ion exchange column (section 2.2.18.1) and fractions analysed for protein (Figure 9). Protein was not detected in the fractions collected after eluting the column with 500 mM or 1 M NaCl.

Figure 9 shows that those proteins which did not bind well or at all to the column were eluted in 100 mM NaCl. Elution with the five higher salt concentrations 150, 200, 250, 300 and 400 mM NaCl split the total protein sample roughly into five fractions based on their ionic strength. No protein could be detected in fractions eluted with either 500 mM or 1 M NaCl. The proteins represented by each peak in Figure 9 were pooled and prepared for IEF (section 2.2.16.4). 300 μg of protein from each sample was loaded and electrophoresed on IEF (section

FIGURE 9

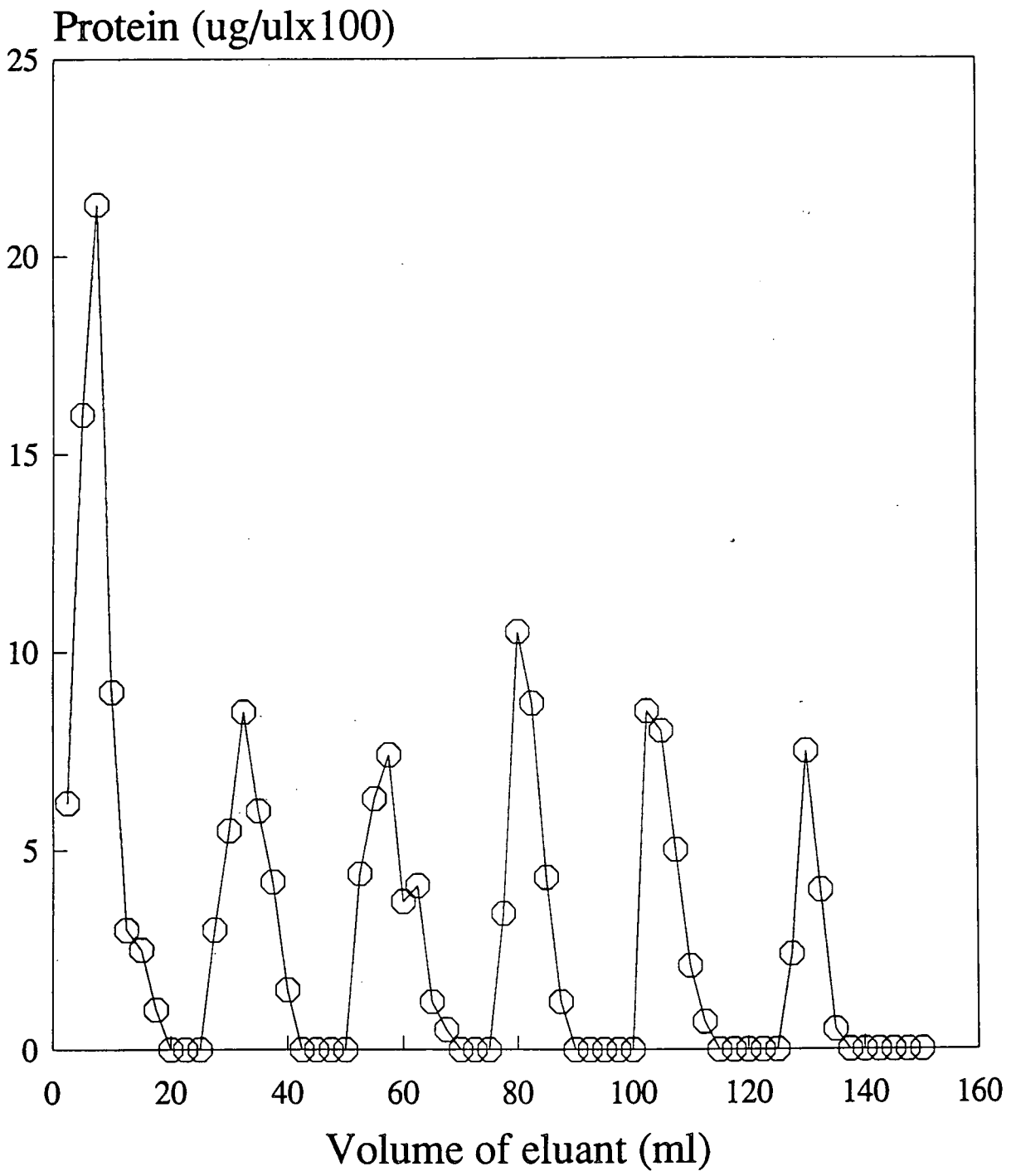
Anion exchange fractionation of *D. innoxia* protein

A Biorad Econo-column was packed with DEAE cellulose (Whatman). Protein was eluted by stepped elutions with various NaCl concentrations. 2.5 ml fractions were collected. Protein content was estimated by Bradford assay (Bradford, 1976).

Eluant volume (ml)	25	25-50	50-75	75-100	100-125	125-500
NaCl concentration (mM)	100	150	200	250	300	400

No protein could be detected in the fractions collected after eluting the column with 500mM or 1 M NaCl

DEAE cellulose column.



2.2.16.5).

After electrofocusing, proteins from each fraction precipitated on the surface of the gel.

3.1.3.4 Incorporation of protein in the gel mixture

In an attempt to cirumvent the problem of protein precipitating on the gel surface protein was incorporated in the IEF gel mixture and the gel was then cast. The gel recipe was the same as that described in section 2.2.16.5 with the exception that the volume of H_2O was reduced to account for the volume of protein sample added. The total volume of both the H_2O and protein was 6 ml. 300 μg of protein extracted from Cd300 cultures exposed to 125 μM Cd for 4 h, and similarly from the sister cultures inoculated with H_2O (section 2.2.16.1), was incorporated into the gel. Following a period to allow polymerisation the rods, holding the IEF gels, were unsealed. The gels which contained protein extracted from Cd-exposed cells had not polymerised whereas those IEF gels which incorporated protein from non-exposed cells polymerised fully.

To investigate if a particular protein species was responsible for inhibiting gel polymerisation the proteins recovered from Cd-exposed (125 μM, 8h) Cd300 cultures (section 2.2.16.1) were separated by ion exchange chromatography (section 2.2.20.1). A similar result was obtained to that shown in Figure 9 (section 3.5.3). Each fraction of protein was collected and prepared for IEF (section 2.2.16.4). 300 μg of protein from each fraction was incorporated into an IEF gel. After allowing time for polymerisation none of the IEF gels were fully polymerised.

It was proposed that Cd-induced Cd300 cultures would have synthesised PCs and it may have been these thiol rich polypeptides which had inhibited IEF gel polymerisation. To test this hypothesis PCs were removed from a Cd300 Cd-exposed (125 μM, 8 h) protein sample (section 2.2.16.1) by gel filtration chromatography (section 2.2.20.2). The result of analysing each sample for protein and Cd is shown in Figure 10. The second peak representing Cd containing fractions has been shown to be associated with PCs (Delhaize *et al.*, 1989a). There appears to be good, baseline resolution of the PC bound cadmium away from the protein containing fractions (Figure 10). These protein containing fractions (13-21) were collected, pooled and prepared for IEF (section 2.2.16.4). 300 μg of protein was incorporated into the gel. Upon unsealing the tubes all the IEF gels had fully polymerised.

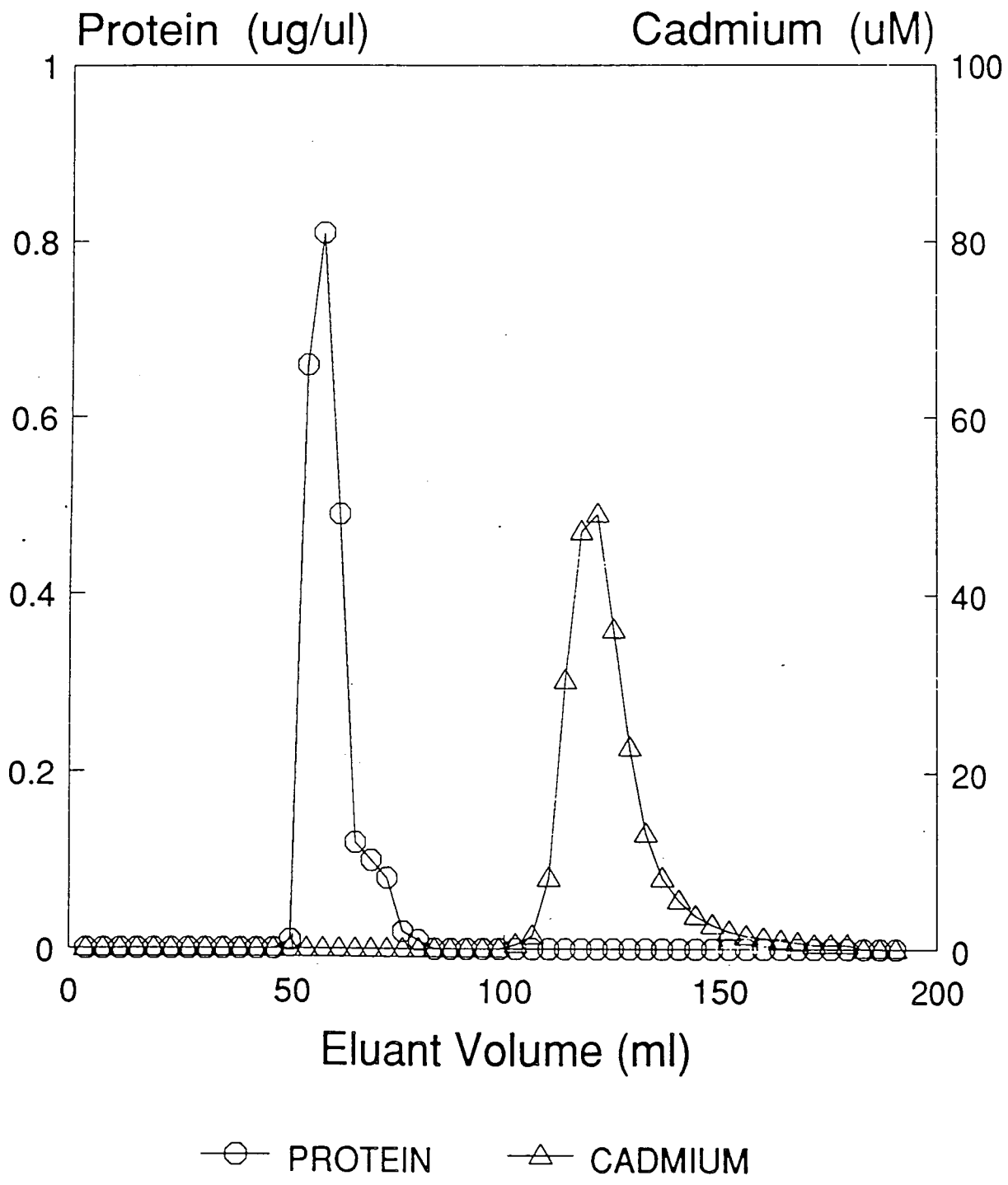
On running the IEF dimensions (section 2.2.16.5) no precipitation was detected. In the second dimension, SDS PAGE (section 2.2.16.2), individual polypeptides were observed following visualisation with Coomassie blue staining (section 2.2.16.3).

FIGURE 10

Separation of protein and Cd-containing fractions by gel filtration chromatography

A Pharmacia C-column was packed with Sephadex G50 (Sigma). Protein was eluted with homogenisation buffer (0.0625 M Tris pH 6.8) at a flow rate of 15 ml h^{-1} (Gilson minipulse 3). 3.5 ml samples were collected. Protein content was estimated by Bradford assay (Bradford, 1976) and Cd-content by atomic absorption spectrophotometry (Perkin and Elmer 5000).

Sephadex G50 Column.



Electrophoresis of more than 300 μg of protein in 2D PAGE resulted in a decrease in the resolution. If more than 0.5 mg of protein was loaded then certain protein species precipitated out at particular pIs, in the IEF dimension. If the IEF gel was run on the SDS PAGE dimension a complete loss of resolution was observed, very few individual polypeptides being identified.

3.1.3.5 Effects of metal exposure and HS on protein expression in *D. innoxia*

D. innoxia cultures were subjected to different treatments and the proteins analysed by 2D PAGE. All protein extracts were prepared as in section 2.2.16.1, fractionated (section 2.2.20.2) and prepared for IEF (section 2.2.16.4) prior to loading the sample in the IEF gel (section 3.1.3.4) and performing 2D PAGE (section 2.2.16.5). In all studies the sister culture to that exposed to Cd was "spiked" with an equivalent volume of water. The protein extracted from the sister cultures underwent the same preparative procedures as that extracted from Cd exposed cultures.

3.1.3.5.1 Cd300 cells exposed to 125 μM Cd for 8 h

Cd300 cells were exposed to 125 μM Cd for 8 h. After a number of 2D PAGE gels had been collected it was apparent that two polypeptides were present in those cultures exposed to Cd and absent in the non-exposed cultures. In replicate gels the appearance of these two polypeptides, designated Cd-1 and Cd-2, was shown to be highly reproducible (Figure 11). The protein labelled 3 in Figure 11, designated Protein-3, is a constitutively expressed protein and was designated to be used as a control in later investigations (described in section 4.2.2). Cd-1 and Cd-2 appear to be greatly induced by exposure to this particular Cd treatment. There would appear to be only marginal Coomassie staining, if any, apparent in the corresponding area of those gels showing protein from non-exposed cultures. The two proteins have an apparent molecular weight of approximately 20,000, as determined by SDS PAGE. The pI of each protein was measured, calibrating the pH at particular points in the IEF gel, using a micro-pH probe (Horiba C-1). The pIs of Cd-1 and Cd-2 were determined to be 4.35 and 4.4. Protein-3 has a Mr of 20,000 and pI of 4.5.

The overall polypeptide pattern can be seen to be highly reproducible between different cultures. This would suggest that protein degradation was not taking place during preparation of the samples. The intensely stained region in the bottom left corner indicates the presence of residual ampholines from the IEF dimension. This can be removed by incubating the IEF in SDS PAGE sample buffer for a minimum of 8 h. However, this procedure also elutes some of the lower molecular weight proteins from the gel. To ensure sufficient protein was present to enable identified polypeptides to be sequenced in subsequent studies (section 2.2.21), polypeptides were

FIGURE 11

Analysis of protein extracted from Cd300 cells exposed to 125 μM Cd for 8 h

Each horizontal pair represent sister cultures. The gels to the left show polypeptides obtained from Cd-exposed cells. Those on the right from non-exposed cells.

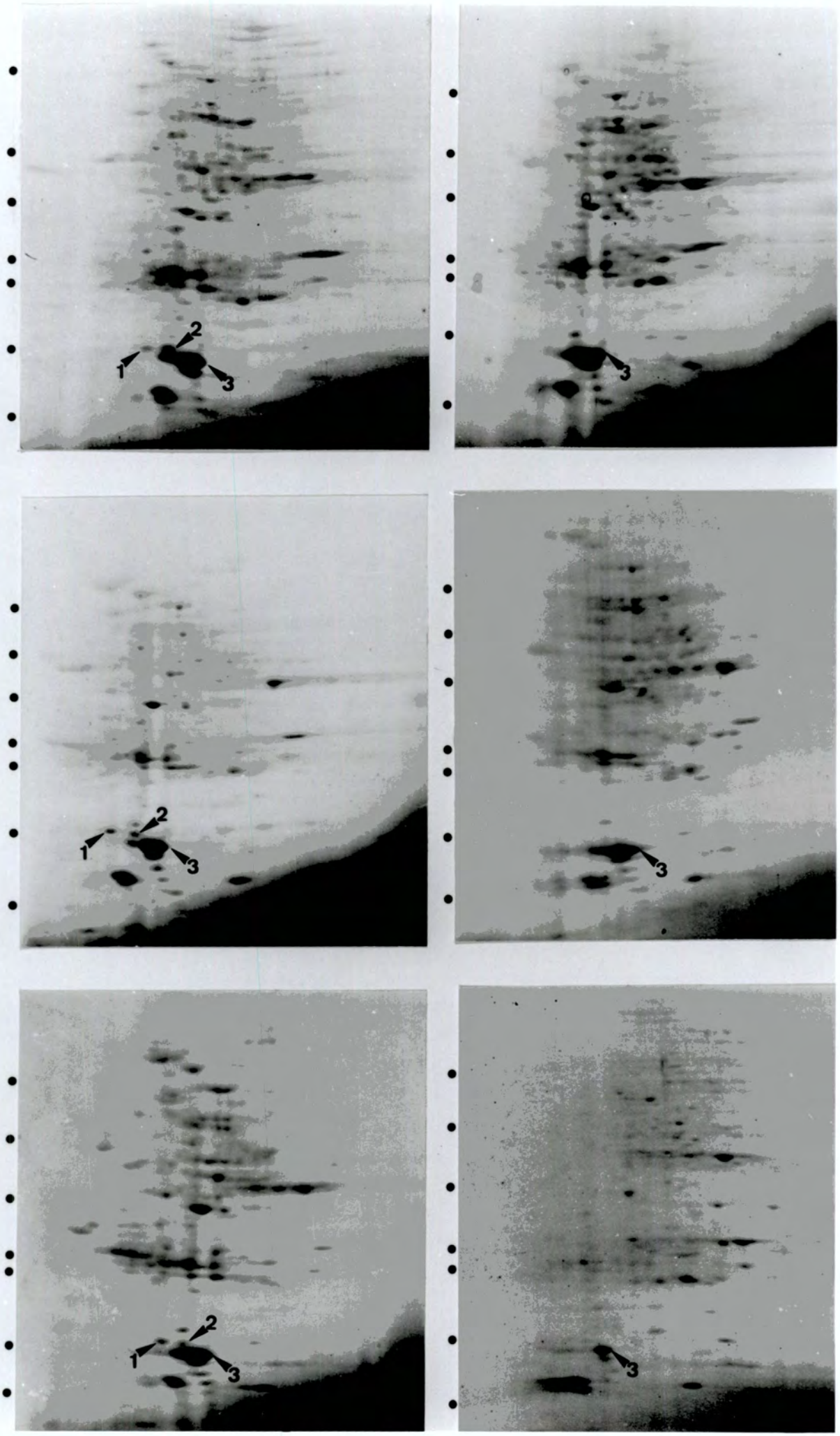
SDS PAGE size markers are indicated by small circles to the left of each gel. The Mr of the markers from the highest at the top are: 60,000 45,000 36,000 29,000 24,000 20,100 14,200.

Arrows:

1= Cd-1, a Cd-induced polypeptide, Mr 20,000, pI 4.35

2= Cd-2, a Cd-induced polypeptide, Mr 20,000, pI 4.4

3= A constitutive polypeptide to be used as a control in later studies, Mr 20,000, pI 4.45


1
2
3
1
2
3
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2
3
1
2
3
3

visualised with Coomassie blue (section 2.2.16.3).

3.1.3.5.2 Cd300 cells exposed to 125 μM Cd for 4 h

A typical effect of exposing Cd300 cells to 125 μM Cd for 4 h is shown in Figure 12. After close examination of a number of 2D PAGE gels no differences could be detected in polypeptide expression between cells exposed to 125 μM Cd for 4 h and non-exposed Cd300 cells. The most striking difference in polypeptide expression of these cultures, compared to those shown in Figure 11 was the lack of induction of Cd-1 and Cd-2.

3.1.3.5.3 Cd300 cells exposed to 250 μM Cd for 4 h

Having failed to identify the presence of any induced polypeptides after 4 h with an extracellular Cd concentration of 125 μM, the Cd-concentration was increased to 250 μM Cd. A representative result is shown in Figure 13. The induction of Cd-1 and Cd-2 was reproducibly observed in response to this Cd-treatment. The induction of a further two polypeptides (Mr 43,000 and 45,000) was also observed in the same cells treated in this manner. Unfortunately the appearance of these two polypeptides was not reproducible (4 and 5 in Figure 13). This particular exposure (250 μM Cd for 4 h) does not seem to induce Cd-1 and Cd-2 to as great an extent as exposure to 125 μM Cd for 8 h (section 3.1.3.5.1) as judged by staining intensity.

3.1.3.5.4 Cd300 cells exposed to 250 μM Cd for 8 h

A representative result of exposing Cd300 cells to 250 μM Cd for 8 h is shown in Figure 14. The appearance of the two proteins 4 and 5 (above) was not observed in any of the cells exposed to 250 μM Cd for 8 h. With the exception of Cd-1 and Cd-2 no other proteins were induced by this treatment. Both Cd-1 and Cd-2 were induced strongly by this Cd treatment as observed by staining intensity.

3.1.3.5.5 Cd300 cells exposed to 42 ^{o}C for 4 h

Cd-1 and Cd-2 were induced in Cd300 cells exposed to 42 ^{o}C for 4 h as observed by intensity of staining. A representative result is shown in Figure 15. No other polypeptide species was seen to be induced by heat shock.

FIGURE 12

Analysis of protein extracted from Cd300 cells exposed to 125 μM Cd for 4 h

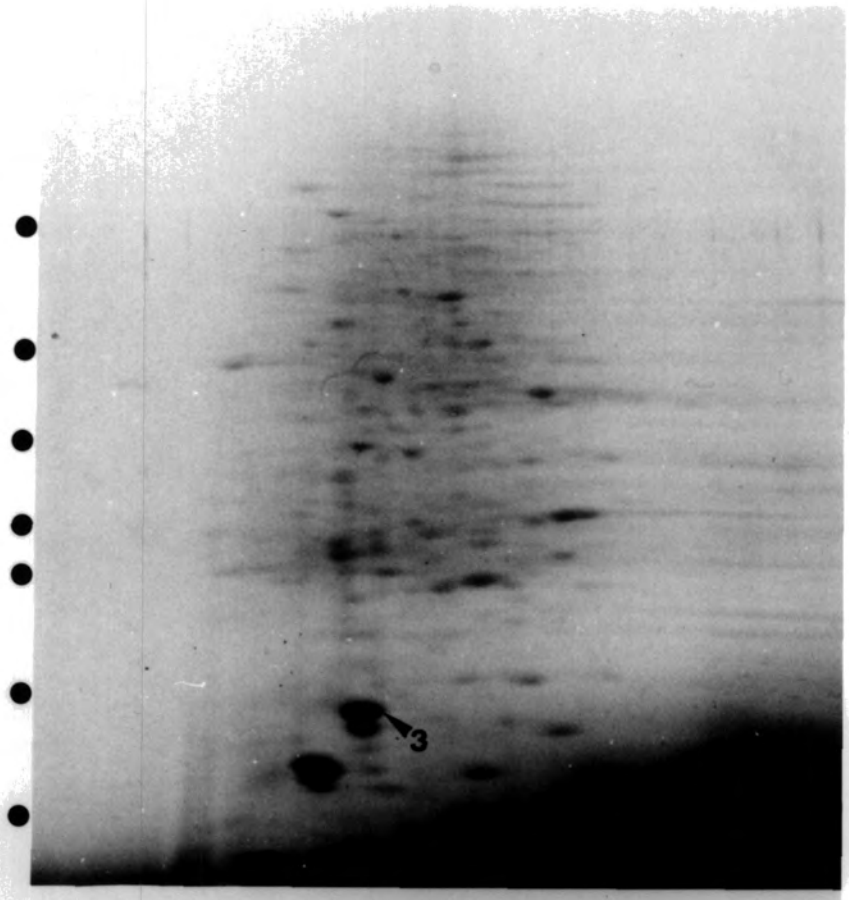
The gel at the top of the page shows polypeptides obtained from Cd-exposed cells. The gel at the bottom of the page shows polypeptides obtained from non-exposed cells.

SDS PAGE size markers are indicated by small circles to the left of each gel. The Mr of the markers from the highest at the top are: 66,000 45,000 36,000 29,000 24,000 20,100 14,200.

Arrows:

3= A constitutive polypeptide to be used as a control in later studies Mr 20,000, pI 4.45.

Note the absence of Cd-1 and Cd-2 in those cells exposed to Cd.


3

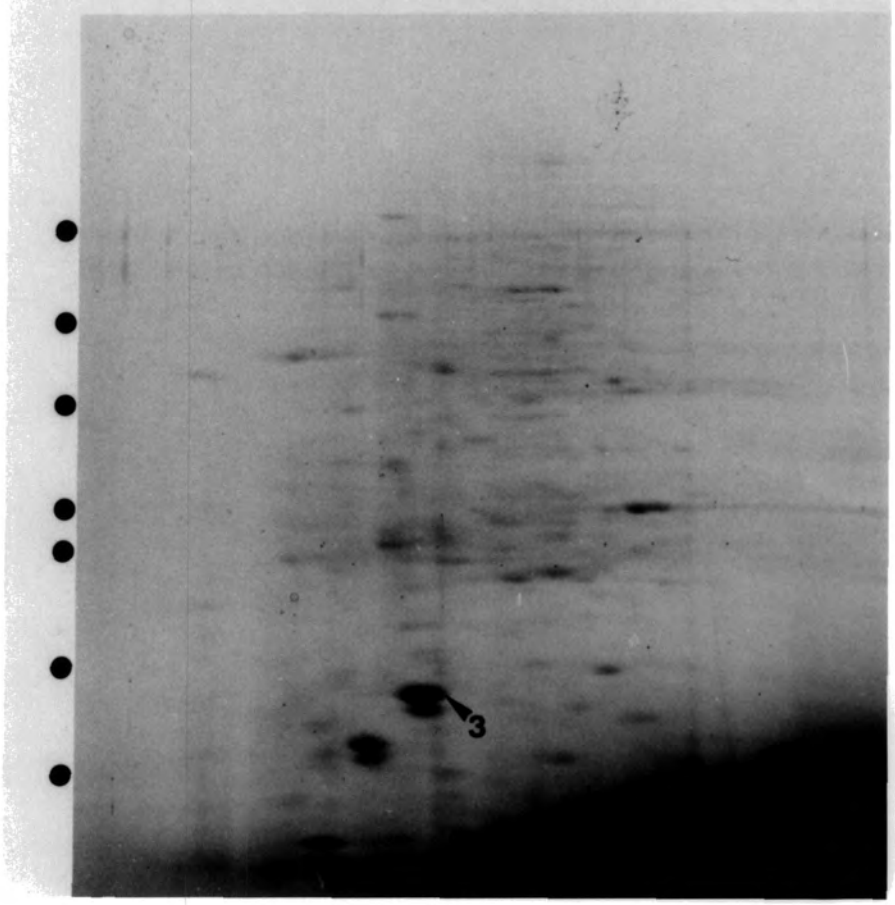

3

FIGURE 14

Analysis of protein extracted from Cd300 cells exposed to 250 μM Cd for 8 h

The gel at the top of the page shows polypeptides obtained from Cd-exposed cells. The gel at the bottom of the page shows polypeptides from non-exposed cells.

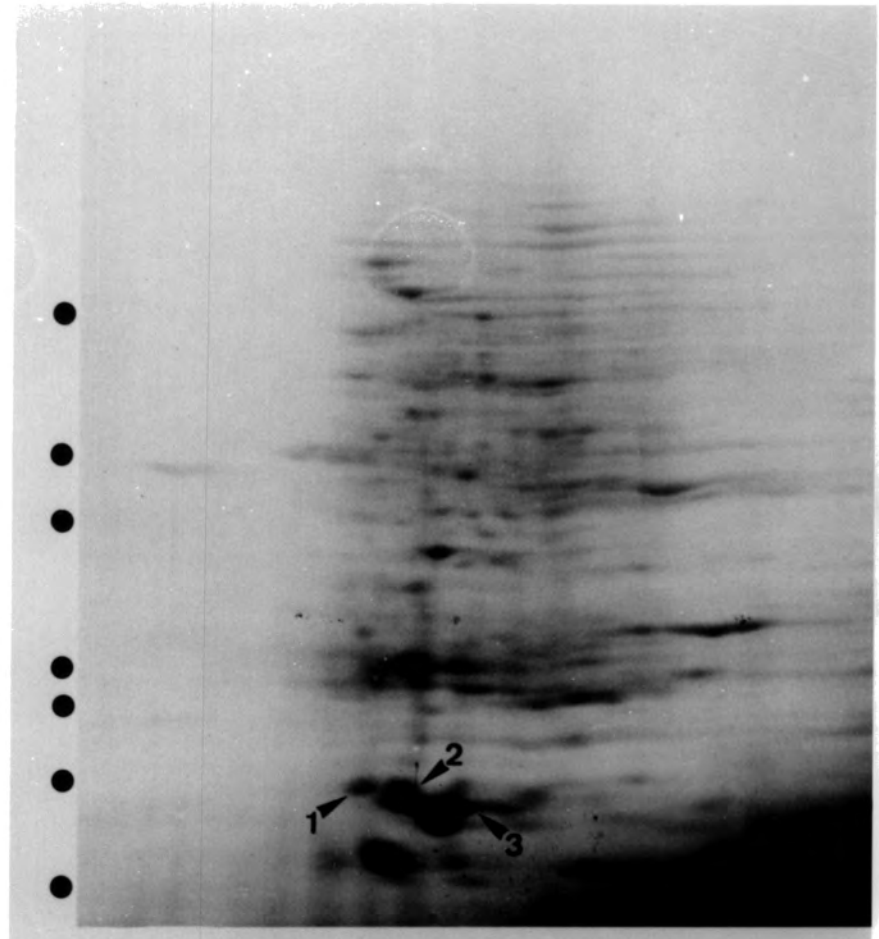
SDS PAGE size markers are indicated by small circles to the left of each gel. The Mr of the markers from the highest at the top are: 66,000 45,000 36,000 29,000 24,000 20,100 14,200

Arrows:

1= Cd-1, a Cd-induced polypeptide Mr 20,000, pI 4.35

2= Cd-2, a Cd-induced polypeptide Mr 20,000, pI 4.4

3= A constitutively expressed polypeptide to be used as a control in later studies Mr 20,000, pI 4.45


1
2
3

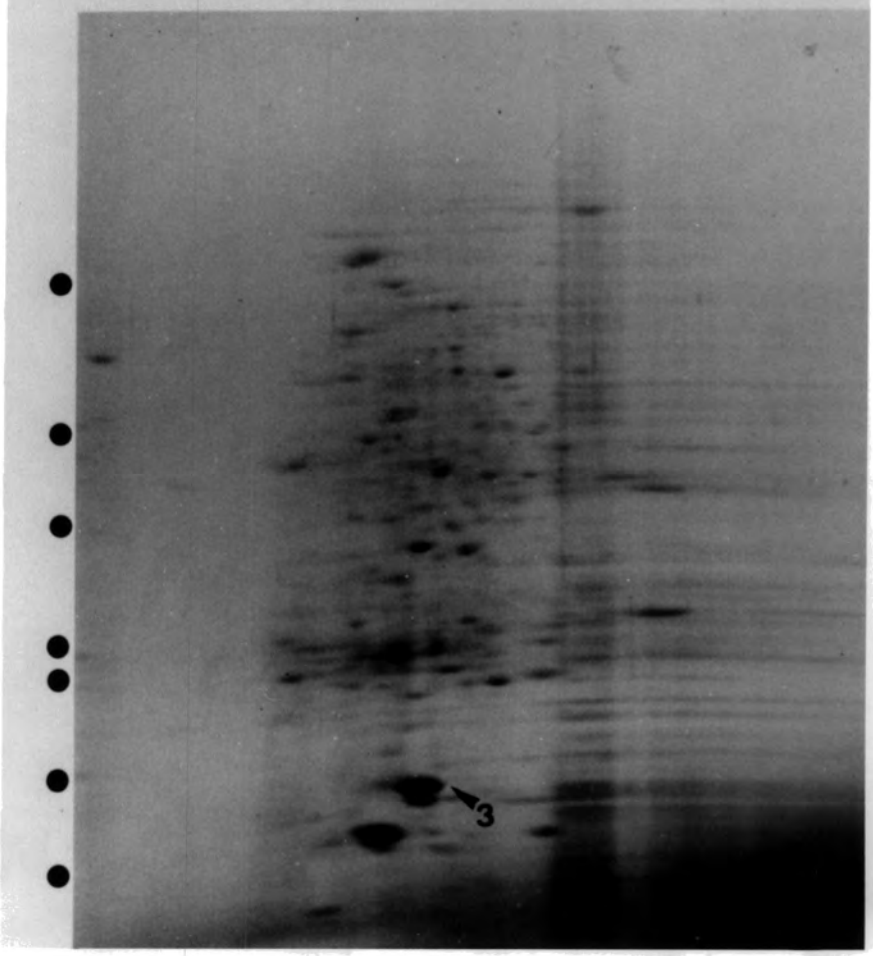

3

FIGURE 15

Analysis of protein from Cd300 cells exposed to 42 ^{o}C for 4 h

The gel at the top of the page shows polypeptides extracted from heat shocked cells. The gel at the bottom of the page shows polypeptides extracted from non-stressed cells.

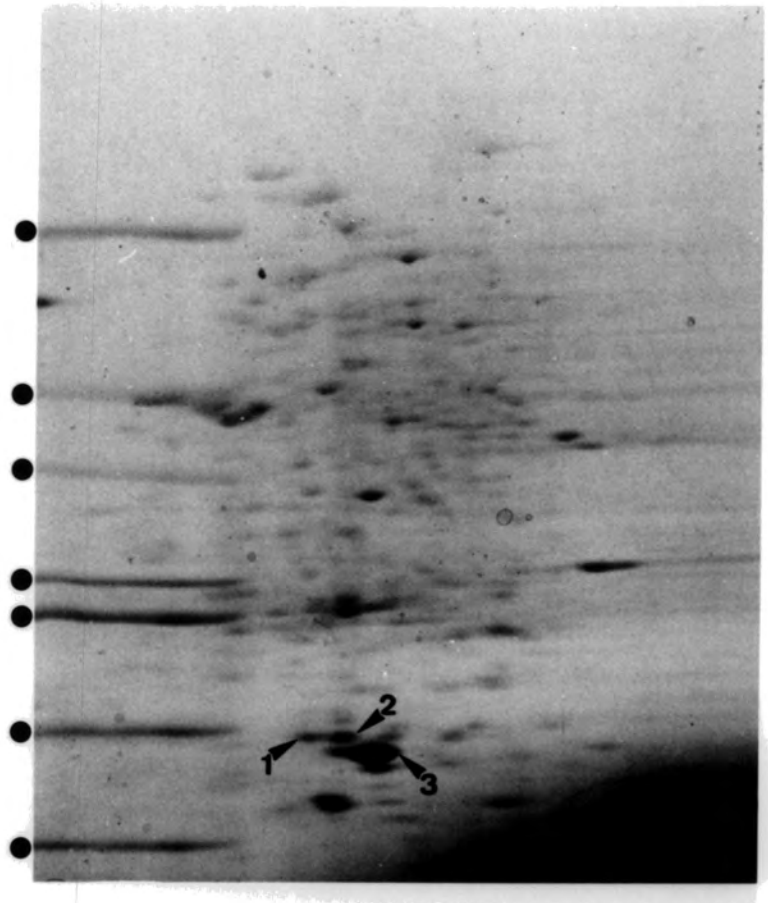
SDS PAGE size markers are indicated by small circles to the left of each gel. The Mr of the markers, from the highest at the top are: 66,000 45,000 36,000 29,000 24,000 20,100 14,200.

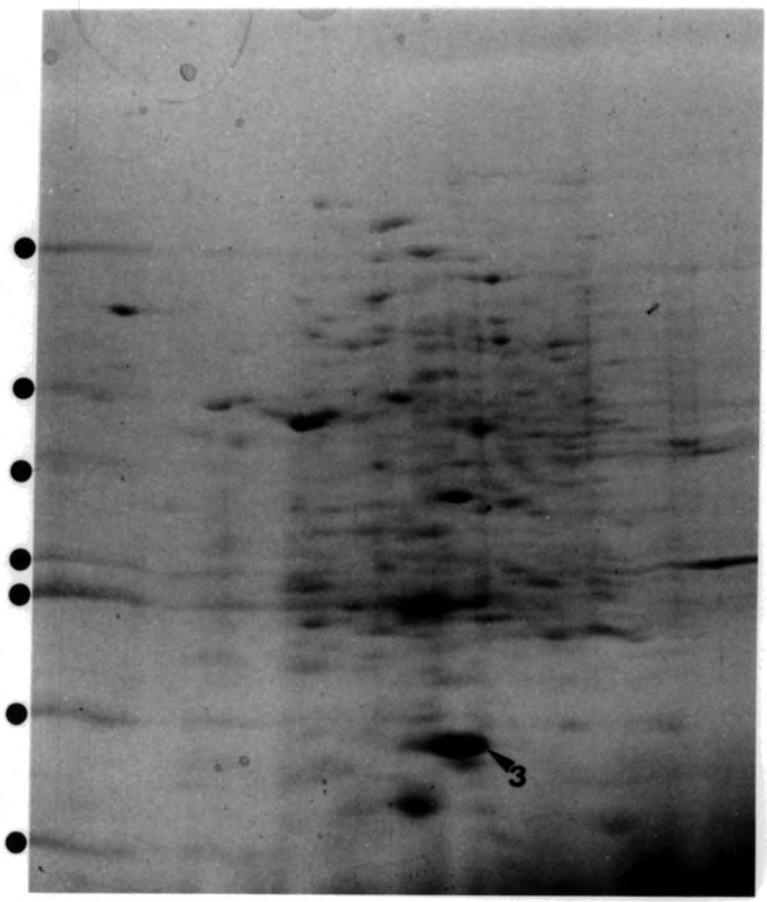
Arrows:

1= Cd-1, a Cd-induced protein Mr 20,000, pI 4.35

2= Cd-2, a Cd-induced protein Mr20,000, pI 4.4

3= A constitutively expressed polypeptide to be used as a control in future studies Mr 20,000, pI 4.45


1
2
3


3

3.1.3.5.6 WDI cells exposed to 125 μM Cd for 8 h

Samples were extracted from WDI cells exposed to 125 μM Cd for 8 h and non-exposed sister cultures and a number of gels resolved, a typical pair are shown in Figure 16. Cd-1 and Cd-2 cannot be observed from cells exposed to 125 μM Cd for 8 h.

3.1.3.5.7 Cd300 cells exposed to Cu and Zn

Typical results from analysis of extracts from Cu and Zn exposed cells are shown in Figures 17 and 18 respectively. Cd-1 and Cd-2 are visible in extracts from Zn and Cu exposed cells. These results would indicate that in addition to exposure to Cd (a non-essential metal) and heat shock, supraoptimal concentrations of essential metals Cu and Zn also induce the same two polypeptides.

3.1.4 Quantification of Cd-1 and Cd-2

To estimate the amount of protein represented by a Coomassie blue spot on a 2D PAGE gel of Cd-1 and Cd-2, known amounts of protein were electrophoresed and staining intensities compared. 200 ng, 400 ng, 600 ng, 800 ng, 1 μg and 2 μg of ovalbumin (Mr 45,000), trypsin inhibitor (Mr 20,100) and α-chymotrypsin (Mr 24,000) were electrophoresed by SDS PAGE (section 2.2.16.2). A 20 well comb was used to obtain bands of similar width to the Cd-1 and Cd-2 spots observed by 2D PAGE. The proteins were visualized by Coomassie staining (section 2.2.16.3). On analysing the gels no difference in staining intensity could be established between the identical quantities of known proteins. Comparing the known proteins with Cd-1 and Cd-2 reveals the latter two proteins have staining intensity similar to 1 μg of all the known proteins. As Cd-1 and Cd-2 have an Mr of approximately 20,000, one spot observed on 2D PAGE was estimated to represent 50 pmoles of protein.

3.1.5 Protein sequencing

In attempting to obtain amino acid sequence of Cd-1 and Cd-2 both peptides were electroblotted onto Problot (section 2.2.17). The electroblotted peptides (500 pmoles) were visualised (section 2.2.17.1) and loaded onto the sequencing block. No sequence information from either Cd-1 or Cd-2 was obtained. To investigate the possibility of Cd-1 or Cd-2 being N-terminally blocked, immobilized Cd-1 and Cd-2 were cleaved with CNBr (section 2.2.17.2). The use of cyanogen bromide (CNBr) for digestion of proteins into fragments suitable for amino acid sequencing is a well established technique (Gross, 1967). Since methionine, the target amino acid

FIGURE 16

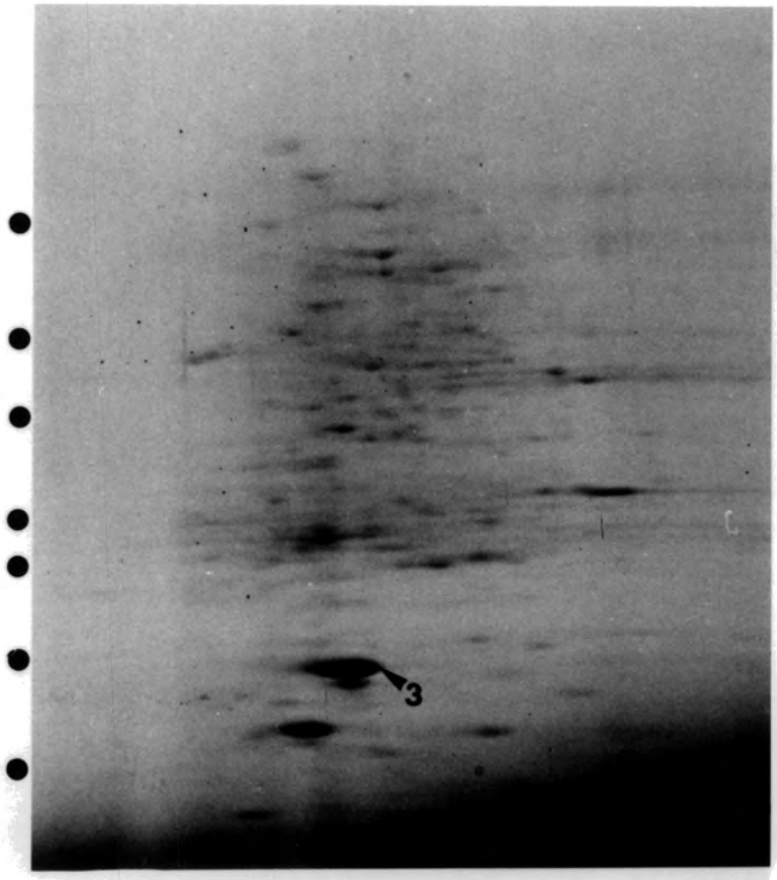
Analysis of protein extracted from WDI cells exposed to 125 μM Cd for 8 h

The gel at the top of the page shows polypeptides obtained from Cd-exposed cells. The gel at the bottom of the page shows polypeptides from non-exposed cells.

SDS PAGE size markers are indicated by small circles to the left of each gel. The Mr of the markers from the highest at the top are: 66,000 45,000 36,000 29,000 24,000 20,100 14,200

Arrows

3= A constitutively expressed polypeptide to be used as a control in later studies Mr 20,000, pI 4.45


3

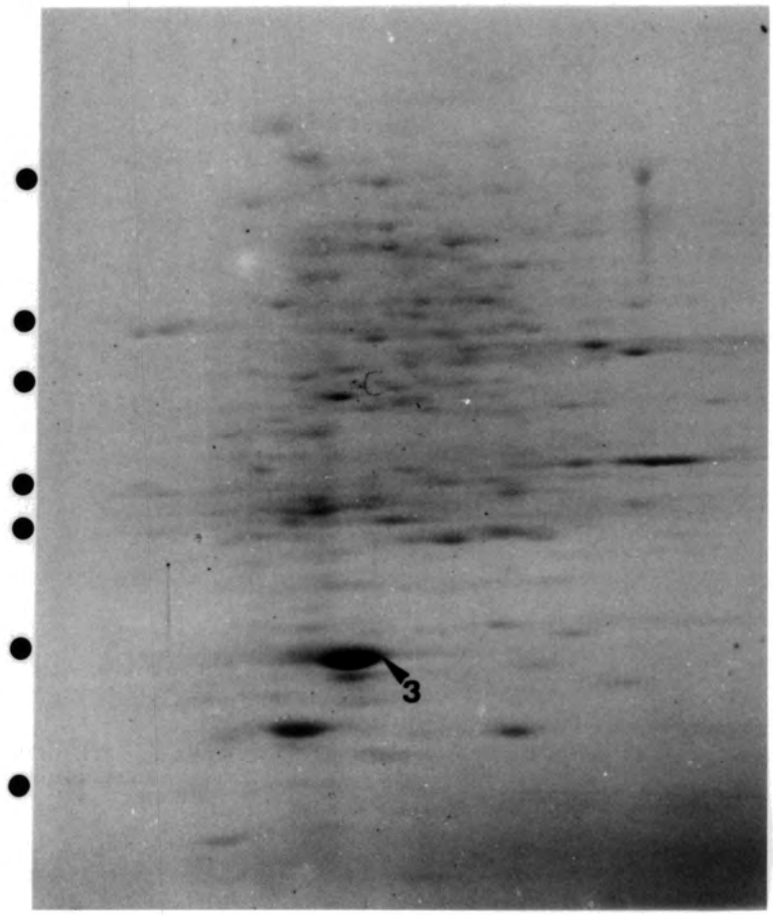

3

FIGURE 17

Analysis of protein extracted from Cd300 cells exposed to 125 μM Cu for 8 h

The gel at the top of the page shows polypeptides obtained from Cu-exposed cells. The gel at the bottom of the page shows polypeptides from non-exposed cells.

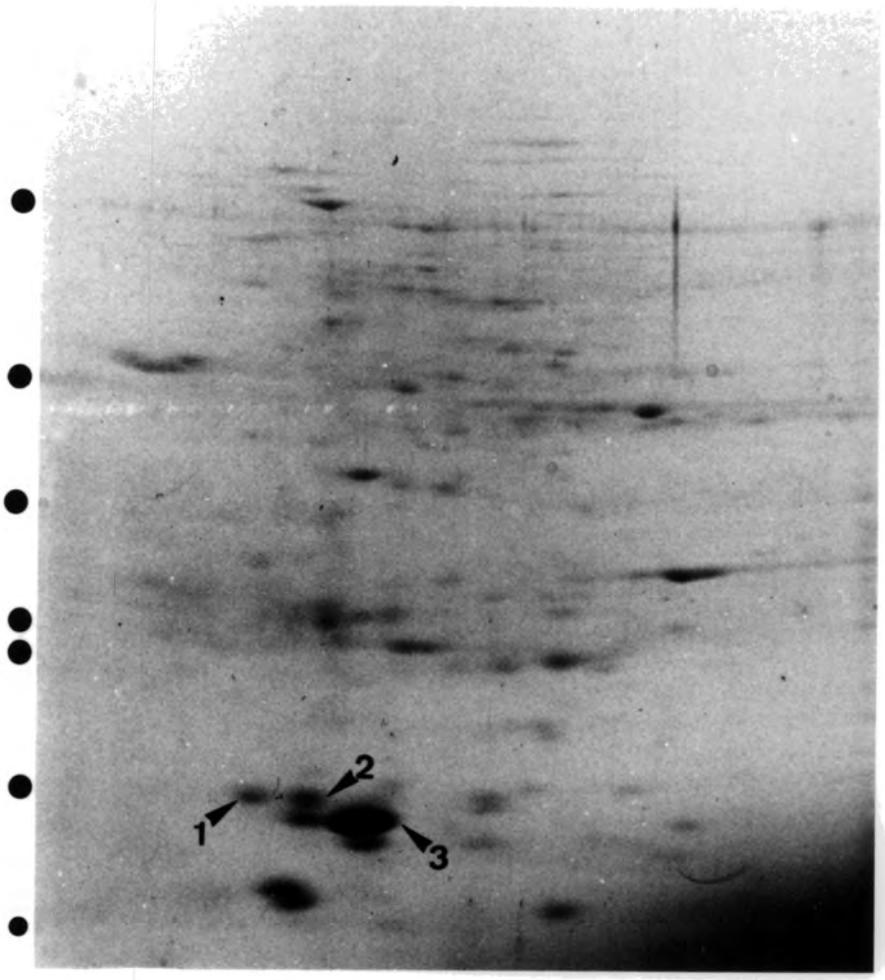
SDS PAGE size markers are indicated by small circles to the left of each gel. The Mr of the markers from the highest at the top are: 66,000 45,000 36,000 29,000 24,000 20,100 14,200.

Arrows

1= Cd-1, a Cd-induced polypeptide Mr 20,000, pI 4.35

2= Cd-2, a Cd-induced polypeptide Mr 20,000, pI 4.4

3= A constitutively expressed polypeptide to be used as a control in later studies Mr 20,000, pI 4.45


1
2
3

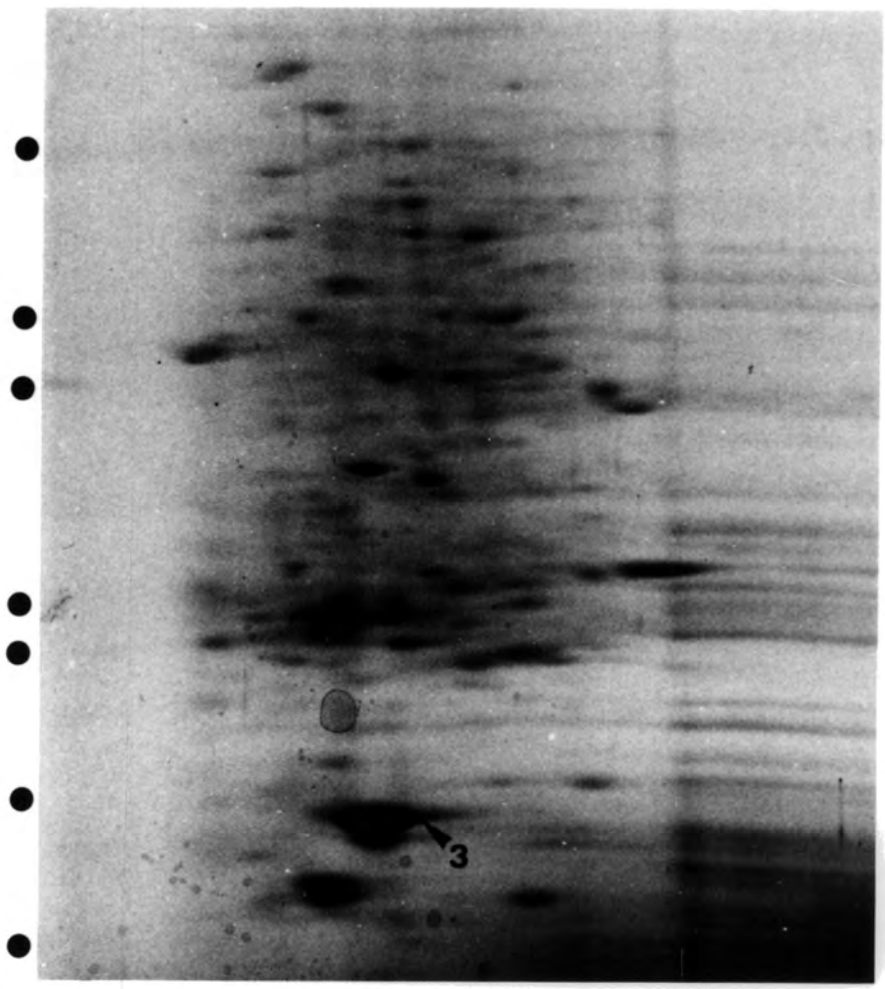

3

FIGURE 18

Analysis of protein extracted from Cd300 cells exposed to 125 μM Zn for 8 h

The gel at the top of the page shows polypeptides obtained from Zn-exposed cells. The gel at the bottom of the page shows polypeptides from non-exposed cells.

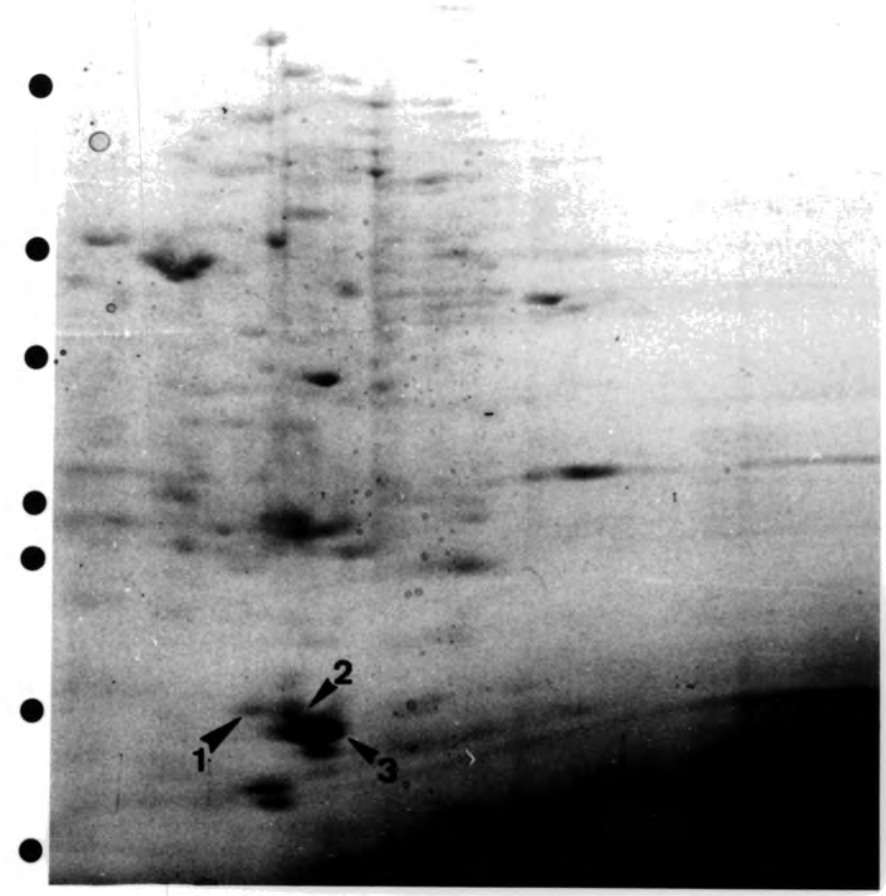
SDS PAGE size markers are indicated by small circles to the left of each gel. The Mr of the markers from the highest at the top are: 66,000 45,000 36,000 29,000 24,000 20,100 14,200.

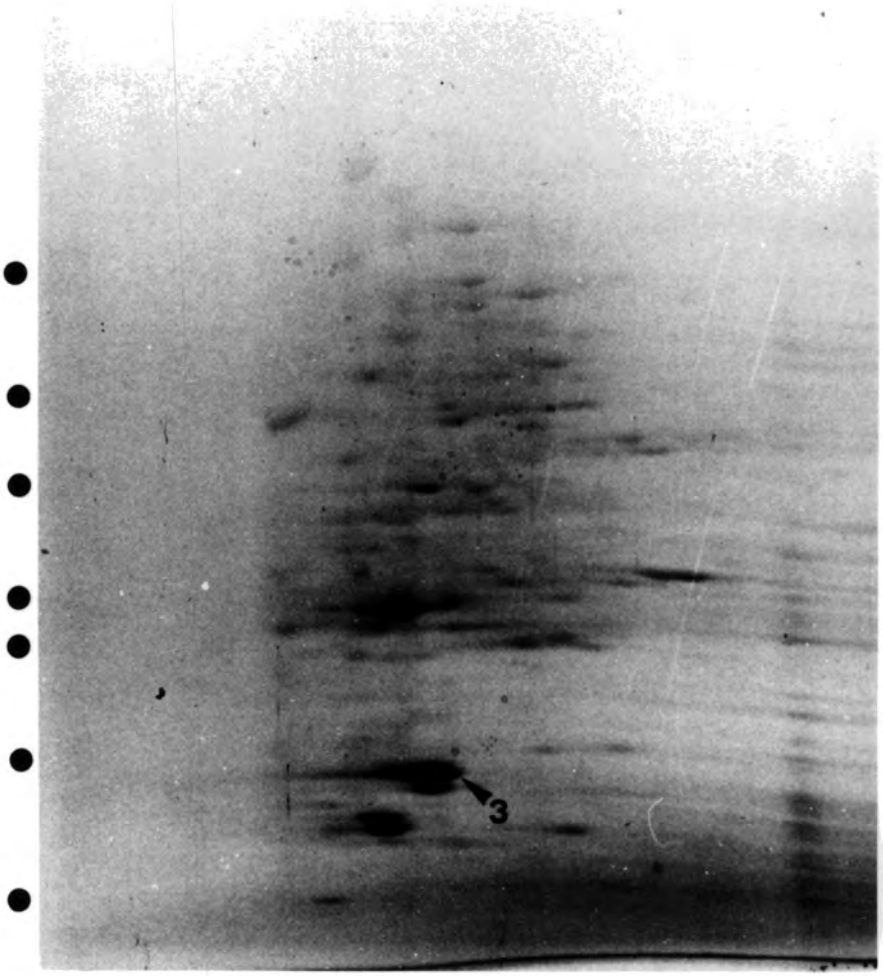
Arrows

1= Cd-1, a Cd-induced polypeptide Mr 20,000, pI 4.35

2= Cd-2, a Cd-induced polypeptide Mr 20,000, pI 4.4

3= A constitutively expressed polypeptide to be used as a control in later studies Mr 20,000, pI 4.45


1
2
3


3

for CNBr is relatively uncommon in proteins, the digestion usually produces long peptides. CNBr breaks peptide bonds on the carboxyl side of methonine residues. Upon attempting to sequence the CNBr cleaved Cd-1 and Cd-2 amino acids were detected in the the microsequencer column eluant. This indicated that CNBr cleaved both Cd-1 and Cd-2. It was therefore necessary to separate the fragments resulting from the cleavage. The individual fragments could then be sequenced.

Sequencing Protein-3 gave reproducible sequence of the N-terminal 30 amino acids. This sequence is shown in Figure 19. On comparing the sequence to the NBRF data base it was shown that Protein-3 had 69 % similarity to the Birch pollen allergen *Betv*1 (Breitender *et al.*, 1989) and pathogenesis related proteins from parsley (Somssich *et al.*, 1988).

3.1.6 Electrophoretic separation of cleavage products

In order to separate the peptide fragments generated by CNBr cleavage of Cd-l and Cd-2 the cleaved products were electrophoresed on 20 % SDS PAGE (as described for 7.5-15% gel section 2.2.16.2). On staining the gel with silver (section 2.2.16.3) only a smear could be seen in the lane loaded with the cleavage products. Urea SDS-PAGE and tricine-SDS PAGE (section 2.2.16.2), both designed to separate small polypeptides, also proved to give inadequate resolution to allow detection of individual cleaved peptides.

3.1.7 HLPC separations involving β-lactoglobulin

To increase resolution, reverse phase HPLC was employed to separate the cleavage products of Cd-1 and Cd-2 (section 2.2.20.3). In order to block the unspecific binding sites on the column, lysozyme was passed through the column. A control sample (100 µl of 2 % (w/v) SDS, 20 mM DTT) gave a single small peak. The two channels of the recorder were set to 0.1 Abs., 10 mV and 0.5 Abs., 10 mV. Lysozyme was dissolved in 2 % (w/v) SDS, 20 mM DTT and 200 pmoles eluted through the column. In addition to the small peak detected as a consequence of passing SDS, DTT solution through the column, a large single peak was detected on the recorder. This procedure was repeated several times.

3.1.7.1 Elution of β-lactoglobulin

200 pmoles of β–lactoglobulin were eluted through the column using the gradient designed to elute intact proteins (section 2.2.20.3). Two peaks were observed, one which had the same retention time as a component detected in the 2 % (w/v) SDS, 20 mM DTT buffer. The peak

FIGURE 19

Amino acid and cDNA sequence of Protein-3

The upper line denotes the cDNA sequence and the lower line the amino acid sequence.

Amino acid sequence was determined using a 447A protein microsequencer (Applied Biosystems) with a 120 PTH-amino acid analyser (Applied Biosystems).

The corresponding cDNA was amplified using PCR. Primers were designed to the sequences shown in bold type. The primer towards the N-terminus had the sequence, 5' CAA/G ACI TAT/C ACI CAT/C GA 3' and the primer towards the C-terminus had the sequence, 5' AC IAA/G A/GTT A/GTC A/GAA A/GTC 3'. A Hybaid "Intelligent heating block" was programmed to have a denaturing step of 92 ^{O}C for 1.5 min, a primer annealing step of 45 ^{O}C for 1.5 min and an extension step of 72 ^{O}C for 4 min, this cycle being repeated x32. A final single cycle with a denaturing step of 92 ^{O}C for 1.5 min, annealing step of 45 ^{O}C for 1.5 min and an extension step of 72 ^{O}C for 4 min was programmed.

A primer was made to correspond to the middle region of sequence (underlined) with which to probe multiple amplification products. Its sequence was, 5' T/CTI TTT/C AAA/G GCI T/CTI GT 3'.

The PCR product was electroeluted from an agarose gel and cloned into the *Hinc* II site of pUC19. Sequence was obtained in both directions using an automated DNA sequencer (370A Applied Biosystems)

```
          CAG  ACG  TAT  ACG  CAT  GAA
Gly  Val  Gln  Thr  Tyr  Thr  His  Glu

TCC  ACA  ACC  ACA  ATT  TCC  CCA  ACA
Ser  Thr  Thr  Thr  Ile  Ser  Pro  Thr

ACG  CTA  TTC  AAA  GCT  TTG  GTT  CTT
Arg  Leu  Phe  Lys  Ala  Leu  Val  Leu

GAT  TTT  GAC  AAC  CTC  G
Asp  Phe  Asp  Asn  Leu  Val
```

representing the β-lactoglobulin was collected and microsequenced to estimate the yield after HPLC (section 3.1.8).

3.1.7.2 Elution of β-lactoglobulin electroblotted and released from Problot

200 pmoles of β-lactoglobulin were electrophoresed by 1D SDS PAGE (section 2.2.16.2), electroblotted (section 2.2.17), stained (section 2.2.17.1) and released from the membrane (section 2.2.17.3). The β-lactoglobulin released from the Problot was eluted through the HPLC column using a programme to elute intact proteins (section 2.2.20.3). A chromatogram similar to that described for the elution of β-lactoglobulin (section 3.1.7.1) was obtained. The fraction of eluant containing the β-lactoglobulin was collected and sequenced (section 3.1.8) to estimate the yield after electroblotting, elution off Problot and passage through HPLC.

3.1.7.3 Elution of β-lactoglobulin electroblotted, cleaved and released from Problot

The study described in section 3.1.7.2 was repeated using β-lactoglobulin which had undergone the additional step of CNBr cleavage (section 2.2.17.2) prior to being released from Problot (section 2.2.17.3). This sample was eluted through the column using the gradient to separate small peptides (section 2.2.20.3). Prior to separating the sample, 100 µl of buffer (2 % (w/v) SDS, 20 mM DTT) was eluted through the column after a smooth base line was established. No deviation from the base line was observed. Two channels were set on the recorder to 0.1 Abs and 0.5 Abs, 5 mV and 10 mV respectively. Five peaks were observed on the chromatogram. The five peaks were considered to represent peptide fragments of β-lactoglobulin, this is consistent with the observation that β-lactoglobulin contains four methionine residues, sites of CNBr cleavage. Fractions of eluant represented by these peaks were collected and microsequenced to establish the yield of a sample after such treatment. Following this separation, a piece of Problot which had been used to electroblot the β-lactoglobulin but which had no protein bound to it as observed by Coomassie blue staining (section 2.2.17.1) was subjected to the same conditions used to release protein from problot as the previous sample (section 3.1.7.2). The solution obtained from that step was eluted through HPLC in the same manner as the cleaved products (above). Small deviations were seen from the base line position, however these were also observed in the cleaved β-lactoglobulin chromatogram in addition to the five major peaks. All samples were collected from HPLC for microsequencing and were dried down to a small volume and resuspended in 0.1 % (v/v) TFA.

3.1.8 Sequencing of fractions collected from HPLC

200 pmoles of pure β-lactoglobulin were sequenced. The repetitive yield of the PTH-amino acids was recorded as 100 pmoles. This is consistent with observed repetitive amino acid yields obtained in protein microsequencing, where yields are recorded as approximately half the amount of protein loaded into the sequencer.

200 pmoles of β-lactoglobulin eluted off the HPLC (section 3.1.7.1) was microsequenced and yields of 100 pmoles were recorded for repetitive yield of PTH-amino acids detected.

On microsequencing 200 pmoles of β-lactoglobulin on PVDF membrane, repetitive yields of PTH amino acids were determined as 100 pmoles. This indicated little loss of protein due to electroblotting and that blotting did not make the intact protein refractory to Edman degradation.

The 200 pmoles of β-lactoglobulin which had been blotted and released from Problot and then passed through HPLC (section 3.1.7.2) showed reduced yields of initially detected PTH amino acids of approximately 60 pmoles. This represents a 40 % loss of protein on releasing the protein from Problot.

When the electroblotted sample had been cleaved with CNBr and eluted off Problot and the cleavage products passed through HPLC (section 3.1.7.3) the repetitive yields of PTH-amino acids detected by the sequencer were again lower. Two CNBr fragments were sequenced. The first had a sequence corresponding to the N-terminus of β-lactoglobulin. The sequence of the second fragment was found to correspond to internal sequence of β-lactoglobulin. Yields were approximately of the order of 15 pmoles indicating a loss of 85 % over yields obtained from electroblotted β-lactoglobulin.

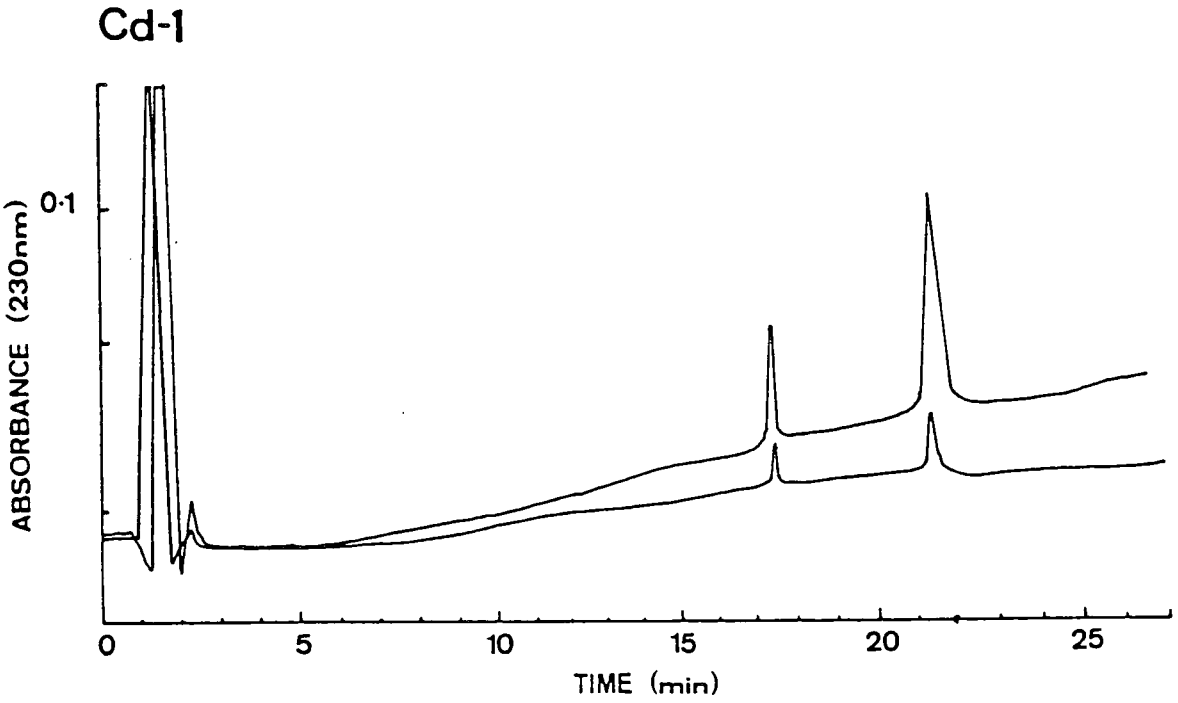
3.1.9 HPLC separation of Cd-1 and Cd-2 CNBr cleavage products

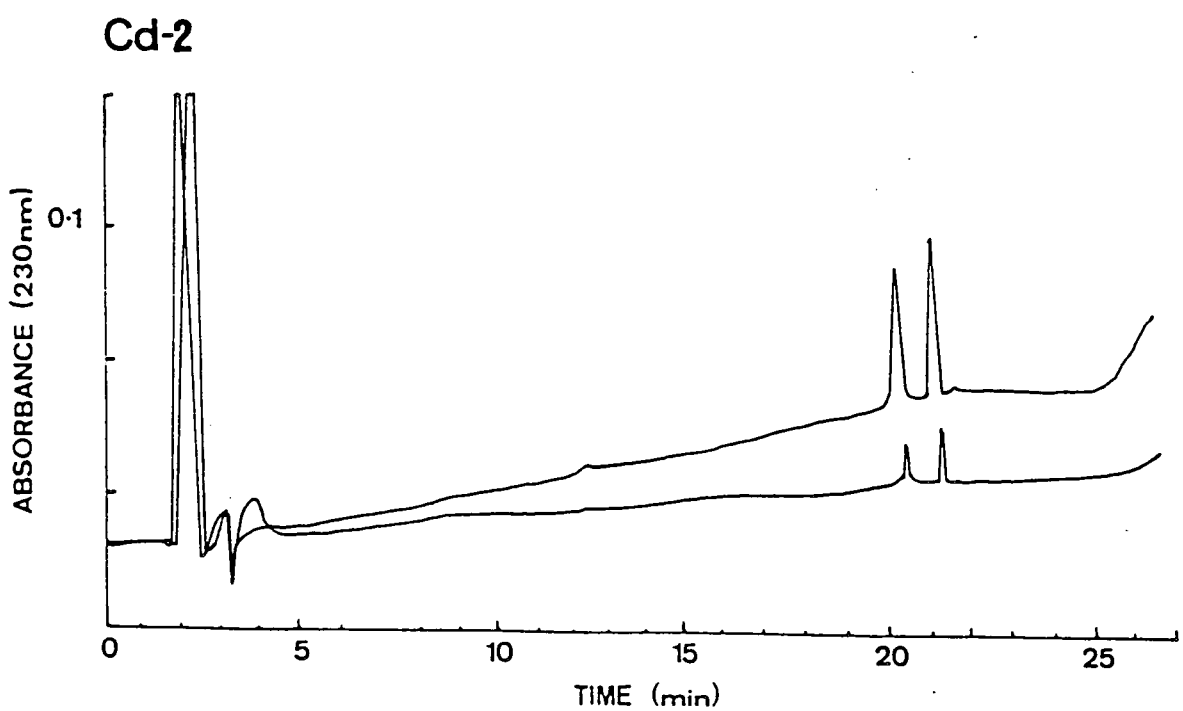
Having demonstrated that the procedures used to separate CNBr cleavage products were effective for peptide fragments of β-lactoglobulin, the fragments collected from HPLC being used to determine protein sequence (section 3.1.9), identical procedures were used to obtain individual peptide fragments resulting from CNBr cleavage of Cd-1 and Cd-2. Ten blots of both Cd-1 and Cd-2, approximately 500 pmoles, were used to generate the two samples. The results of the HPLC elutions are shown in Figure 20. The second peak observed in each of the two chromatograms has the same retention time (20.5 min). This may indicate that the two proteins Cd-1 and Cd-2 are very similar. The control experiment using Problot with no protein bound to it, which had been subjected to identical procedures, resulted in no major deviations from the base line, any minor deviations which were observed were paralleled in the chromatograms obtained

FIGURE 20

CNBr cleavage products of Cd-1 and Cd-2 separated by reverse phase HPLC

Cd-1 and Cd-2 were electroblotted onto Problot, CNBr cleaved, released from Problot and the cleavage products eluted through reverse phase HPLC. HPLC elution buffers were 0.1 % (v/v) TFA (A) and 80 % (v/v) acetonitrile, 0.1 % (v/v) TFA, (B). The gradient was: 5 % (v/v) B to 0.1 min, to 40 % (v/v) B at 35 min to 100 % (v/v) B at 42 min and then a decline to 5 % (v/v) B at 45 min for 5 min.


Cd-1
ABSORBANCE (230nm)
0·1
0
5
10
15
20
25
TIME (min)


Cd-2
ABSORBANCE (230nm)
0·1
0
5
10
15
20
25
TIME (min)

from Cd-1 and Cd-2.

3.1.10 Sequencing of Cd-1 and Cd-2 CNBr cleavage products

Fractions of eluant corresponding to the peaks observed in section 3.1.9 were collected and individually applied to the microsequencer. No sequence information was determined for either Cd-1 or Cd-2.

3.1.11 Amplification of genomic DNA corresponding to Protein-3

Two redundant oligonucleotide primers were designed from the 30 amino acid sequence obtained from Protein-3 which corresponded to two regions of the protein shown underlined in Figure 19. The sequence of the primer corresponding to the N-terminal region (A) was 5' CAA/G ACI TAT/C ACI CAT/C, GA 3' and an inverse complementary primer designed toward the C-terminal end of the protein sequenced (B) was 5' AC IAA/G A/GTT A/GTC A/GAA A/GTC 3'. These primers were used to amplify the corresponding region of genomic DNA (section 2.2.13.1). On analysing the PCR products on a 1 % (w/v) agarose gel (section 2.2.6) a number of unspecific amplification products were observed. The PCR reaction was repeated using annealing temperatures of 50 ^{o}C and 55 ^{o}C. No reduction in the number of non specific amplification products was seen using a primer annealing temperature of 50 ^{o}C and at 55 ^{o}C no amplification products were observed at all. A probe (C) was designed to correspond to the middle region of the obtained protein sequence underlined (broken line) in Figure 19. The sequence of this probe was 5' T/CTI TTT/C AAA/G GCI T/CTI GT 3'. This was used as a probe to identify the PCR amplification products which corresponded to the Protein-3 amino acid sequence from amongst the non-specific amplification products. This oligonucleotide was end-labelled with ^{32}P-ATP (section 2.2.8) and used to probe a Southern blot of the gel on which the numerous PCR amplification products had been separated (section 2.2.9/10). The filters were washed to a final stringency of 1 x SSC at room temperature for 15 min before being exposed to X-ray film. Even following such a low stringency washing no signal was detected, indicating that the probe designed from the middle region of the protein did not hybridise to any of the PCR amplification products.

3.1.12 Amplification of cDNA corresponding to Protein-3

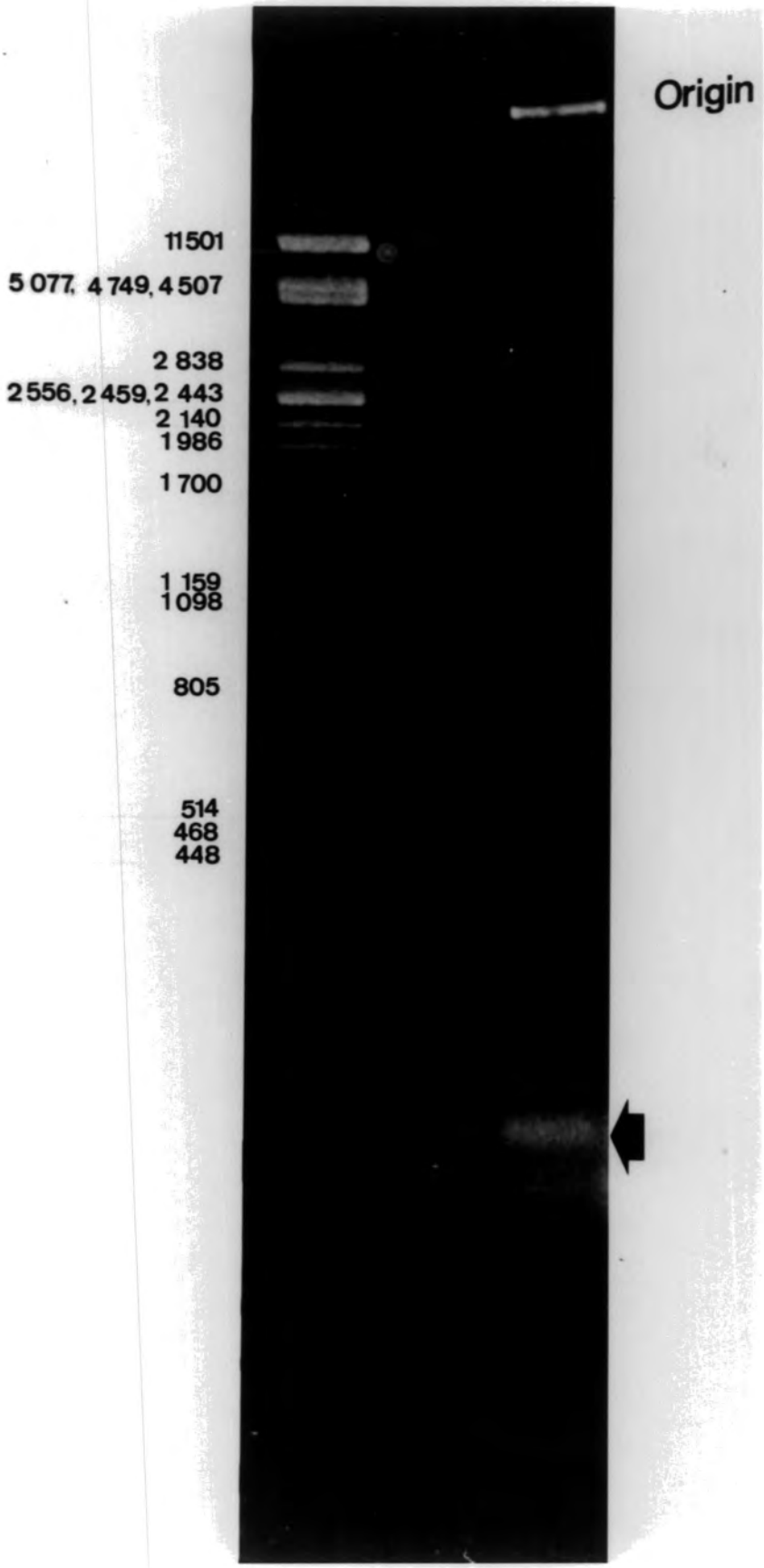
The same two primers A and B (section 3.1.11) were used to amplify a fragment from total cDNA (section 2.2.13.2). The reaction products separated on a 2 % (w/v) agarose gel (section 2.2.6) are shown in Figure 21. A single PCR amplification product of the expected size is

FIGURE 21

Amplification of cDNA corresponding to the N-terminal protein sequence of Protein-3

Lane 1= Size markers, λ DNA restricted with *Pst* 1

Lane 2= Amplification product.


Origin
11501
5 077, 4 749, 4 507
2 838
2 556, 2 459, 2 443
2 140
1 986
1 700
1 159
1 098
805
514
468
448

observed. Following isolation from the gel (section 2.2.7) the DNA fragment was blunt ended (section 2.2.5.5) and ligated (section 2.2.5.6) into pUC19 restricted with *Hinc* II to generate blunt ends (2.2.5.2). *E. coli* were transformed (section 2.2.4.2), colonies harbouring the recombinant plasmid were detected by *in situ* hybridisation (section 2.2.11) using the original 82 bp fragment labelled with ^{32}P-dCTP (section 2.2.8) as a probe. The plasmid was recovered (section 2.2.3.4) and sequenced (section 2.2.14). The sequence is shown in Figure 19. This sequence can be seen to contain an ORF which exactly aligns to the protein sequence.

The 82 bp fragment amplified above was labelled with [α-^{32}P]dCTP (section 2.2.8) and used to probe a Southern blot of *D. innoxia* genomic DNA. Genomic DNA was isolated from *D innoxia* (section 2.2.3.1), restricted (section 2.2.5.2.1), separated on a 0.8 % (w/v) agarose gel (section 2.2.6), blotted onto nylon (section 2.2.9) and probed (section 2.2.10). However, upon developing the autoradiogram the probe had not hybridised to the genomic DNA. After several unsuccessful attempts it was decided to try and amplify a longer cDNA corresponding to Protein-3, by RACE PCR.

3.1.13 Amplification of a full length cDNA

Rapid amplification of cDNA ends (RACE) PCR was carried out as described by Frohman *et al.* (1988). PCR was performed using a primer designed from the N-terminal amino acid sequence of Protein-3 (A) (section 3.1.11) and a third primer, 5 ' GCG ATA TCT CGA TCC $(T)_{17}$ 3 ' (gift, J. Gatehouse) designed to bind to the polyA tail of the cDNA. The heating block was programmed in an identical manner to that described in section 2.2.13.2. Multiple amplification products were observed on analysing the PCR products on a 1 % (w/v) agarose gel (section 2.2.6). The 82bp cDNA clone obtained previously (section 3.1.12) was labelled with ^{32}P-dCTP (section 2.2.8) and used to probe the amplification products. On analysing the autoradiograph this was seen to have hybridised to a single band of approximately 850 bp. These results are shown in Figure 22.

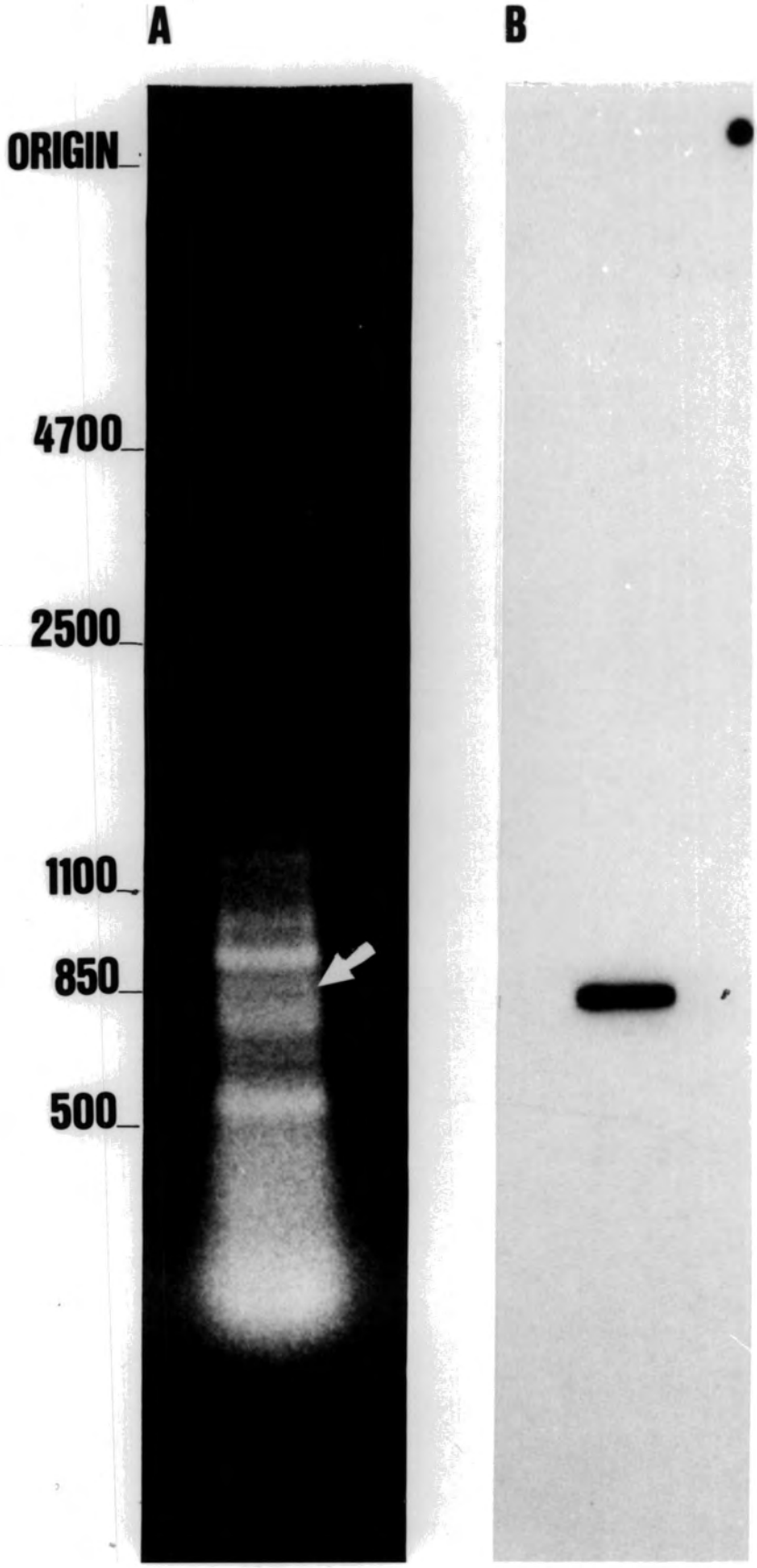
Attempts were made to clone the RACE product. The primer designed to hybridise to the polyA tail of the cDNA from which the RACE product was amplified contained the recognition sites for the restriction enzymes *EcoR* V, *Bam* HI and *Xho* I. After isolation of the fragment from the gel (section 2.2.7) the ends were blunted with T4 polymerase (section 2.2.5.5). The fragment was then restricted with one of the above enzymes (section 2.2.5.2) and ligated into pUC19, restricted with *Sma* I, to generate a blunt end, and also the same enzyme as that used to restrict the isolated fragment. *E. coli* were transformed (section 2.2.4.2) and in order to isolate cells harbouring the recombinant plasmid *in situ* hybridisations were carried out using [^{32}P]dCTP-labelled 82 bp sequence corresponding to the N-terminal region of Protein-3 as a probe.

FIGURE 22

Amplification of full length cDNA corresponding to Protein-3

A. RACE amplification was performed using the two primers, N-terminal primer 5'ACA/G ACI TAT/C ACI CAT/C GA 3' and a primer designed to bind to the polyA tail of the cDNA 5' GCG ATA TCT CGA TCC $(T)_{17}$ 3'. A Hybaid "Intelligent heating block" was programmed to have a denaturing step of 92 ^{O}C for 1.5 min, a primer annealing step of 45 ^{O}C for 1.5 min and an extension step of 72 ^{O}C for 4 min, this cycle being repeated x32. A final single cycle with a denaturing step of 92 ^{O}C for 1.5 min, annealing step of 45 ^{O}C for 1.5 min and an extension step of 72 ^{O}C for 4 min was programmed.

B. The PCR amplification products were electrophoresed on a 1 % (w/v) agarose gel, Southern blotted and probed with the 82 bp cDNA clone corresponding to the N-terminus of Protein-3.


A
B
ORIGIN
4700
2500
1100
850
500

However, despite numerous attempts, cloning of the RACE product proved unsuccessful.

Taq polymerase has a template-independent terminal transferase activity, which results in the addition of a single nucleotide at the 3' end of the fragment. This fragment is almost exclusively an adenosine due to the strong preference of the polymerase for dATP. Adding a dTTP to the 3' end of a blunt ended vector creates complimentary 3' overhangs, which allows ligation with the efficiency of "sticky end" cloning (refs. in Marchuk *et al.*, 1991). A T-vector was constructed as described by Marchuk *et al.* (1991) using *EcoR* V restricted pUC19. The RACE product was isolated from the gel (section 2.2.7) and ligated into the T-vector (section 2.2.5.6) and *E. coli* were transformed (section 2.2.4.2). To identify cells harbouring the recombinant plasmid *in situ* hybridisations were carried out using [^{32}P]dCTP-labelled 82 bp sequence corresponding to the N-terminal region of Protein-3 as a probe. This cloning strategy also proved unsuccessful.

3.2 ISOLATION OF Cd-INDUCED cDNA FROM A *D. innoxia* cDNA LIBRARY

3.2.1 Production of antibodies against Cd-1 and Cd-2

The peptides Cd-1 and Cd-2, identified by 2D PAGE (section 3.1.3.5) were collected and used to raise an immune response, as described in section 2.2.22.

3.2.1.1 Antibody specificity

Western blots of *D. innoxia* protein were probed with antiserum raised against Cd-1 and Cd-2 (section 2.2.24) to establish the specificity of the antibodies. To establish a background level of antigenicity western blots were also incubated with serum from the preimmune bleed. On western blots from *D. innoxia* protein separated by 1D SDS PAGE incubated with the pre-immune serum three very faint bands were observed on development of the colour reaction. When western blots of protein separated by 2D PAGE were incubated with pre-immune serum, no reaction of any protein to the serum was detected by observable colour development. The reaction seen on western blots of protein separated by 1D SDS PAGE may have been due to a complex of proteins which when separated into individual components were no longer recognisable by the antibodies present in the pre-immune serum. Analysis of the antiserum raised against Cd-1 and Cd-2 was performed separately in an identical manner. On analysing the antigenicity of the antiserum by its reaction with proteins on a western blot of a 2D PAGE gel, no antigenicity could be detected against any individual protein species including Cd-1 and Cd-2. In an attempt to detect an antigenic response the dilution of the antisera was lowered; 1 in 750, 1 in 500 and 1 in 250 dilutions were made of the antisera. On probing western blots no increase in the specific antigenic response was detected. Antisera raised against Cd-1 and Cd-2 did not react against the respective proteins on western blots.

3.2.1.2 Purification of the immune fraction from antisera

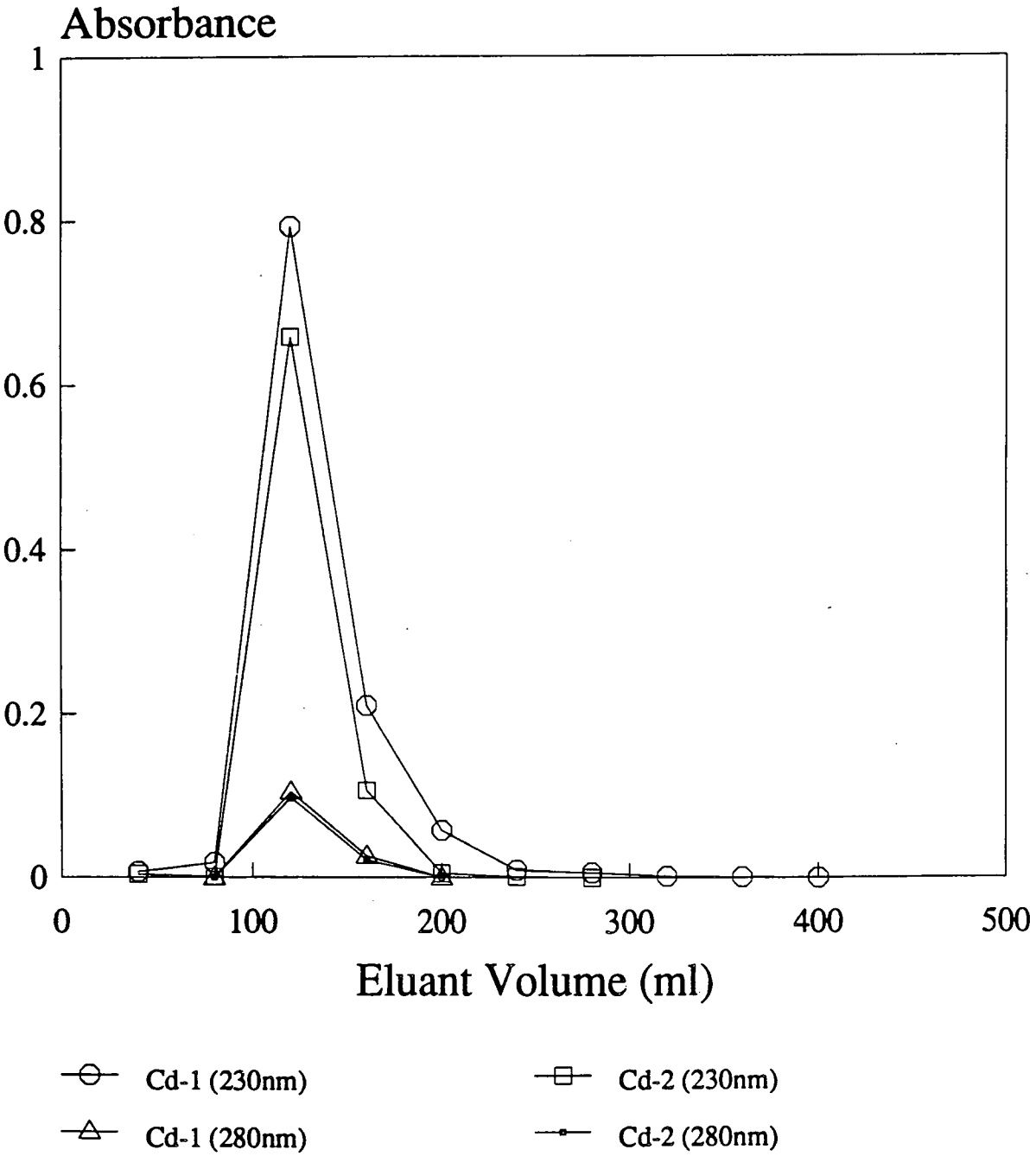
Immunoglobulins were purified from the antisera using protein-A bound on porous glass beads, Prosep-A (section 2.2.23). Prosep-A binds IgG, IgM and IgA classes of immunoglobulin in addition to some subclasses. The controlled pore glass (CPG) used to immobilise the Protein-A is a rigid and durable matrix which can sustain the high pressures without compacting. Prosep was designed to purify antibodies on a large preparative, industrial scale. The result of spectrophotometric analysis of fractions collected from Prosep-A is shown in Figure 23. The graph shows clearly that the immunoglobulin elutes off the column in 120 µl of eluant volume. Absorbance at 280 nm was only detected in the two fractions corresponding to an eluant volume

FIGURE 23

Purification of the immune fraction from antisera

Protein-A bound to porous glass beads, Prosep-A, was used to purify the immunoglobulin fraction from antisera raised against Cd-1 and Cd-2. Prosep-A was packed into an OPC, the serum was loaded onto the column and eluted with 0.1 M glycine pH3. 40 µl fractions were collected and their absorbance determined, as shown in the graph opposite.

Affinity Chromatography
Protein-A glass beads



of 120 and 160 µl indicating that the immunoglobulins elute from the column with the eluant buffer front.

The two fractions of both antisera which had high absorbance readings were pooled and tested against western blots (as described for serum in section 2.2.24). Using dilutions of 1 in 1000 no antigencity could be detected on western blots derived from 1D or 2D PAGE. Again in an attempt to detect an antigenic response the dilution of the purified immunoglobulin fraction was lowered to 1 in 750, 1 in 500 and 1 in 250. However, after testing these dilutions against western blots the purified immunoglobulins of both antisera were shown to have no antigenicity to Cd-1 or Cd-2.

3.2.2 Construction and analysis of a λZAP cDNA library

A λZAP library was constructed from mRNA of the Cd300 cell line of *D. innoxia* exposed to Cd (section 2.2.15). RNA which had been isolated from Cd300 *D. innoxia* cultures exposed to 125 µM Cd for 4 h (80 ml culture), 125 µM Cd for 8 h (160 ml culture), 250 µM for 4 h (80 ml culture) and 250 µM for 8 h (80 ml culture) was pooled and used for library production. To analyse the size distribution of the cDNA clones phagemid recoveries were performed on eight plaques selected at random (section 2.2.15.1). The phagemids were restricted with *Eco*R I and *Xho* I (section 2.2.5.2) and the products of the restriction digest were electrophoresed on a 0.8 % (w/v) agarose gel (section 2.2.6). The result is shown in Figure 24. All the recombinant phage contained inserts ranging in size from approximately 300 bp to 2,000 bp.

3.2.2.1 Differential screen of *D. innoxia* cDNA library

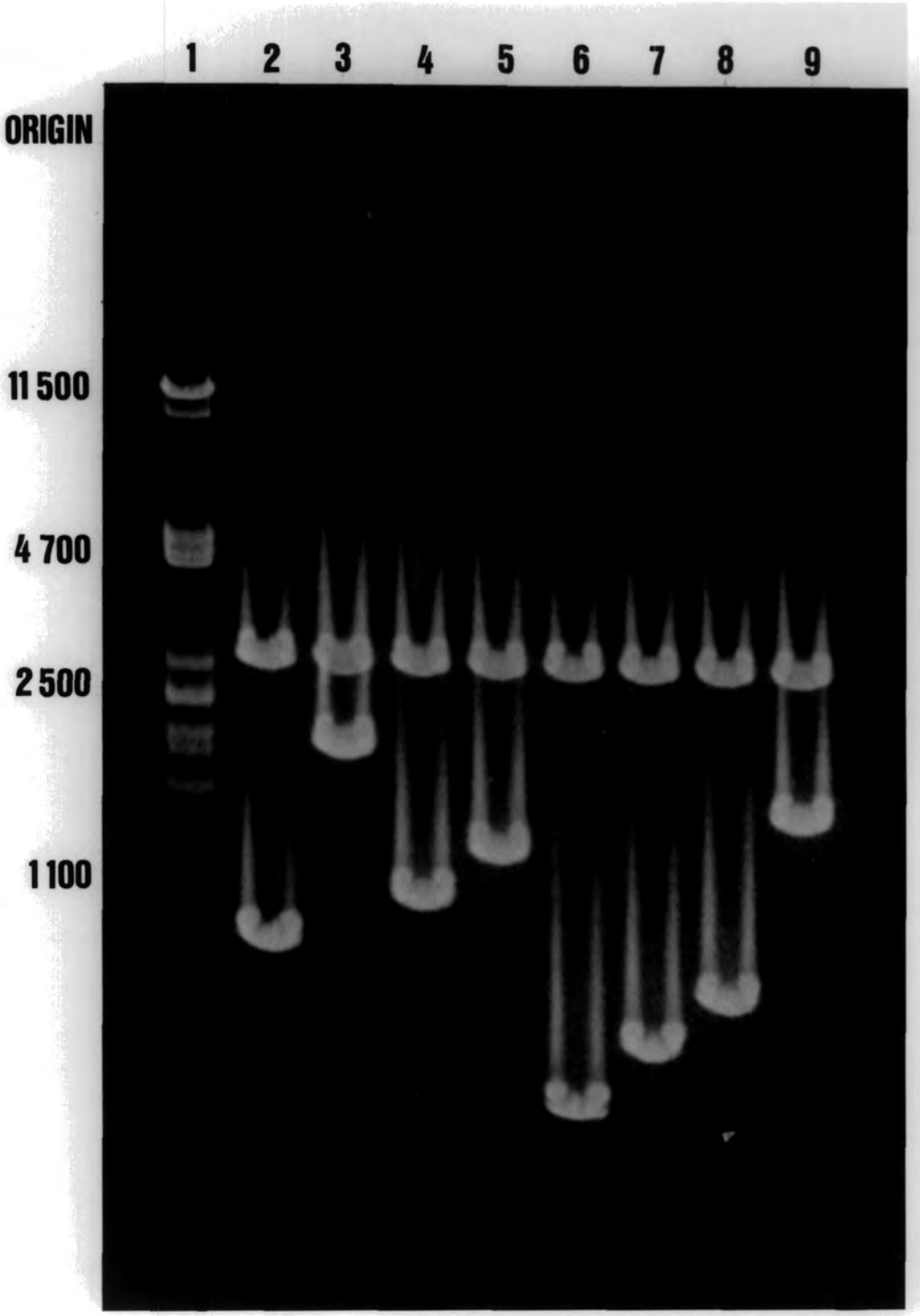
As no antibodies against Cd-1 and Cd-2 were available to screen the λ-ZAP cDNA library it was decided to differentially screen the library as described by Olszewski *et al.* (1989) (section 2.2.15.2). This method does not detect those cDNAs which have been repressed or enhanced, only cDNAs which have been induced. As both films are exposed to the same filter, alignment and hence identification of the recombinant phage containing Cd-induced cDNAs is very easy. The autoradiographs from a secondary screen in which the plaques were derived from those present in a plug taken from a plate after a primary screen are shown in Figure 25. The ratio of spots representing recombinant phage containing Cd-induced cDNA to spots representing recombinant phage containing non-induced cDNA corresponds to the number of phage taken in a plug after a primary screen, one of which would contain the Cd-induced cDNA. In the tertiary screen all the plaques on a plate showed hybridisation to the [α-^{35}S]dATP-labelled probe but not to the [α-^{32}P]dCTP-labelled probe. This was demonstrated when film placed adjacent to the filter showing localised darkening, whilst the film placed behind the piece of card was completely

FIGURE 24

Analysis of λZAP cDNA inserts

Following phagemid recoveries of eight plaques selected at random the cDNA inserts were recovered by restricting the recombinant phagemid with *Eco*R I and *Xho* I. The products were electrophoresed on a 0.8 % (w/v) agarose gel.

Lane 1= Size markers, λ DNA restricted with *Pst* 1.
Lanes 2-9= Restricted recombinant phagemids.

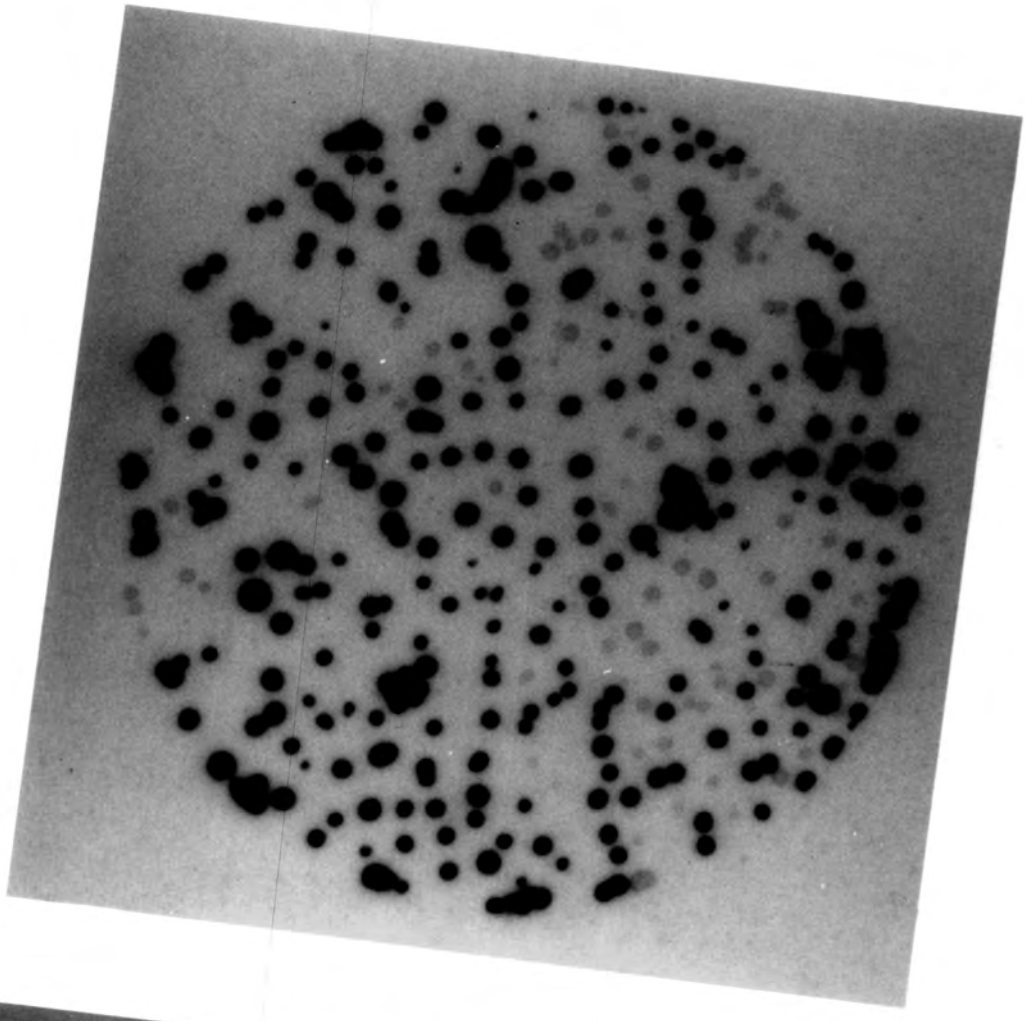

1
2
3
4
5
6
7
8
9
ORIGIN
11 500
4 700
2 500
1 100

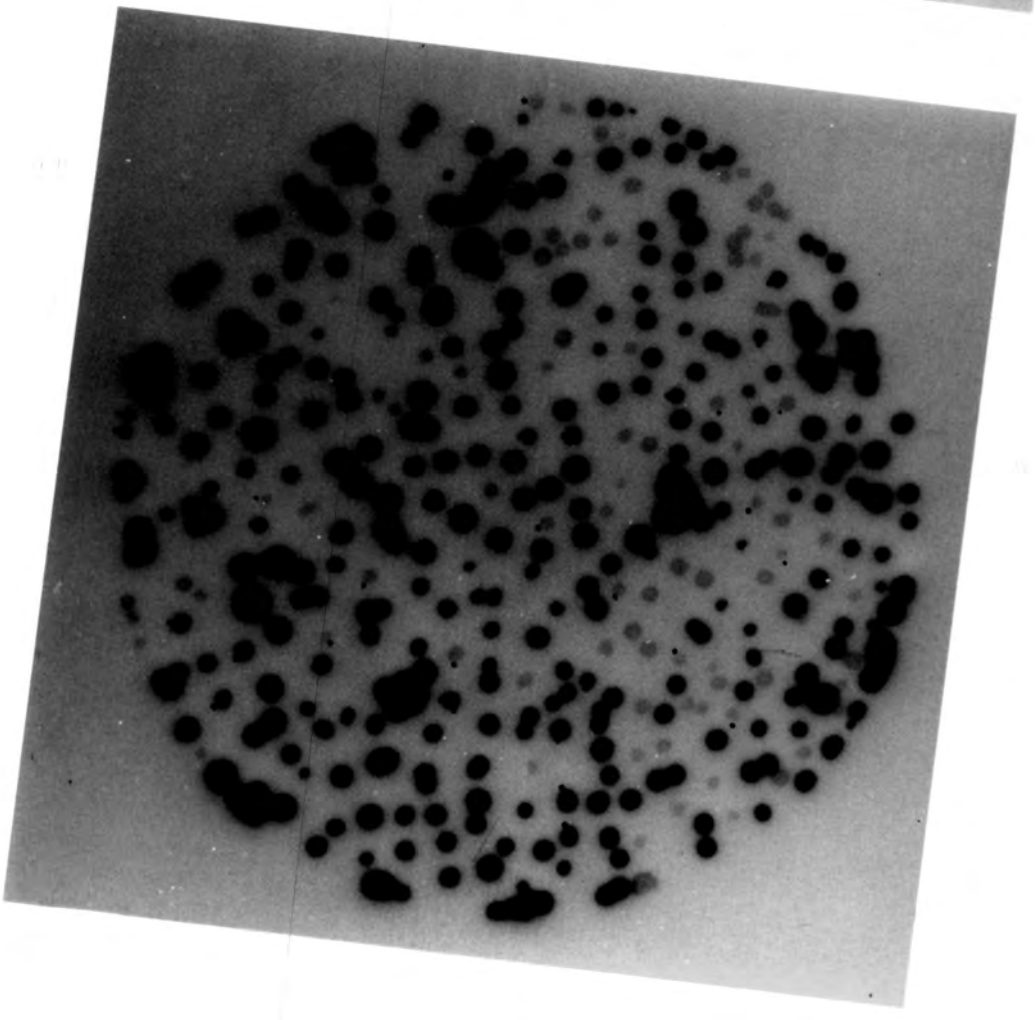
Cd6 or Cd8?

FIGURE 25

Autoradiographs of a secondary screen of a *D. innoxia* λZAP library

A filter from a plaque lift was screened with both ^{35}S-labelled probe (constructed fom Cd-induced RNA) and a ^{32}P-labelled probe (constructed fom non-induced RNA). The upper autoradiograph was placed behind a piece of card, separating it from the filter. The lower autoradiograph was placed adjacent to the filter. The extremely small dots on the lower autoradiogram are adjacent to recombinant plaques which have only hybridised to the ^{35}S-labelled probe.





clear.

After performing a tertiary screen two recombinant phage were shown to contain cDNA sequences which were induced by Cd. The phagemid Bluescript SK$^-$ was recovered (section 2.2.15.1) and the size of the two clones determined by excision of the cDNA insert by restriction with *Eco*R I and *Xho* I (section 2.2.5.2). The products of the restriction digest were electrophoresed on a 1 % (w/v) agarose gel (section 2.2.6). The two clones designated Cd-6 and Cd-8 were determined to be approximately 950 bp and 650 bp respectively.

3.2.3 Sequencing of Cd-6 and Cd-8

The two clones were sequenced as described in section 2.2.14 using T7 and T3 primers. In order to determine complete sequence in both directions the original clones were reduced in length and certain regions were subcloned (Figures 26 and 27).

A *Hind* III restriction site was identified at one end of the Cd-6 insert. To ensure no other *Hind* III restriction sites were present in that part of the clone for which no sequence information was available the recombinant phagemid was restricted (section 2.2.5.2) and the products separated on 0.8 % (w/v) agarose gel (section 2.2.6). Two bands were apparent indicating that only one *Hind* III site was present in the cloned cDNA, the second site being in the multicloning site of SK$^-$. Cd-6 was restricted with *Hind* III to remove a terminal region of cDNA and after dilution the recombinant phagemid was religated (section 2.2.5.6). The phagemid was transformed into *E. coli* (section 2.2.4.2) and subsequently recovered as described in section 2.2.3.4. The shortened Cd-6 clone was sequenced in both directions using M13 primers (section 2.2.14). The *Hind* III restriction described above was repeated. The products of the restriction were electrophoresed on a 1% agarose gel (section 2.2.6). The smaller fragment was recovered from the gel (section 2.2.7) and ligated (section 2.2.5.6) into SK$^-$ which had been restricted with *Hind* III (section 2.2.5.2). The resulting clone was transformed into *E. coli* (section 2.2.4.2), the phagemids recovered (section 2.2.3.4) and those shown to contain inserts of the correct size, by electrophoresis on 1 % (w/v) agarose gel (section 2.2.6) were sequenced in both directions using M13 primers (section 2.2.14). A unique *Hinc* II site was detected and the clone was restricted with *Hinc* II (section 2.2.5.2) to remove a large fragment, diluted and religated (section 2.2.5.6) prior to sequencing in both directions (section 2.2.13). Sequence information also indicated that a unique *Pst* I site was present at the opposite end of the fragment to the *Hind* III site in the cloned cDNA, Cd-6. An identical procedure was used to that described above to remove a *Pst* I fragment from the opposite end of the original recombinant phagemid. This clone was recovered and sequenced in both directions using M13 primers (section 2.2.14). This strategy is shown diagramatically in Figure 26 and the sequences obtained are shown in Figure 28A. The complete

FIGURE 26

Sequencing strategy to obtain full length, double stranded sequence of Cd-6

The shaded region indicates the cDNA insert. Restriction enzymes are abreviated as E, *Eco*R I; H, *Hind* III; Hc, *Hinc* II; P, *Pst* I. The diagram is not drawn to scale.

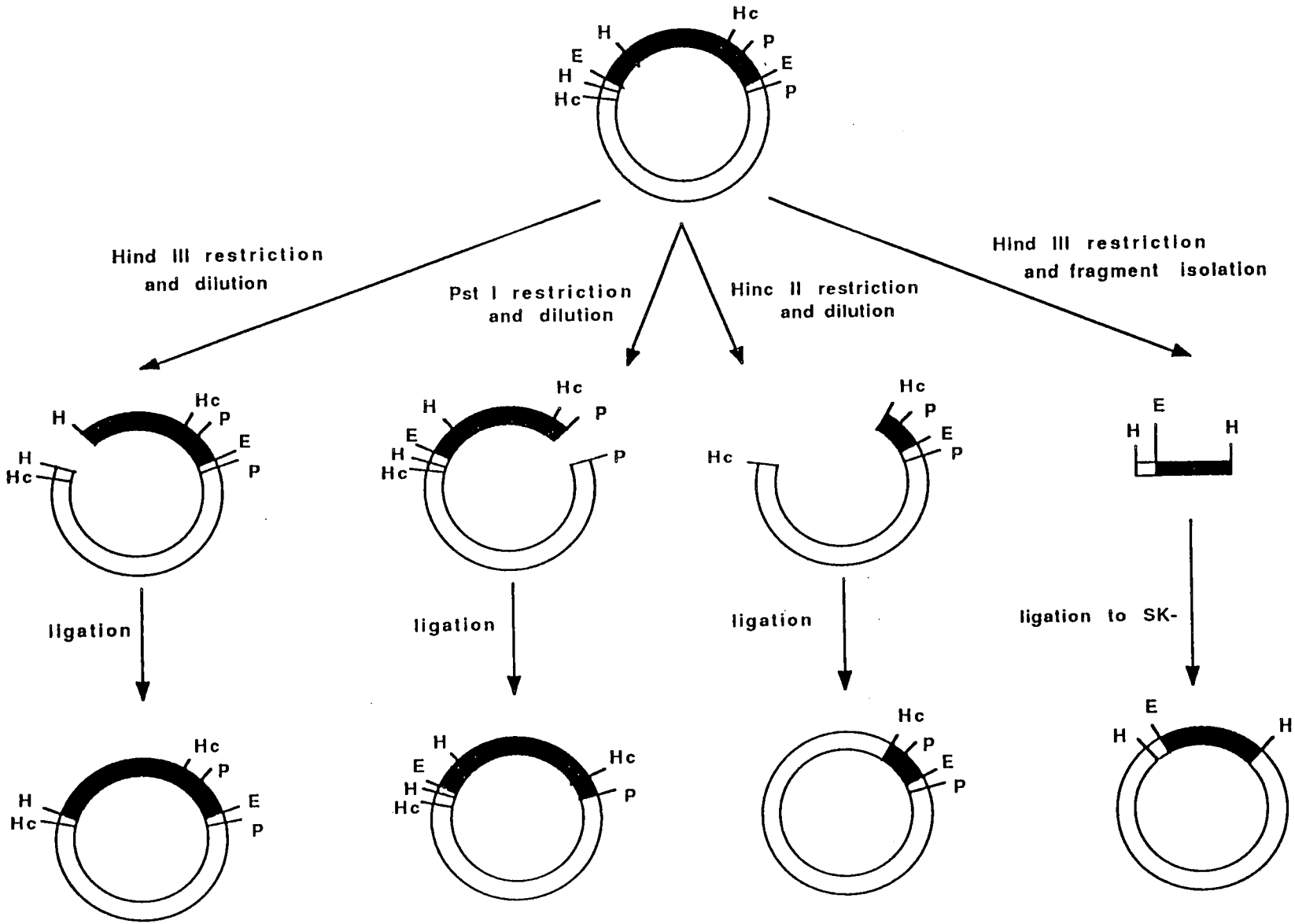

Hc
H
P
E
H
E
Hc
P
Hind III restriction
and dilution
Pst I restriction
and dilution
Hinc II restriction
and dilution
Hind III restriction
and fragment isolation
ligation
ligation
ligation
ligation to SK-

FIGURE 27

Sequencing strategy to obtain full length, double stranded sequence of Cd-8

The shaded region indicates the cDNA insert. The diagram is not drawn to scale.

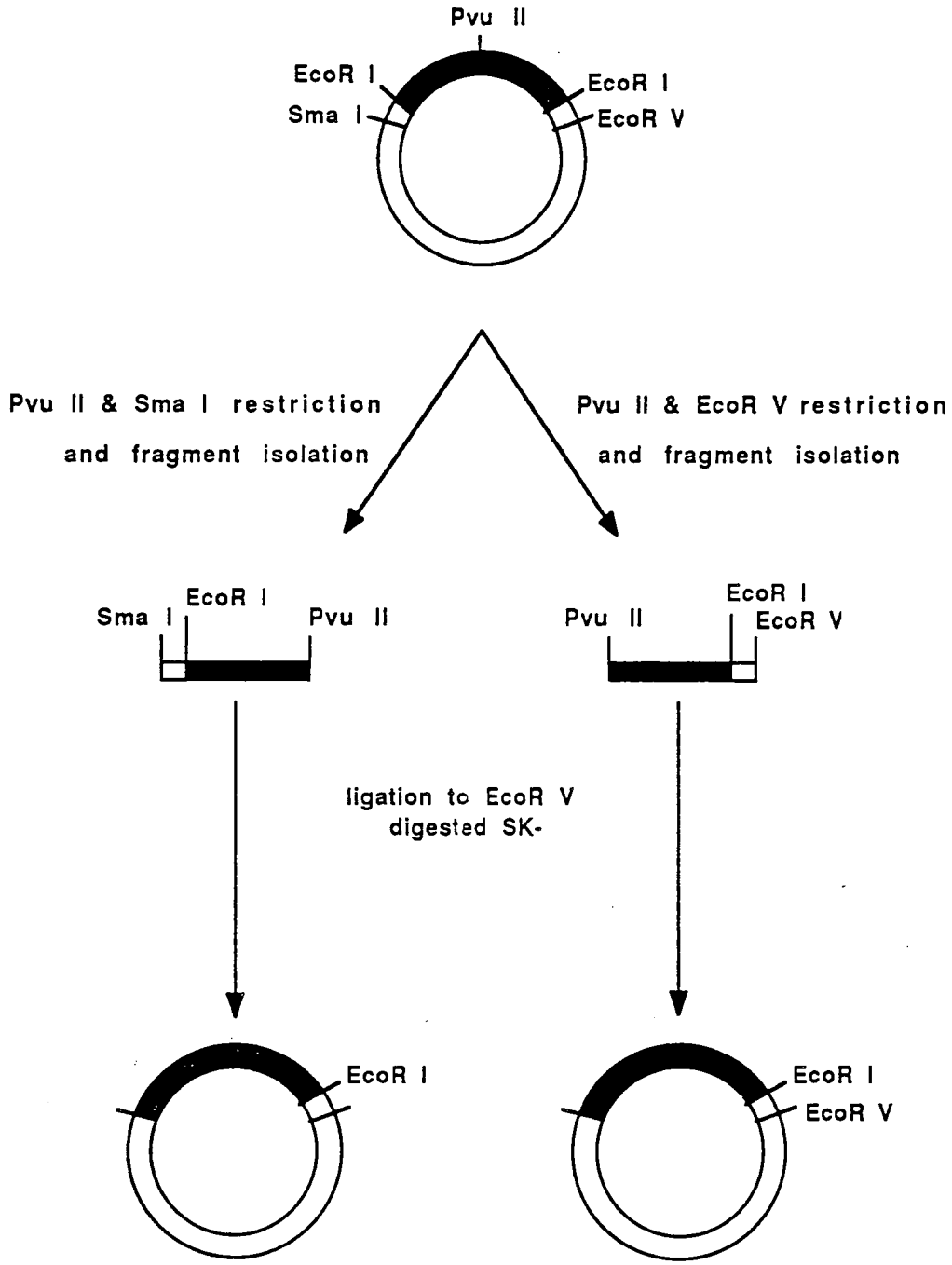
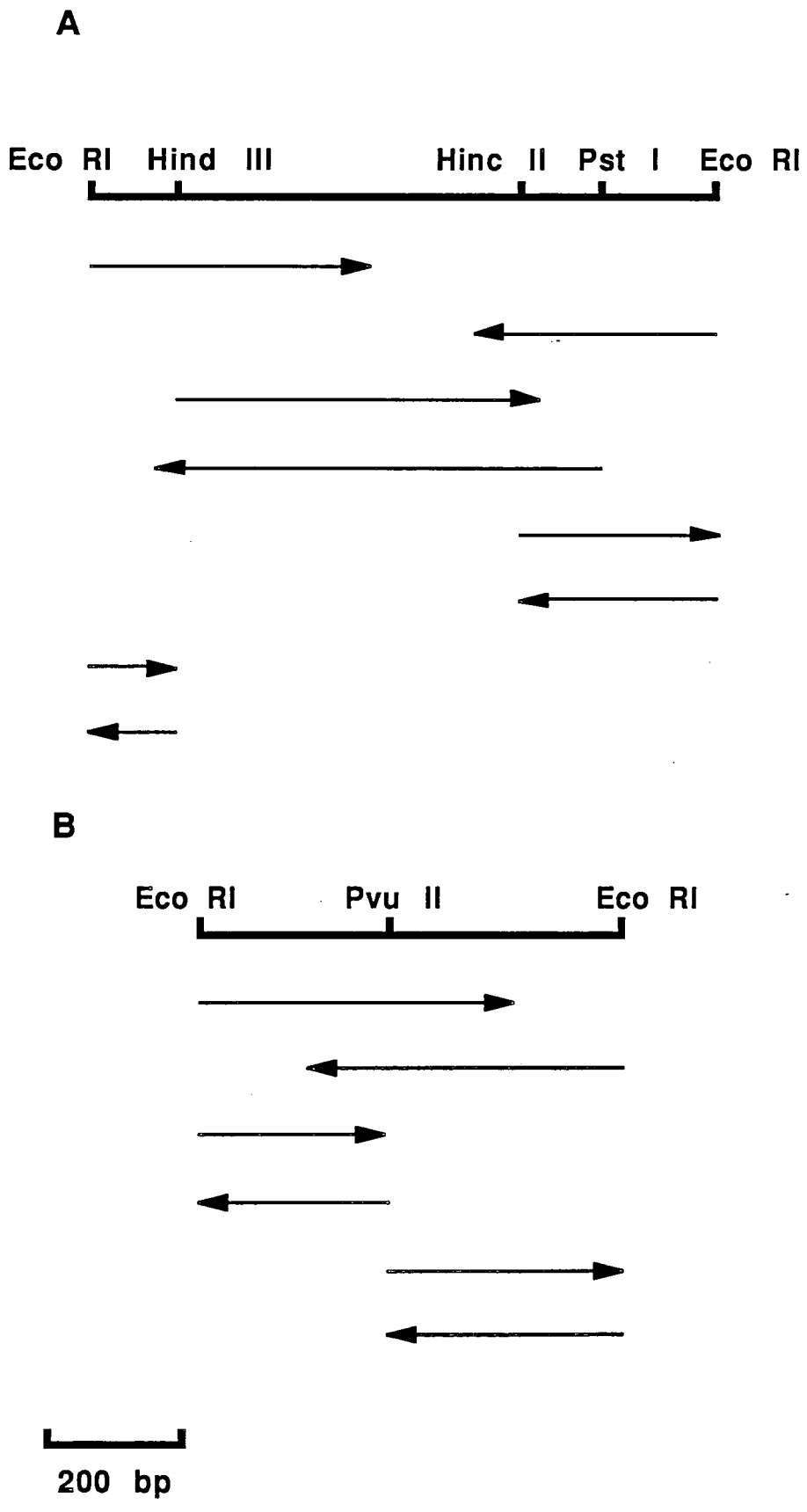

Pvu II
EcoR I
Sma I
EcoR I
EcoR V
Pvu II & Sma I restriction
and fragment isolation
Pvu II & EcoR V restriction
and fragment isolation
Sma I
EcoR I
Pvu II
Pvu II
EcoR I
EcoR V
ligation to EcoR V
digested SK-
EcoR I
EcoR I
EcoR V

FIGURE 28

Regions of Cd-6 and Cd-8 which were sequenced


A
Eco RI
Hind III
Hinc II
Pst I
Eco RI
B
Eco RI
Pvu II
Eco RI
200 bp

sequence of Cd-6 (949 bp) is shown in Figure 29.

From initial sequence information it was determined that Cd-8 contained a *Pvu* II restriction site. The multicloning site of the SK$^-$ contains a *Sma* I site to one side of the insert and a *Eco*R V site to the other. Both *Sma* I and *Eco*R V generate blunt ends on restriction and are therefore compatible with blunt ends generated by *Pvu* II. Cd-8 was restricted with *Pvu* II and *Sma* I and with *Pvu* II and *Eco*R V. The products of these restriction digests were electrophoresed on a 1 % (w/v) agarose gel (section 2.2.6). The smaller fragment resulting from each digest was recovered from the gel (section 2.2.7) and ligated (section 2.2.5.6) into SK$^-$ which had been restricted with *Eco*R V (section 2.2.5.2). Both of the resulting clones were transformed into *E. coli* (section 2.2.4.2), grown up, the phagemids recovered (section 2.2.3.4) and those shown to be of the correct size, by electrophoresis on 0.8 % (w/v) agarose gel (section 2.2.6) were sequenced in both directions using M13 primers (section 2.2.14). This strategy is shown diagramatically in Figure 27 and the sequences obtained are shown in Figure 28B. The complete sequence of Cd-8 (659 bp) is shown in Figure 30.

The sequence information indicated that the original inserts were cloned into the *Eco*R I site of the λ-ZAP vector and not in the *Eco*R I : *Xho* I sites as would be expected. To determine if this was indicative of the whole library, inserts were again digested (section 2.2.5.2) from recovered phagemids (section 2.2.15.1) using both *Eco*R I and *Xho* I digestions and *Eco*R I digests only. The results from *Eco*R I : *Xho* I digestions were similar to those described in section 3.2.2. Digestion with *Eco*R I resulted in linearised recombinant phagemids but did not release the cDNA inserts.

3.2.4 Northern blot analysis

The two cDNA sequences were recovered from the recombinant phagemids by restriction with *Hin*d III and *Bam* HI (section 2.2.5.2), labelled with [α-^{32}P]dCTP by random priming (section 2.2.8) and used to probe northern blots (section 2.2.10). The mRNA extracted from Cd300 *D. innoxia* cells exposed to 125 μM Cd for 8 h, 250 μM Cd for 4 h, 250 μM Cd for 8 h, and in addition RNA extracted from non-exposed cells was separated on a formaldehyde agarose gel (section 2.2.6) which was blotted (section 2.2.9). 7.5 μg of each RNA population, as determined by absorbance at 260 nm, was loaded on the gel (section 2.2.5.1). The results of probing such a northern blot with [α-^{32}P]dCTP-labelled Cd-6 and Cd-8 are shown in Figure 31A. The northern blot was probed with Cd-6 and exposed to film after which the blot was stripped (section 2.2.10). The filter was exposed to film to ensure no signal remained and then reprobed with Cd-8. Both cDNAs hybridised to the mRNA populations from Cd-exposed cells and both Cd-6 and Cd-8 correlate to transcripts of approximately 900 bp. The two cDNAs did

FIGURE 29

Full length nucleotide sequence of Cd-6 and putative amino acid sequence

A putative metal-binding motif (which is similar to a motif in the α-domain of animal MT) and 3 other cysteine residues are underlined.

```
ttgtcaaggaccgcagacgggtgaagcgtgaatatgatgaatttaaggttcgaatcaat

agcctccctgattcaattcgtcggcgttctgatgcctataatgctcgagaagaaatcaa

ggccatgaagcttcagcgaagaggcagctggggatgagcttttggaacccatcaagata

acaaaggccacttgg ATG GCA GAT GGG ACC CAC TGG CCT GGC ACT TGG
                M   A   D   G   T   H   W   P   G   T   W

ATG GTT TCT GCT CCT GAG CAC TCT AGG GGT GAT CAT GCA GGA ATC
M   V   S   A   P   E   H   S   R   G   D   H   A   G   I

ATA CAG GTT ATG TTA AAA CCT CCA AGT GAT GAA CCC TTA CAT GGA
I   Q   V   M   L   K   P   P   S   D   E   P   L   H   G

ACC TCT GCT GAT GAT GGT CTA ATT GAT TTA ACA GAA GTT GAT ATT
T   S   A   D   D   G   L   I   D   L   T   E   V   D   I

CGG CTT CCC TTG CTG GTA TAT GTC TCT CGT GAG AAG CGT CCT GGC
R   L   P   L   L   V   Y   V   S   R   E   K   R   P   G

TAT GAT CAT AAC AAG AAG GCT GGA GCC ATG AAT GCC TTG GTC CGA
Y   D   H   N   K   K   A   G   A   M   N   A   L   V   R

GCT TCA GCC ATC ATG TCT AAT GGC CCC TTT ATT CTC AAT CTT GAT
A   S   A   I   M   S   N   G   P   F   I   L   N   L   D

TGT GAT CAC TAC ATC TAC AAC TCG CAG GCA ATG AGG GAG GGA ATG
C   D   H   Y   I   Y   N   S   Q   A   M   R   E   G   M

TGT TTT ATG ATG GAC CGA GGG GGG GAT CGA ATC TGT TAT GTT CAG
C   F   M   M   D   R   G   G   D   R   I   C   Y   V   Q

TTT CCT CAA CGT TTT GAG GGA ATT GAT CCT TCC GAC CGT TAT GCA
F   P   Q   R   F   E   G   I   D   P   S   D   R   Y   A

AAT CAC AAT ACT GTT TTC TTT GAT GTC AAC ATG CGT GCC CTT GAT
N   H   N   T   V   F   F   D   V   N   M   R   A   L   D

GGA CTT CAA GGT CCA ATG TAT GTT GGG ACC GGT TGC CTC TTC CGC
G   L   Q   G   P   M   Y   V   G   T   G   C   L   F   R

AGG ACT GCT CTT TAT GGT TTT GAC CCC CCA CGA ACC AAG GAA TAC
R   T   A   L   Y   G   F   D   P   P   R   T   K   E   Y

CAC CCT GGT TGC TGC AGC TGT TGT TTT GGT CGG CGC AAG AAT AAT
H   P   G   C   C   S   C   C   F   G   R   R   K   N   N

GCC ACT GTG TCT TCT GTC TCT GAT GAC AAC AAG GCA CTT AGA ATG
A   T   V   S   S   V   S   D   D   N   K   A   L   R   M

GGG GAT TTT GAT GAT GAG GAG ATG AAT CTT GCT TCG TTT CCT AAA
G   D   F   D   D   E   E   M   N   L   A   S   F   P   K

AGG TTT GGT AAT TCA AGT TTC CTT ATT GAT TCG ATT CCG GTC GCC
R   F   G   N   S   S   F   L   I   D   S   I   P   V   A
```

FIGURE 30

Full length nucleotide sequence of Cd-8 and putative amino acid sequence

```
GCC AAG GGG ATG CAT CTT GGT CAA GCT GCA GTC TCC CAA GAA CCA
A   K   G   M   H   L   G   Q   A   A   V   S   Q   E   P

TTG TCT GGA GTG AGA AGA CCA AGC CAG ATC TCA AAT CTG AAT CCC
L   S   G   V   R   R   P   S   Q   I   S   N   L   N   P

AGT ATG TCT CAG AGA CCA ACG GGG CAA ACT CAA GCA TTT TTA CCT
S   M   S   Q   R   P   T   G   Q   T   Q   A   F   L   P

GGA GAG GTA GGA AGC CCT CCT TCA GGT CAT GAG CCA CAT GCT GGA
G   E   V   G   S   P   P   S   G   H   E   P   H   A   G

TAC ATG CAC ACA ATG GCA AAT CCA TCA TAT TTA ATG CCT CAT TTT
Y   M   H   T   M   A   N   P   S   Y   L   M   P   H   F

GCA CAA AGT TCT CCA AGC CGC TTG GGA CAG CAG CCT CTT TAC CGA
A   Q   S   S   P   S   R   L   G   Q   Q   P   L   Y   R

TTT AAC CAG GGG AGA CCA GCT GTT CAT TAC AAT GAA AGT CAT GCC
F   N   Q   G   R   P   A   V   H   Y   N   E   S   H   A

AAG GCG CAG TCA TCT CAT TCT ACC TAT AAT ACA GAT GCT CCA AAT
K   A   Q   S   S   H   S   T   Y   N   T   D   A   P   N

TCT GCT CCA AGG AAT GGT GCA TCA TGG GGG CGT AGA GGA AGC AAT
S   A   P   R   N   G   A   S   W   G   R   R   G   S   N

CCT ATT CCA AAC ATT CCA CCA ACT TCC AGG ACA AGA AAA GAC TAC
P   I   P   N   I   P   P   T   S   R   T   R   K   D   Y

AAG AGA GTT GTT TAA cttttttgtagtcttcatcgacgattagttcgataagcagt
K   R   V   V   *

tcagacaagtgaaacaaagcgggtaagcctgctttagtggcgtgcgttacaaatcctcat

cgacgttcttttgtagtgcttgcataaatgttaatttaacaccgccatgatctggtcatg

cctgtttgtcatcaccatgctcgatctgggagct
```

FIGURE 31

Induction of Cd-6 and Cd-8 transcripts in response to Cd-exposure in Cd300 cells

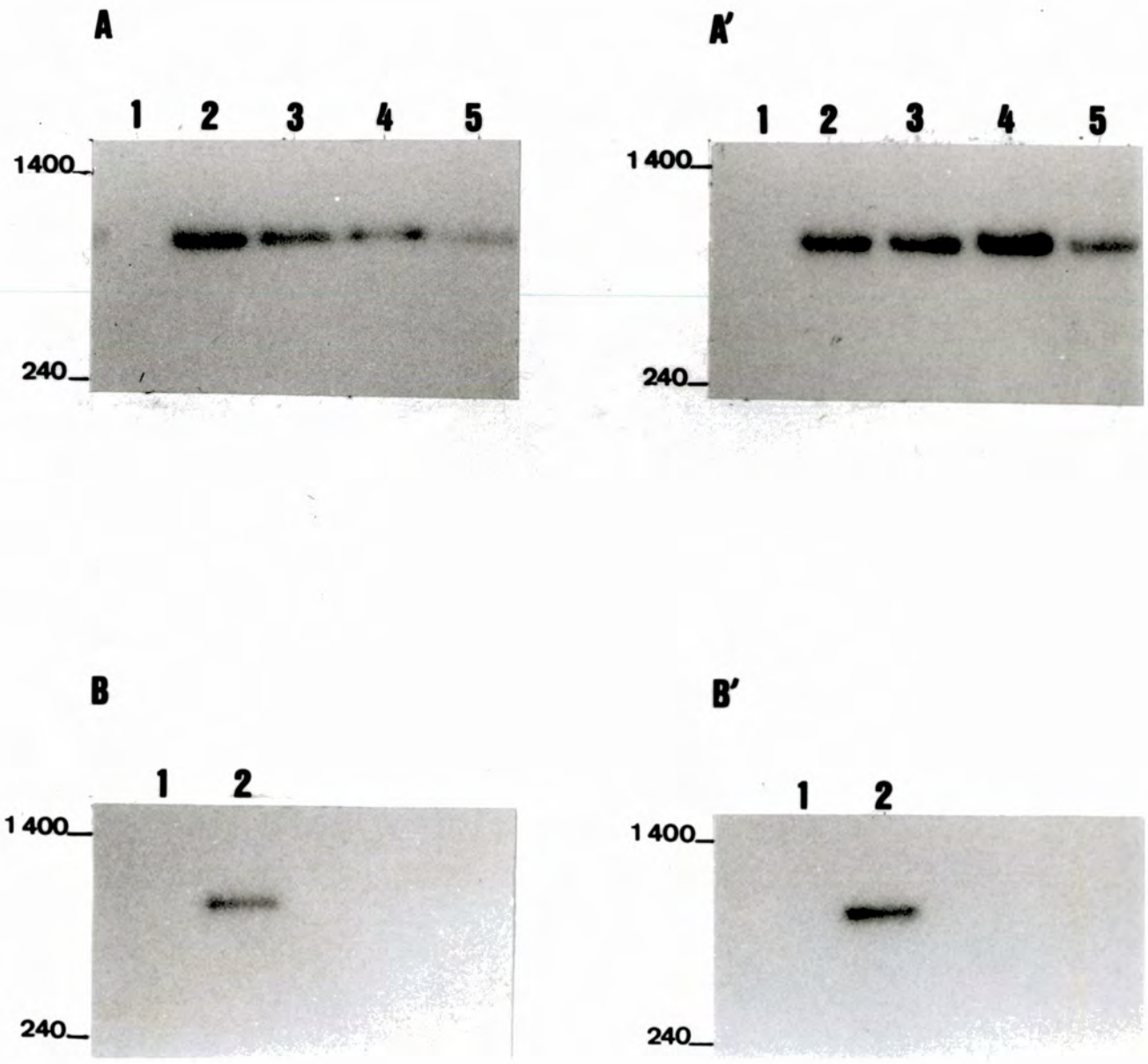
Total RNA.

In both **A** and **A'** the lanes contain
Lane 1= mRNA (7.5 μg) prepared from non-exposed Cd300 cells.
Lane 2= mRNA (7.5 μg) prepared from Cd300 cells exposed to 125 μM Cd for 4 h.
Lane 3= mRNA (7.5 μg) prepared from Cd300 cells exposed to 125 μM Cd for 8 h.
Lane 4= mRNA (7.5 μg) prepared from Cd300 cells exposed to 250 μM Cd for 4 h.
Lane 5= mRNA (7.5 μg) prepared from Cd300 cells exposed to 250 μM Cd for 8 h.

The northern blot in **A** was probed with Cd-6.
The northern blot in **A'** was probed with Cd-8.

In both **B** and **B'** the lanes contain
Lane 1= mRNA (7.5 μg) prepared from non-exposed Cd300 cells.
Lane 2= mRNA (7.5 μg) prepared from Cd300 cells exposed to 125 μM Cd for 8 h.

The northern blot in **B** was probed with Cd-6.
The northern blot in **B'** was probed with Cd-8.


A
1 2 3 4 5
1400
240
A'
1 2 3 4 5
1400
240
B
1 2
1400
240
B'
1 2
1400
240

not hybridise to mRNA isolated from non exposed cells. To ensure that the differential expression was reproducible mRNA was again isolated from Cd-exposed (125 μM Cd for 8 h) and non-exposed cells. The northern blot analysis was repeated using mRNA extracted from non-exposed and Cd-exposed (125 μM 8 h) Cd300 cells. The results of this investigation are shown in Figure 31(B). Both Cd-6 and Cd-8 hybridised to the mRNA isolated from Cd exposed cells but not to mRNA from non-exposed cells. The transcript sizes shown to hybridise to Cd-6 and Cd-8 were again both determined to be 900 bp.

Investigations were performed to determine if similar transcripts were induced in the WDI *D. innoxia* cell line and in the Cd300 cell line in response to heat shock. mRNA was isolated from WDI cells exposed to 125 μM Cd for 8 h and from Cd300 cells exposed to 42 ^{o}C for 4 h (section 2.2.3.2). 7.5 μg of mRNA from both populations was northern blotted and probed with [α-^{32}P]dCTP-labelled Cd-6 and Cd-8 (section 2.2.10). The northern blot was stripped between probing with Cd-6 and Cd-8 as described above. The results of these studies are shown in Figure 32. It was observed that a transcript of 900 bp present in mRNA extracted from Cd300 cells exposed to elevated temperatures hybridised to both Cd-6 and Cd-8.

3.2.5 Southern blot analysis

Genomic DNA was isolated from Cd300 cells and restricted with *Eco*R I, *Bam* H I and *Xho* I (section 2.2.5.2.1). The products were separated on a 0.7 % (w/v) agarose gel and Southern blotted (section 2.2.9). The blots were then analysed with [α-^{32}P]dCTP-labelled Cd-6 and Cd-8 (section 2.2.10) (Figure 33). Both Cd-6 and Cd-8 hybridised to the restricted *D. innoxia* DNA giving hybridising multiple bands.

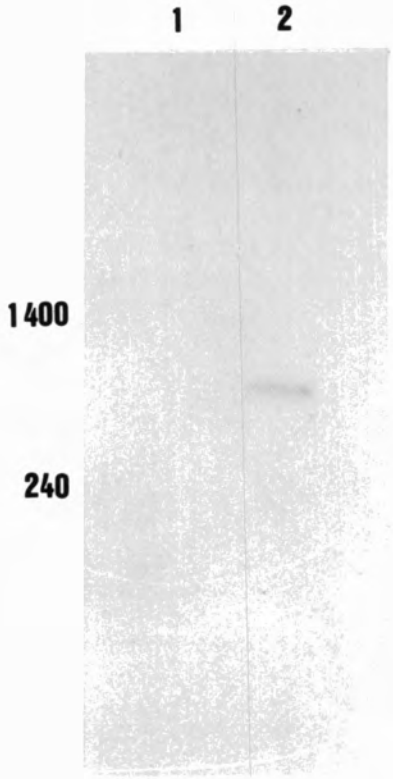
FIGURE 32

Induction of Cd-6 and Cd-8 transcripts in WDI cells in response to Cd-exposure and in Cd300 cells in response to heat shock.

A shows RNA separated on a formaldehyde agarose gel, the lanes contain:
Lane 1= mRNA (7.5 μg) prepared from WDI cells exposed to 125 μM Cd for 4 h.
Lane 2= mRNA (7.5 μg) prepared from Cd300 cells exposed to 42 ^{O}C for 4 h.

B shows the northern blot probed with Cd-6
B' shows the northern blot probed with Cd-8


1
2
ORIGIN
1 400
240


1
2
1 400
240

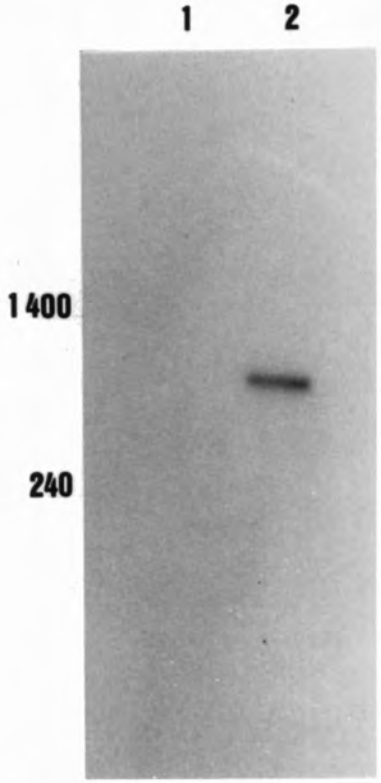

1
2
1 400
240

FIGURE 33

Southern blot analysis of Cd-6 and Cd-8

In both **A** and **B** the lanes contain:

Lane 1= Size markers, λ DNA restricted with *Pst* I.

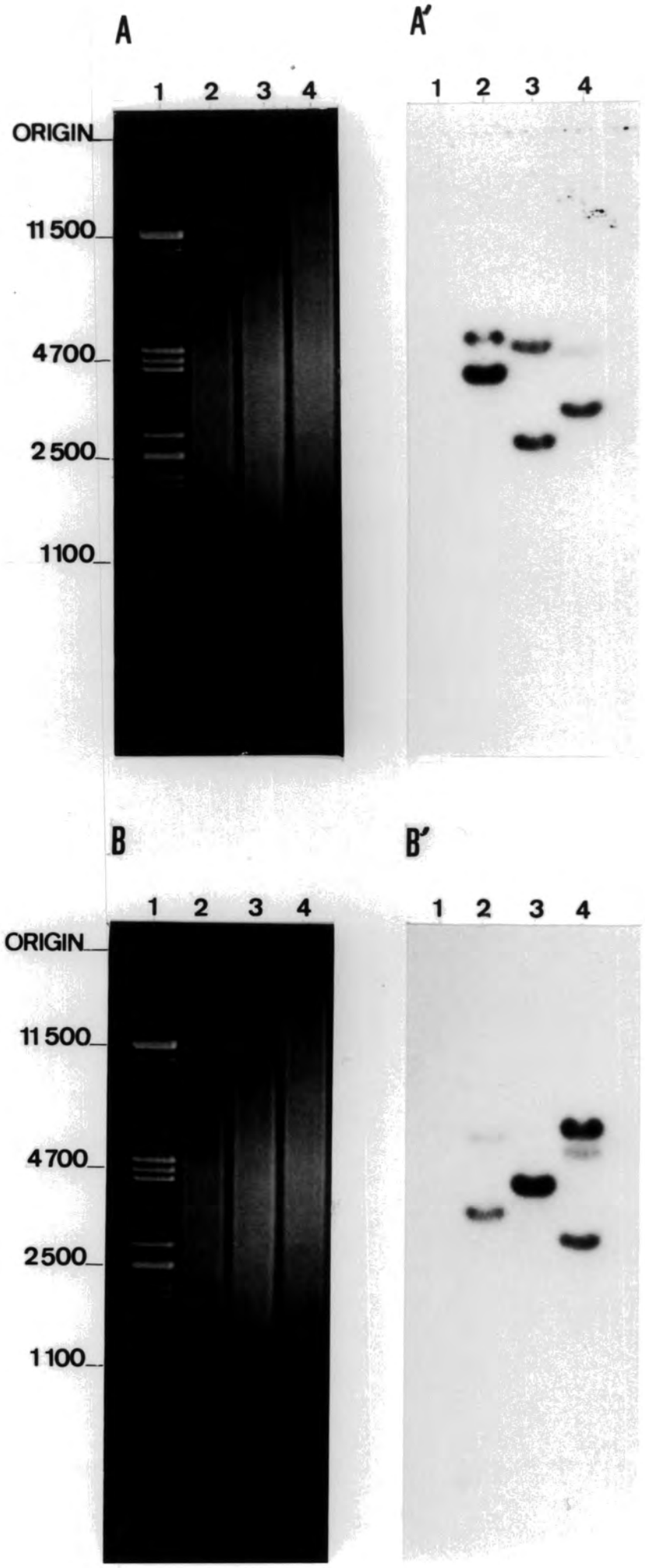
Lane 2= *D. innoxia* DNA restricted with *EcoR* I.

Lane 3= *D. innoxia* DNA restricted with *Bam* HI.

Lane 4= *D. innoxia* DNA restricted with *Xho* I.

A' indicates a Southern blot of **A** probed with Cd-6.

B' indicates a Southern blot of **B** probed with Cd-8.


A
A'
1 2 3 4
ORIGIN
11500
4700
2500
1100
B
B'
1 2 3 4
ORIGIN
11500
4700
2500
1100

CHAPTER 4

DISCUSSION

4.1 GROWTH OF *D. innoxia* SUSPENSION CULTURES

The results obtained in section 3.1.1 indicate that *D. innoxia* suspension cultures were maintained almost continuously in exponential growth phase (Figure 4). Cultures were exposed to Cd and other treatments at a similar stage of the culture cycle. The rapidly dividing cultures presented a readily available source of relatively uniform biological material with which to work.

The biochemistry of the two cell lines, Cd-tolerant (Cd300) and non-tolerant (WDI), of *D. innoxia* (Jackson *et al.*, 1984) used is well documented (section 1.4) and lends the two cell lines as a good model system in which to isolate Cd-induced genes and to identify the differential effects of Cd on tolerant and non-tolerant plant cells. Previous studies of Cd-tolerant, non-tolerant and Cd-sensitive plants or cultures have included the use of plants collected from metal-polluted environments, e.g. *Silene vulgaris* (Verkleij *et al.*, 1990), or alternatively, plants adapted from wild type cultured cells e.g. *D. innoxia* (Jackson *et al.*, 1984) (used here), *Lycopersicon esculentum* Mill. cv VFNT-cherry (Steffens *et al.*, 1986: Gupta and Goldsbrough, 1991) or Cd-sensitive plants selected by mutation from the wild type e.g. Cd-sensitive *A. thaliana* (Cobbett, 1992). Other adapted cell lines of *D. innoxia* have also been derived from the wild type line. Among these, a cell line has been derived which is resistant to sulphonylurea which was used to study herbicide resistance (Saxena and King, 1988) and a lactose adapted cell line has been used to study lactose metabolism (Herouart *et al.*, 1991).

4.1.1 Effect of trace metals and heat shock on the growth of *D. innoxia* suspension cultures

The results shown in Figure 5 demonstrate that exposure of Cd300 *D. innoxia* cultures to either 125 or 250 μM Cd for 4 or 8 h has no significant effect on the growth of those cultures compared to non-exposed cultures. Similarly exposure of Cd300 cultures to 125 μM Cu or Zn for 8 h had no effect on the growth of the cultures (Figure 6), the PCVs of metal-exposed cultures not being significantly different from those of non-exposed cultures. These results indicate that exposure of Cd300 cultures to these elevated metal concentrations, used in studies to identify induced peptides, would not result in the accumulation of peptides merely as a result of the cells becoming necrotic. By way of contrast to elevated metal exposure, heat shock (HS) (42 ^{o}C 4h) resulted in a small but significant decrease in the PCV of HS-exposed cultures compared to non-exposed cultures. The PCVs of WDI cultures exposed to 125 μM Cd for 8 h also showed a lower, but significantly different PCV compared to non-exposed cultures (Figure 6). It is considered unlikely that such small differences in the PCV measurements represent cell death. The lower PCVs may be the result of initial differences in the cell densities of the two cultures

used to study each treatment, or may be due to the treatments merely causing a reduction in the growth rate of the cultures.

4.2 IDENTIFICATION OF Cd-INDUCED PROTEINS BY 1D AND 2D PAGE

As shown in Figure 7 1D SDS PAGE indicated that proteins were induced by Cd in *D. innoxia* cells. The induced proteins were of similar M_r to some of those shown to be induced by Cd in the studies of Delhaize *et al.* (1989b). The proteins which were induced by Cd were observed after 2 h. This observation was also paralleled in the results described by Delhaize *et al.* (1989b).

In an effort to increase resolution 2D PAGE was attempted. Precipitation of *D. innoxia* protein on the surface of the IEF gel following electrophoresis initially prevented this. This phenomenon did not occur with protein extracted from salt grass cells, used as a control, indicating that the precipitation was due to an inherent characteristic of the *D. innoxia* sample.

Extracts from cultures exposed to Cd at 24, 48 and 72 h all resulted in precipitation. This indicated that the precipitation was not caused by a cellular component which accumulated at a particular point in the growth of a culture.

Precipitation occurred even at lower loadings of protein (30 μg), shown in Figure 8. Electrophoresis of IEF gels on which protein had precipitated both on the surface, and as it migrated into the gel, resulted in smears being observed in the gel following staining. IEF gels in which precipitation had occurred could not be used for subsequent electrophoresis to identify individual polypeptides.

By reversing the two running buffers used in electrofocusing (section 3.1.3.1), with respect to the anode and cathode, it was observed that precipitation occurred in both acidic and basic conditions. This meant that the necessary pH gradient could not be established in either orientation without the problem of precipitation occurring.

Fractionating *D. innoxia* protein by various $(NH_4)_2SO_4$ precipitations still resulted in precipitation at the IEF gel surface. This demonstrated that contaminants, such as carbohydrate or nucleic acids, which would have been removed by this procedure were not the cause of precipitation.

Fractionation by ion exchange chromatography using a stepped elution generated six protein containing fractions (Figure 9). All of these fractions when loaded onto IEF and electrofocused precipitated at the gel surface. It was concluded that precipitation was not due to a particular protein or association of proteins which could be separated by this method.

In attempting to circumvent the problem of precipitation the samples were loaded in the IEF gel mixture prior to polymerisation. IEF gels prepared in such a manner, containing protein extracted from Cd-exposed Cd300 cells, did not polymerise fully. Removal of PCs, Cd or some other component that was resolved from void volume on Sephadex G50 gel filtration chromatography (Figure 10) allowed the main protein containing fractions to be incorporated in the IEF gel mixture and did not inhibit subsequent polymerisation. This protocol was therefore used to prepare all samples prior to analysis by 2D PAGE.

4.2.1 Identification of Cd-induced peptides by 2D PAGE

Analysis of protein extracted from Cd300 cells exposed to 125 μM Cd for 8 h identified two Cd-induced peptides, designated Cd-1 and Cd-2 (Figure 11). In attempts to observe other peptides which were induced by Cd, *D. innoxia* cells were exposed to different Cd-treatments. It was possible that any peptides being synthesised soon after Cd-exposure had been degraded before 8 h. In halving the period of exposure to 4 h (125 μM Cd) it was hoped to identify any proteins expressed during the early period of exposure. However, as Figure 12 shows, no peptides, including Cd-1 and Cd-2, were seen to be induced by this particular treatment. (The apparent disparity between the results of 2D and 1D-PAGE (Figure 7) analysis of samples collected from cells exposed to 125 μM Cd for 4 h may reflect inherent variability in the cultures. Alternatively it could be explained due to the loss of protein in the preparative procedures carried out on samples prior to 2D PAGE). To identify Cd-induced peptides after 4 h exposure to Cd, the Cd concentration was increased to 250 μM (4 h). As shown in Figure 13, this led to the induction of Cd-1 and Cd-2, to a similar extent as exposure to 125 μM Cd for 8 h, and also to the induction of two other peptides. However these two peptides, unlike Cd-1 and Cd-2, were not reproducibly observed. In an attempt to achieve reproducible synthesis of these two peptides the length of Cd-exposure was increased to 8 h (250 μM Cd). Although Cd-1 and Cd-2 were apparently strongly induced by this Cd-exposure no other induced peptides were observed (Figure 14). The exposure to 250 μM Cd for 8 h was the most toxic treatment the cells were exposed to. This probably changed the intracellular environment to a greater extent than the other exposures, thus leading to greater accumulation of Cd-1 and Cd-2.

In addition to exposure to Cd, exposure to Cu (Figure 17) and Zn (Figure 18) (both to 125 μM for 8 h) led to the induction of Cd-1 and Cd-2 indicating that whatever the intracellular signal that leads to the induction of the two peptides, it responds to elevated concentrations of the non-essential metal, Cd, and to essential metals Cu and Zn. Cu has previously been shown to induce the synthesis of a number of proteins in plants, including superoxide dismutase (SOD) (Chongpraditnum *et al.*, 1992) and plastocyanin in addition to stimulating the formation of PCs

(Salt *et al.*, 1989). Zn has also been shown to induce PCs, but far less strongly than Cd or Cu (Steffens, 1990).

Figure 15 shows that Cd-1 and Cd-2 are also co-induced by HS (42 ^{o}C 4 h). HS has previously been shown to induce many of the same cellular responses as Cd. Delhaize *et al.* (1989b) showed, using 1D PAGE, that both Cd and HS induced the same proteins in *D. innoxia*, and certain polyA^{+} RNA species have been shown to be induced to a similar degree by both Cd and HS (Edelman *et al.*, 1988; Czarnecka *et al.*, 1984). Czarnecka *et al.* (1984) reported that Cu induced a set of polyA^{+} RNA species much more weakly than did Cd or HS. The 2D PAGE analysis described in sections 3.1.3.5.5 and 3.1.3.5.7 indicates that Cd-1 and Cd-2 are induced to an equal degree by both HS (Figure 15) and Cu (Figure 17) as determined by staining intensity.

Cd-1 and Cd-2 may correspond to known heat shock proteins (HSPs). HSPs are categorised by their molecular weights as HSP110 (Mr 110,000), HSP90s (Mr 90,000), HSP70s (Mr 70,000), HSP60s (Mr 60,000) and the low molecular weight (LMW) HSPs (Mr 17-28,000). Using this classification Cd-1 and Cd-2 would be classified as LMW HSPs. Genes which encode LMW HSPs have been isolated from a number of plant species including soybean (Schoffl and Key, 1983), pea (Lauzon *et al.*, 1990), *Arabidopsis* (Helm and Vierling, 1990), carrot (Zimmerman *et al.*, 1989), petunia (Chen and Vierling, 1991), wheat (McEwain and Spike, 1989), lily (Bouchard, 1990) and *Chlamydomonas* (Grimm *et al.*, 1989). Analysis of these genes shows that LMW HSPs fall into four multi gene families. Two of these families encode cytoplasmic proteins, one encodes a chloroplast localised protein and one an endomembrane protein (Vierling, 1991). LMW HSPs are among the most abundant proteins induced by HS (Mansfield and Key, 1987). Unlike the situation in the HSP70 class, no constitutively expressed proteins have been identified. Comparison of the amino acid sequences of LMW HSPs indicates there is greatest sequence conservation in the C-terminal portion of the proteins inferring a functional constraint on this region. The function of most LMW HSPs remains unknown. However, the genes encoding the ubiquitin conjugating enzymes in *S. cerevisiae*, UBC4 and UBC5, have been shown to be induced by heat shock (Seufert and Jentsch, 1992). These are two enzymes, of low molecular weight, known to mediate selective degradation of short-lived and abnormal proteins.

To establish if Cd induced Cd-1 and Cd-2 in the wild type cell line, the cells were exposed to 125 μM Cd for 8 h. The least toxic exposure known to induce Cd-1 and Cd-2 synthesis was used because the WDI cell line is Cd-sensitive. As Figure 16 shows, no Cd-induced peptides were observed in extracts from these Cd-exposed cells. It is possible that the synthesis of Cd-1 and Cd-2 is induced in WDI but only at much lower Cd concentrations than those used here.

Possible mechanisms by which Cd, Cu, Zn and HS treatments may induce the synthesis of different categories of proteins are discussed in section 4.5.

4.2.2 Further analysis by 2D PAGE

It would have been possible to continue analysis of protein expression by 2D PAGE. Experimental design could have been refined to allow greater numbers of polypeptides induced by Cd to be detected. Various Cd treatments could have been used to study expression of Cd-1 and Cd-2 in the WDI cell line. It was decided instead to characterise Cd-1 and Cd-2 more specifically and attempt to isolate corresponding cDNAs and ultimately genes. Radiolabelled cDNAs or genes for Cd-1 and Cd-2 could be used to probe northern blots to establish whether or not there was also mRNA induction following exposure to Cd in both the Cd300 and WDI cell line. In such investigations the transcripts encoding the constitutively expressed protein, Protein-3, may be used as a non-inducible control.

4.3 PROTEIN MICROSEQUENCING

Having attempted to microsequence an estimated 500 pmoles (by comparative Coomassie staining) of both uncleaved Cd-1 and Cd-2, no sequence information was obtained. As a control, the correct protein sequence was determined for 200 pmoles of β-lactoglobulin which had been electrophoresed, electroblotted, cleaved with CNBr and the cleavage fragments released from the membrane and then eluted through HPLC prior to microsequencing. Due to the manipulative procedures the sample was subjected to, the repetitive yields of this sample showed an overall loss of 85 %. These losses indicate that the cleaved peptides were not being recovered with high efficiency from Problot membranes or they were not being efficiently recovered from HPLC. It is most likely that losses occurred to some extent in both steps and the additive loss recorded in the protein sequencer. However, even with such low yields, protein sequence was still correctly determined from the remaining peptide quantities.

Identical procedures used with an estimated 500 pmoles (based on staining intensity) of both Cd-1 and Cd-2 were unsuccessful. If Cd-1 and Cd-2 bind Coomassie stain very efficiently it is possible that insufficient material was used from which to obtain sequence information. It is equally possible that the physical and chemical properties of Cd-1 and Cd-2 were markedly different from those of β-lactoglobulin, used in the control experiments. The peptide fragments generated by CNBr cleavage may bind more strongly to the Problot and be released less efficiently than those in the control study. Those peptide fragments which were released from Problot may have been very hydrophobic and therefore not eluted efficiently through HPLC (Figure 20). The decrease in efficiency in these two steps may have resulted in an insufficient

amount of each peptide fragment being recovered to enable amino acid sequence to be determined. It is also possible that the peptide fragments of Cd-1 and Cd-2 had become N-terminally blocked in one of the steps prior to microsequencing, resulting in their being refractory to Edman degradation. The peaks observed following HPLC separation of Cd-1 and Cd-2 cleavage fragments (Figure 20) may not represent actual peptide fragments but may be artifactual. This latter suggestion is considered unlikely as no peaks were seen in the control experiments. Additionally, if the peaks had resulted from practical procedures it would be expected that peaks resulting from both samples would have the same retention times.

Although no sequence information was established for Cd-1 and Cd-2, the first 30 N-terminal amino acids were determined for Protein-3 (Figure 19). When this sequence was compared to a data base it was found to have 69 % identity to the Birch pollen allergen, *Betv*1 (Brietender *et al.*, 1989). The alignment of Protein-3 with the N-termini of Betv1 and the parsley PR proteins, PR1 and PR3 is shown in Table 6. Pollen of the whiteBirch (*Betvia verrucoza*) is one of the main causes of Type 1 allergic reactions such as rhinoconjunctivitis and allergic bronchial asthma in Middle and Northern Europe, Northern America and the USSR. Betv1 is an allergic protein with an Mr of 17,000. It is a constituent of the pollen of white birch and is responsible for IgG binding in pollen allergic patients.

*Betv*1 shows 55 % sequence identity with a pea disease resistance response gene, I49. The mRNA *Betv*1 is found in several somatic tissues and it is possible that it is involved with the pathogenesis related response (Brietender *et al.*, 1989). It has been suggested that the PR proteins of *Phaseolus vulgaris*, PvPR1 and PvPR2, pSTHR of potato, PcPR1-1 of parsley, *Betv*1 of white birch and pI49 of pea be classified as a separate group of PR proteins, distinct from the four other recognised groups (PR-1; PR-2,N,O; PR-P,Q and PR-R,S (Kauffman *et al.*, 1990)) (Walter *et al.*, 1990). Protein-3 showed 54 % identity to PR proteins from parsley, the alignment of which is shown in Table 6. Protein-3 may be a constitutively expressed member of the new group of PR proteins proposed by Walter *et al.* (1990). Constitutively expressed PRs have been identified in tobacco explants during flower formation. This suggests a role for PRs in normal developmental processes in healthy plants (Neale *et al.*, 1990). The constitutive appearance of Protein-3 may be due to its induction by growth regulators, such as 2,4-D, present in the medium (section 2.2.1.2). 2,4-D has been shown to induce members of the PR-1 family in tobacco callus, intact leaves (Antoniw *et al.*, 1982) and suspension cultures (Ohashi and Matsuaka, 1987).

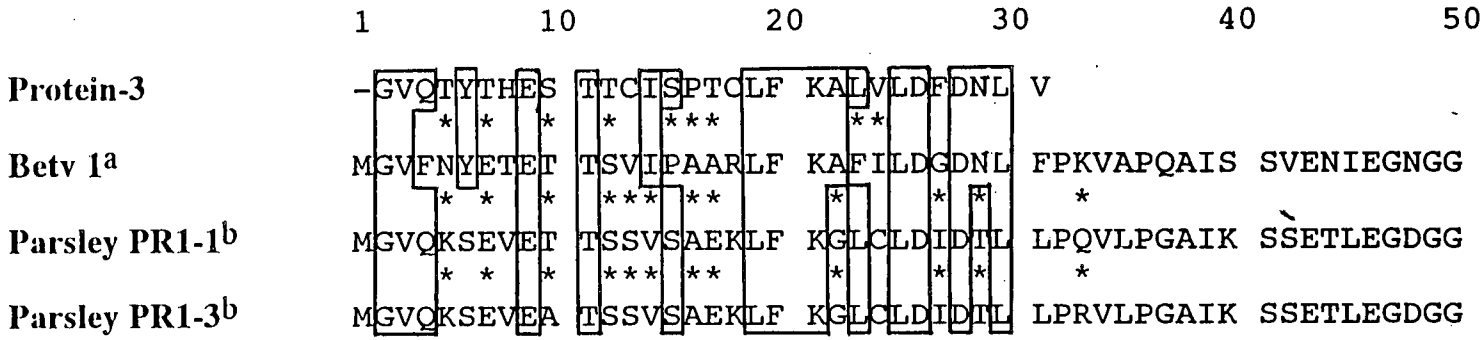
TABLE 6

Alignment of Protein-3 with the N-termini of Betv1of white birch and PR proteins from parsley

a) Brietender *et al.* (1989)

b) Somssich *et al.*(1988)

Protein-3 is used as a reference. A space at the begining of the sequence is used to obtain maximal alignment. Identical amino acids (to those in protein-3) are boxed. An * above a residue indicates a conservative change at that position.


1
10
20
30
40
50
Protein-3
-GVQTYTHES TTCISPTCLF KALVLDFDNL V
Betv 1[a]
MGVFNYETET TSVIPAARLF KAFILDGDNL FPKVAPQAIS SVENIEGNGG
Parsley PR1-1[b]
MGVQKSEVET TSSVSAEKLF KGLCLDIDIL LPQVLPGAIK SSETLEGDGG
Parsley PR1-3[b]
MGVQKSEVEA TSSVSAEKLF KGLCLDIDIL LPRVLPGAIK SSETLEGDGG

4.3.1 Amplification of nucleotide sequence corresponding to Protein-3

The amino acid sequence of Protein-3 was used to construct two oligonucleotides to amplify the corresponding nucleic acid sequence by PCR (section 3.1.11). Although the amplification from genomic sequence was unsuccessful (section 3.1.11) the cDNA corresponding to Protein-3 was amplified from total cDNA from *D. innoxia* (Figure 21) (the sequence of which is shown in Figure 19). On labelling this (short) amplification product with ^{32}P[dCTP] and using it to probe Southern blots no signal was detected on developed autoradiograms. Following several unsuccessful attempts using the 82 bp cDNA sequence in Southern blot analysis an alternative PCR strategy, RACE PCR, was used to amplify a longer cDNA sequence corresponding to Protein-3, to be used in such analysis. A number of cDNAs were amplified, one of which hybridised to the 82 bp cDNA clone previously obtained (Figure 22). Attempts at cloning this cDNA proved unsuccessful, however these results clearly indicate that the chosen strategy of 2D PAGE identification of individual polypeptides, microsequencing and amplification of corresponding nucleic acid sequences from *D. innoxia* cultures was a feasible one.

4.4 ISOLATION OF Cd-INDUCED cDNA FROM A *D. innoxia* cDNA LIBRARY

Due to the inability to obtain sequence information for Cd-1 and Cd-2, in order to identify cDNA sequences that encoded Cd-1 and Cd-2, a cDNA library was constructed which was to be screened with antibodies raised against the two peptides. Such screening would lead to the isolation of cDNAs representing Cd-induced Poly A^+ RNA species.

Following construction of the library the length of cDNA inserts was analysed. Figure 24 shows that of eight phagemids recovered the insert sizes range from 0.4 to 2.0 Kb.

Neither the antisera nor the immune fractions purified from the antisera (Figure 23) raised against Cd-1 or Cd-2 reacted against western blots of *D. innoxia* proteins. As can be detected from comparative Coomassie staining (section 3.1.4) it was considered that sufficient material had been used to raise an antibody (approximately 500 pmoles). Coomassie R250 may bind very strongly to Cd-1 and Cd-2 and comparative staining analysis may have led to an over estimation of the amount of protein present. If only a small amount of protein was used, intraperitoneal injections may have led to antibodies being raised against the two proteins, however these were not attempted in this study. It is also possible that the two proteins Cd-1 and Cd-2 were not sufficiently antigenic to raise an immune response.

As described above no antigenic response could be detected with antisera raised against Cd-1 and Cd-2, as analysed by western blots, therefore it was decided to differentially screen the library. The method of screening was chosen, as following exposure of the cells to Cd only those

sequences which are induced are detected, due to the dual labelling method used to construct the probes. In addition the procedures entailed the least complex manipulations. Differential screening led to the isolation of two cDNA clones, designated Cd-6 and Cd-8.

Both clones required subcloning in order to allow full length sequence to be determined in both directions (Figures 26, 27 and 28). On analysing the sequence it was observed that it was possible to impose an open reading frame of 826 bp on the Cd-6 sequence . Similarly, Cd-8 was shown to contain an ORF which encompassed 489 bp of sequence before the ORF was terminated by a stop codon. No other ORF can be seen in either sequence. The predicted amino acid sequences from these ORFs are shown in Figures 29 and 30. These amino acid sequences were compared to a protein database (Daresbury). Cd-8 did not show significant similarity to any protein in the database. It did have 25-30 % identity with extensins from tobacco, carrot and tomato. However, these scores were due to Cd-8 having a relatively high proline (12.3 %) and serine (13 %) content. The amino acids are not arranged in any motifs such as the Ser-$HPro_4$ repeat found in hydroxyproline rich glycoproteins (HRGP) but are distributed evenly throughout the protein and therefore this slight similarity to extensins was not considered to be significant.

The predicted product of Cd-6 was shown to have no significant homology to any protein sequence in the data base when comparison was made to the full length ORF of Cd-6. When partial alignments were made, a cysteine rich motif, Cys-Cys-Ser-Cys-Cys, was found to exist within the ORF. This motif is shown underlined in Figure 29 and it is noted that a similar motif is present in MTs. This raises the interesting possibility that the Cd-6 product may contain a Cd-binding domain.

Neilson *et al.* (1985) were able to determine the relative binding affinities for metal MT complexes to be in the order: Hg (II) > Cu (I) > Cd (II) > Zn (II) > Ni (II), Co (II). Metal is co-ordinated in two domains designated α and β. The chelating agent EDTA was used as a competitive chelator to study the domain specificity of Cd^{2+} and Zn^{2+} in rabbit liver MT-2. The results of these studies suggested that the Cd^{2+} bound more strongly to the α-domain than to the β-domain and that Cd and Zn are equally labile in the β-domain with respect to chelation by EDTA (Stillman and Zelazowski, 1987). Reconstitution studies of apo-MT from rabbit liver with Cu salts revealed that Cu ions bind preferentially to the β-domain of the molecule and that the binding stoichiometry of Cu-MT differs from Cd- or Zn-MT. Cu-MT appears to contain 11-12 Cu ions, 6 bound in the β-domain and 5-6 in the α-domain. In contrast, Cd-MT contains 7 Cd ions, 3 bound to the β- and 4 to the α-domain (Neilson and Winge, 1984). The distribution of cysteine residues between the α- and β-domains of MTs is shown below in Figure 34. It is possible that the different arrangement of cysteines in the two domains accounts for the different affinities for Cd, Zn and Cu. Cys-Cys-X-Cys-Cys motifs may play an important role in determining the Cd and Zn binding of the α-domain. Similarly, the presence

of this motif in the putative Cd-6 protein may suggest a preferred involvement of the protein with the metals Cd and Zn rather than Cu.

Figure 34

A consensus sequence for mamalian MT derived from the sequence of 14 mammalian MTs.

MPDNCSCATGGSCTCAGSCKCKECKCTSCKKS**CCSCC**PVGCAKCAQGCVCKGASDKCSCCA
----C-C-----C-C---C-C--C-C--C---**CC-CC**---C--C---C-C------C-CC-

β-domain α-domain

The position of the consensus cysteine signature is shown on the lower line. The CC-CC motif is shown in bold (adapted from Hamer (1986), in which original references are cited).

4.4.1 Northern and Southern analysis of Cd-6 and Cd-8

Figure 31 indicates that the differential expression of Cd-6 and Cd-8 in Cd-exposed and non-exposed cultures was reproducible. The intensity of hybridisation of the two clones differs between the RNA extracted from cells exposed to different Cd-treatments (Figure 30A). This may represent differences in the induction of RNA, corresponding to Cd-6 and Cd-8, by varying Cd-treatments; the less toxic the treatment the greater the induction of Cd-6 and exposure of the cells to 250 μM Cd for 4 h resulting in the greatest induction of Cd-8. However, further analysis is needed to ensure that this observation is reproducible as the high stringency wash used to strip the filters may have resulted in RNA being lost to differing extents in the lanes. Northern blots also showed both Cd-6 and Cd-8 to be coinduced by HS but neither were observed to be induced by Cd in the non-tolerant, WDI, cell line (Figure 32). The difference in hybridisation intensities of the two clones may be due the RNA corresponding to Cd-8 being induced to a greater extent than that of Cd-6 following HS, although the possibility of loss of RNA on stripping the filter cannot be totally excluded (since the experiment has not been replicated). It is possible that Cd treatments other than those tested here may have led to the identification of Cd-induced transcripts in the WDI cell line. Southern blot analysis revealed the presence of 2-3 bands representing hybridisation to the two clones in most digests (Figure 33). This may be indicative of Cd-6 and Cd-8 being members of a small gene family but it is equally conceivable that, as there are so few bands, there were internal restriction sites for restriction enzymes used within the corresponding (unique) genes for both clones.

4.5 CHANGES IN THE ABUNDANCE OF SPECIFIC PROTEINS AND mRNAs FOLLOWING EXPOSURE TO Cd, Cu, Zn OR HS; POSSIBLE MECHANISMS

The abundance of the two peptides Cd-1 and Cd-2 increased following exposure of Cd-tolerant *D. innoxia* cells to Cd, Cu, Zn and HS, whilst increased abundance of RNA species which hybridise to Cd-6 and Cd-8 was detected in response to Cd and HS. The increase in abundance of Cd-6 and Cd-8 mRNA indicates that Cd stimulates transcription of the corresponding genes and/or enhances Cd-6 and Cd-8 mRNA stability. The accumulation of the two novel peptides Cd-1 and Cd-2 may be due to increased transcription, increased translation, modified mRNA, modified protein stability or post-translational protein modification.

Some of the proposed mechanisms which could cause the abundance of particular proteins or mRNAs to change in response to Cd are categorised below. This is followed by further discussion of possible mechanisms which explain the increased abundance of Cd-1 and 2 proteins and Cd-6 and 8 mRNA seen in response to Cd-exposure (and other factors) in *D. innoxia.*

i) Alteration of enzyme activity and compensatory modification of protein synthesis

The ways in which Cd can directly alter the biochemical activity of enzymes are binding to sulphydryl groups involved in the structural or catalytic activity of the enzyme and substitution for the intrinsic metal ions in the enzymes. Table 2 provides a list of some of those enzymes whose activity is affected by Cd. The alteration of enzyme activity by Cd may result in feed back regulation leading to a compensatory alteration in transcription of the gene encoding the affected enzyme. This may be particularly significant for those enzymes inhibited by Cd, with feedback regulation leading to increased transcription and hence *de novo* synthesis of the deficient enzyme. Substitution of Cd for Zn in RNA polymerase has been shown to decrease both its activity and DNA binding ability (Solaiman and Wu, 1985b). The affect of such a substitution would be to reduce the general level of transcription in the cell. More stable proteins and transcripts would thus become relatively more abundant.

Metal substitution in metalloproteins may lead to a change in tertiary protein structure resulting in them being recognised as abnormal proteins which could subsequently be targeted by the ubiquitin system. The ubiquitin system could then mediate the HS induction response as described by Munro and Pelham (1985) (*iii*, below).

ii) Substitution of metal requiring transcription factors

Hirt *et al.* (1989) demonstrated that exposure of tobacco suspension cultures to Cd stimulated RNA synthesis and proposed that this was due to Cd altering the specificity of DNA binding factors. Cd has been shown to be capable of substituting for Zn in all three classes of transcription factor, Zn-fingers, Zn-twists and Zn-clusters (Kuwahara *et al.*, 1991; Sabbath *et al.*, 1987; Gadhavi *et al.*, 1991). Cd substitution was determined to have no effect on the DNA

binding activity of SpI (Kuwahara *et al.*, 1991), a Zn-finger, and similarly in bovine oestrogen receptor, a Zn-twist, Cd substitution resulted in no loss of DNA binding activity (Predki and Sarkar, 1991). Although Cd has been shown to substitute for Zn in the Zn-cluster, GAL4, the effect on its DNA binding activity was not established (Gadhavi *et al.*, 1991). Whether the transcription of particular genes is affected by Cd-substituted transcription factors has still to be established. Where the DNA binding activity of a transcription factor is lost as a result of Cd substitution, or if its effect upon transcription is modified, then this direct interaction between metal and transcription factor may result in the altered expression of a number of genes.

To date Cu has not been shown to bind the transcription factors termed Zn-fingers, however it has been shown that the intrinsic Zn of bovine oestrogen receptor, a transcription factor classified as a Zn-twist, can be replaced by Cd and Cu, although the reconstituted Cu-transcription factor showed no DNA binding activity (Predki and Sarkar, 1991). Gadhavi *et al.* (1991) demonstrated that in the transcription factor GAL4, a Zn-cluster, the Zn could be replaced by Cd. Another transcription factor CUP1 binds Cu in a similar manner and is termed a Zn-fist (Dameron *et al.*, 1991). If such a mechanism as that mentioned above does account for the induction of transcripts, the transcription factor may resemble the Zn-cluster category factors.

iii) Cd-induction of the heat shock response

Cd has been shown to closely parallel the effects of HS. Following exposure to HS normal cell protein synthesis is altered and a set of new proteins is induced. Most of these proteins are co-induced by Cd (Edelman *et al.*, 1988). Transcripts of one particular HSP, ubiquitin, have been shown to be co-induced by Cd (Mueller-Taubenberger *et al.*, 1988). In addition to its known role in the degradation of abnormal and short-lived proteins the ubiquitin system has also been proposed to play a role in activating transcription in response to HS (Munro and Pelham, 1985). Ubiquitin is involved in an ATP-dependent proteolysis pathway in eukaryotes (Hamer, 1986). Proteins targeted for degradation are recognised by ubiquitin-conjugating enzymes which covalently attach ubiquitin to internal lysine residues of the substrate (Jentsch *et al.*, 1990). Ubiquinated substrates are then degraded by the proteosome, a large hollow cylindrical particle composed of multiple protein subunits (Seufert and Jentsch, 1992). The model of Munro and Pelham (1985) suggests that the depletion of the free ubiquitin pool causes the dissociation of ubiquitin from a transcription factor allowing it to bind to the HSEs of genes encoding HSPs. Since ubiquitin is induced by HS this would subsequently replenish the amount of ubiquitin, some of which could bind to the transcription factor rendering it inactive and preventing further transcription of the HSP genes. Since some proteins may be structurally impaired by Cd, as they are by HS, it is also possible that some of the genes encoding proteins involved in the ubiquitin mediated proteolysis may be co-induced by Cd. This could include specific ubiquitin conjugating enzymes.

Brodl (1990) proposed two other ways in which the HS response may be elicited. These mechanisms include changes in the oxidative/ reductive environment of the cell, which it was proposed could account for the induction of certain aspects of the HS response by other factors including Cd. In addition, changes in ion levels, probably Ca^{2+} mediated by a Ca^{2+}-dependent protein kinase, were also proposed as an alternative mode of regulation of the HS response. Burke and Orzech (1988) proposed that HS makes plant cell membranes leaky to toxic metals which otherwise would be excluded from the cell. This may also account for the observation that exposure to Cd co-induces, at least in part, the HS response.

Cross protection to metal toxicity has been reported to occur following HS. Wheat leaf segments acquired protection against metal toxicity following exposure of the seedlings to HS in the dark. The heat shocked tissue acquired an increased protection against Cd, Al and Fe toxicity. The acquired protection against Cd was 400 fold greater than that of leaf segments held at 25 ^{o}C. No changes in the sensitivity of the leaf segments to Zn were observed following HS and only a minor increase in the resistance to Cu-toxicity was recorded (Orzech and Burke, 1988).

Heat shock has been proposed to make the cell membranes leaky to metals (Burke and Orzech, 1988). This would allow an influx of metal previously excluded from the cell which could bind to transcription factors and hence induce transcription by the mechanism described above, or by other mechanisms. Cd-1, 2, 6 and 8 could thus be induced as part of the HS response, if the cell membranes do become leaky to metals following HS, even if their induction is part of the metal homeostatic mechanism (*iv*, below). However, no experimental evidence is available to support the hypothesis of Burke and Orzech (1988).

iv) Stimulation of metal homeostatic mechanisms

Exposure to Cd leads to the stimulation of homeostatic responses which reduce the amount of free intracellular Cd in both plants and animals. The majority of the literature relating to this response in plants centres on PCs. It has been established that PCs bind Cd and other metals and are involved in metal detoxification, however it still remains to be shown that any of the enzymes involved in the biosynthesis of these molecules are induced by Cd. Indeed, data indicate that this response, at least initially following exposure to Cd, is largely regulated at a post-translational level (reviewed in Robinson, 1990b). More recently, MT-like peptides have been reported to occur in plants. Studies carried out by Evans *et al.* (1992) suggest a role for one of these MT-like peptides, $PsMT_A$, in Cu-homeostasis and comparison of the pH of half dissociation constants of other metal-binding compounds suggests that recombinant $PsMT_A$ has a high affinity for Cu and affinities for Cd similar to PCs (section 1.2.3.1.3). However, no evidence currently exists to suggest that these peptides are induced by Cd in the same manner as animal MTs.

Some of the mechanisms discussed above would provide a single means by which Cd, Cu, Zn and HS could account for the transcriptional induction of Cd-1 and Cd-2, and Cd and HS for the induction of Cd-6 and Cd-8. Many different mechanisms could also operate in the cell. The increased accumulation of Cd-6 and Cd-8 following Cd exposure may be due to increased mRNA stability. The peptides Cd-1 and Cd-2 may accumulate following Cd exposure due to increased protein stability, to Cd interacting with factors important in the translational process or by post-translational protein modification, possibly by the Cd-stimulated cleavage of an existing larger protein.

The investigations described here did not lead to the isolation of "MRE-like" *cis*-acting sequences. However, the two cDNAs, Cd-6 and Cd-8, could be used to isolate the corresponding DNA sequences from a genomic library and the 5'-regulatory regions of these sequences could subsequently be analysed for the presence of "MRE-like" motifs. It would be expected that *cis*-acting regulatory sequences identified in the 5'-region of the genes encoding Cd-6 and Cd-8 would interact with *trans*-acting factors stimulated by both Cd and HS.

As discussed above the presence of the motif, Cys-Cys-X-Cys-Cys in the putative Cd-6 protein may suggest metal binding. The isolation of a longer cDNA or genomic clone would allow further database analysis and the possible identification of Cd-6. Future studies on the role of the product of Cd-6 in response to elevated Cd might be of particular interest.

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