ISOLATION AND CHARACTERISATION OF NOVOBIOCIN-BINDING PROTEINS IN THE YEAST *SACCHAROMYCES CEREVISIAE*

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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August 1993

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Acknowledgements

There are a few people who should be mentioned who have helped me complete my thesis. Eli for offering me a position in his lab and his unique form of supervision and some very interesting lab meetings I hope I'm a better scientist for it.

I would also like to thank the "Yeastie boys" (in alphabetical order so no one gets jealous Agus, Amos, Big Will, Ed, Frank, Gary, Lee, Little Will, Mike Murray, Mick Pock, Sean, Steve,) for making life in the lab a pleasure, so much so that they were the only thing that brought me into the lab on some deeply depressing days. Thanks also to other members of the Genetics Department who have helped me over the years I'm afraid they are to numerous to mention individually.

I'm very much indebted to my parents for there help and encouragement over these many years of being a poor student. Thank-you, I hope the end result was worth it!

Last but by no means least; thanks for all your love and help Katrina.

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Abbreviations

One and three letter amino acid abbreviations

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Glu	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
B	Asx	Asp or Asn
Z	Glx	Glu or Gln
X		Undetermined or atypical

Other abbreviations

A (dATP)	2'-deoxyadenosine 5'-triphosphate
ADPNP	5'-adenylyl-β-γ-imidodiphosphate
*m*AMSA	4'-(9-acridinylamino) methanesulphon-*m*-anisidide
APS	Ammonium Persulpate
ATP	Adenosine 5'-triphosphate
bp	base pair
BSA	Bovine serum albumen
C (dCTP)	2'-deoxycytidine 5'-triphosphate
DAPI	4,6-diamino 2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol

EDTA	Diaminoethanetetra-acetic acid disodium salt
EMBL	European Molecular Biology Laboratory
EtBr	Ethidium Bromide
G (dGTP)	2'-deoxyguanosine 5'-triphosphate
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
IgG	Immunoglobulin G
IMS	Industrial methylated spirit
IPTG	Isopropyl-β-D-galactopyranoside
Kb	Kilobase pair
KDa	Kilodalton
MOPS	3-(N-morpholino)propanesulphonic acid
MOPS buffer	[20mM MOPS, 5mM sodium acetate, 100μM EDTA] pH 7.0
OD	Optical density
ORF	Open reading frame
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
SSC	Saline sodium citrate
T (dTPC)	2'-deoxythymidine 5'-triphosphate
TAE	Tris-acetate (40mM Tris-acetate, 1mM EDTA)
TBS	Tris-buffered saline (10mM Tris-HCl pH7.4, 150mM NaCl)
TBE	Tris-borate (45mM Tris-borate, 1mM EDTA)
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
Tris	Tris(hydroxymethyl methylamine) [2-amino-2-(hydroxymethyl)propane-1,3-diol,(tris)]
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YPD	Yeast peptone dextrin

Chapter 1

Introduction

1.1 Novobiocin

Novobiocin is a member of a group of antibiotics known collectively as coumarins (coumarin-glycoside antibiotics). As well as novobiocin, this group also includes dihydronovobiocin, methoxynovobiocin, chlorobiocin and coumermycins.

The name novobiocin was accepted in 1956 for the antibiotic which was independently discovered by at least five laboratories. It was first reported to have been isolated in 1951 by Waga & Takahashi (1951), but the original description (Waga, 1953), under the name griseoflavin, omitted certain physico-chemical data (infra red, ultra violet absorption spectra) and contained several discrepancies (Berger & Batcho, 1978). Kuroya *et al.* (1958) again isolated novobiocin from Waga's culture (*Streptomyces griseoflavus* No. 160) and by direct comparison with a novobiocin sample (Upjohn) demonstrated identity of the antibiotic. Further work, including analysis of UV and IR absorption spectra, optical rotations, antibacterial spectra and bacterial cross-resistance by Welch & Wright (1955), showed that cathomycin produced by *Streptomyces spheroides* (Kaczka *et al.*, 1955) and streptonivicin produced by *Streptomyces niveus* (Hoeksema *et al.*, 1955) were identical to novobiocin.

Structural studies carried out independently by groups at Merck (Kaczka *et al.*, 1955; Spencer *et al.*, 1956; Shunk *et al.*, 1956; Walton *et al.*, 1956; Kaczka *et al.*, 1956) and Upjohn (Hoeksema *et al.*, 1955; Hoeksema *et al.*, 1956; Hinman *et al.*, 1956) established the unique structure of novobiocin as 7-(3,O-carbamoyl-5,5-dimethyl-4-O-methyl-α–L-lyxosyl)-4-hydroxy-3-{4-hydroxy-3-(3-methylbut-2-enyl) benzamido}-8-methylcoumarin (see Fig. 1.1). The antibiotic consists of a substituted benzoic acid moiety (A ring) linked by an amide bond to an aminohydroxycoumarin moiety (B ring), which is bound to a unique sugar, novose, with one hydroxyl carrying a carbamyl group (C ring).

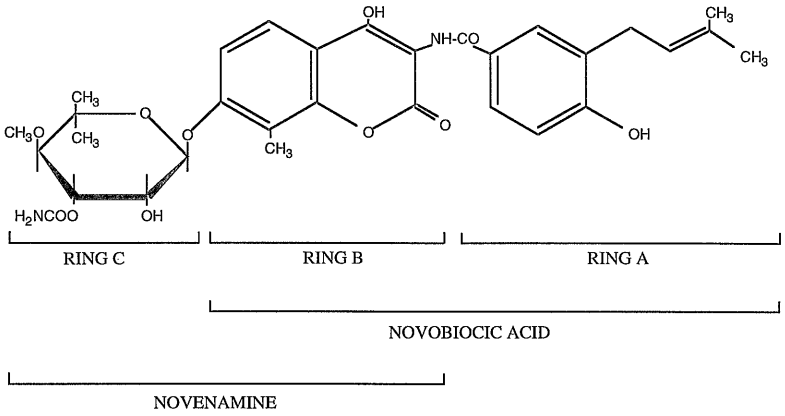


Figure 1.1 Structure of novobiocin

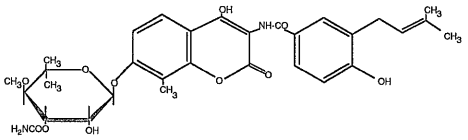
The antibiotic consists of a substituted benzoic acid moiety (A ring) linked by an amide bond to an aminohydroxycoumarin moiety (B ring), which in turn is joined to a unique sugar, novose, with one hydroxyl carrying a carbamyl group (C ring). The A and B ring constitute novobiocic acid; the B and C ring constitute novenamine.

Fig. 1.2 shows a comparison of the structure of novobiocin with that of two other coumarin antibiotics, coumermycin A_1 and chlorobiocin. Coumermycin A_1 is essentially a dimer of novobiocin, yet it has been shown to have a higher anti-microbial activity. However, in spite of its excellent *in vitro* anti-microbial activity, the insoluble nature of coumermycin A_1 made it unsuitable for clinical use (Berger & Batcho, 1978).

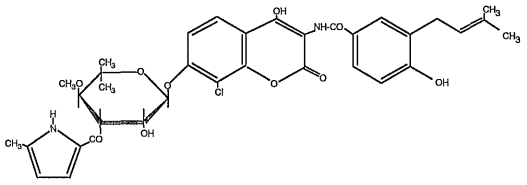
The antimicrobial activity of novobiocin has been evaluated by many investigators. Novobiocin is active against *Staphylococcus aureus*, (including β-lactamase producing and methicillin-resistant strains) *Streptococcus pneumoniae*, *Corynebacterium diphtheriae*, *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Lambert and O'Grady, 1992). Some strains of *Pasteurella*, *Citrobacter* and *Proteus*, particularly *Proteus vulgaris*, are susceptible to moderate concentrations of the antibiotic but other enterobacteria are resistant (Lambert and O'Grady, 1992).

Susceptibility to novobiocin has been used to distinguish *Staphylococcus saprophyticus*, which is almost universally resistant, from other staphylococci, which are almost universally susceptible. Similarly, peptostreptococci, which are susceptible to novobiocin, can be distinguished from peptococci, which are resistant to the antibiotic (Ducate and Floreke-Ebelling, 1983). In general, novobiocin (in common with other coumarins) is more active against Gram-positive than Gram-negative organisms. It appears that Gram-positive bacteria are more permeable to novobiocin than Gram-negative bacteria.

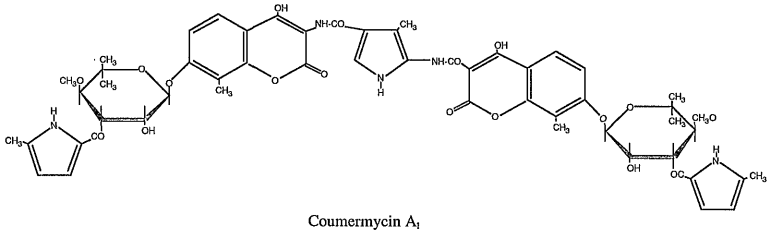
Novobiocin was widely used for some time in human medicine, its major use being in the treatment of staphylococcal infections (Martin *et al.*, 1956). An idea of the widespread use of novobiocin in the early nineteen sixties can be obtained from estimates of the amount of novobiocin produced; for example, in 1961 the total annual production of the drug in the USA alone was estimated to be 15,000Kg (Berger & Batcho, 1978). However, as a result of the frequency of adverse effects, the availability of penicillinase-resistant penicillins and other potent anti-staphylococcal agents, the drug has become more or less obsolete. Novobiocin does not figure in current bacterial therapeutics to the extent that pharmaceutical preparations of the drug are no longer available in the UK (Lambert and O'Grady, 1992). In spite of this drop in its clinical use, interest in the antimicrobial



Novobiocin



Chlorobiocin



Coumermycin A_1

Figure 1.2 Comparison of the structures of coumarin antibiotics

The structures of three coumarin antibiotics novobiocin, chlorobiocin and coumerycin A_1 are shown above.

uses of novobiocin persists, particularly its use in combination with sodium fusidate or rifampicin for the treatment of methicillin-resistant Staphylococcal infections (Jensen, 1968; Drusano *et al.*,1986; Walsh, 1986).

More recently, in the general search for new anti-cancer therapeutic agents, interest in novobiocin has been revived. Novobiocin has been shown to reduce the proliferation of melanoma cells (Nordenberg *et al.*, 1992). In addition, the effects of three different alkylating agents on murine tumour cells is enhanced synergistically by novobiocin (Eder *et al.*, 1989). This study resulted in the drug being entered into clinical trials (Eder *et al.*, 1991). However, the serum levels of novobiocin attained in these trials are within the range found to inhibit T-cell mediated cytotoxicity (Wood and Stansfield, 1992). These studies seem to contain contradictory reports of novobiocin mediated cytotoxicity and a connection between the reports of Eder *et al.* (1989, 1991) and Wood and Stansfield (1992) is not yet clear. It is obvious that the cytotoxic effect of novobiocin is not fully understood and further study of its mode of action is required to understand this and other effects.

1.2 The target of novobiocin in prokaryotes

The mechanism of action of novobiocin in prokaryotes has been studied extensively. Some of the initial work focused on the negative charge associated with novobiocin. Novobiocin was shown to bind magnesium and it was suggested that it might inhibit certain magnesium-dependent enzyme systems *in vitro* by inducing a magnesium deficiency (Brock, 1962b). This hypothesis was reinforced by the fact that magnesium deprivation of *Escherichia coli* ML-35 cells leads to impaired membrane integrity and RNA degradation (Brock, 1962a). On these grounds, Brock proposed that the effects of novobiocin were a consequence of the drug binding intracellular magnesium (Brock, 1962a; Brock, 1962b). However, these effects were later found to be unique to ML strains of *E. coli* (Smith and Davis, 1967) making the binding of magnesium an unlikely explanation for the biological activity of novobiocin.

Treatment of *Staphylococcus aureus* with novobiocin causes the accumulation of cell wall precursors (Strominger and Threnn, 1959; Wishnow *et al.*, 1965) and, for some

time, inhibition of cell wall synthesis was considered to be the primary lethal effect of novobiocin. However, later studies of macromolecule synthesis in novobiocin-treated *Escherichia coli* cells indicated that inhibition of DNA synthesis and, to a lesser extent, RNA synthesis occurred immediately, whereas protein and cell wall synthesis were affected later (Smith and Davis, 1967). Subsequently, novobiocin was shown to inhibit DNA replication in toluene-treated *E. coli* (Staudenbauer, 1975) and to inhibit plasmid ColE1 and phage ϕX174 DNA replication *in vitro* (Staudenbauer, 1976; Sumida-Yasumoto *et al.*, 1976). These observations demonstrated that the inhibition of DNA replication and not cell wall synthesis, was the primary effect of novobiocin in *E. coli*. However, this conclusion was complicated by the discovery that inhibition of purified *E. coli* DNA polymerase and RNA polymerase by coumermycin A_1, a closely related antibiotic, was nonspecific (Ryan and Wells, 1976). In addition, novobiocin inhibits sporulation and it has been suggested that this inhibition is due to its effect on transcription (Vazquez-Ramos & Mandelstam, 1981) since novobiocin inhibits spore out-growth in *Bacillus subtilis*, a process that is independent of DNA synthesis (Ginsberg and Keynan, 1978; Gottfried *et al.*, 1979).

The situation was simplified by the discovery of DNA gyrase (Gellert *et al.*, 1976a) and the demonstration that coumarins strongly inhibited DNA gyrase activity (Gellert *et al.*, 1976b). In cell-free extracts prepared from *E. coli*, DNA gyrase activity was shown to be strongly inhibited by novobiocin at a concentration of 3μg ml^{-1}, whereas enzyme from a novobiocin-resistant mutant of *E. coli* was entirely resistant to the same concentration of the antibiotic. In addition, mutations conferring coumarin-resistance were mapped to the *cou* locus close to the *dnaA* gene in *E. coli* (Ryan, 1976; Orr *et al.*, 1979; Fairweather *et al.*, 1980). The *cou* locus was subsequently found to specify the B subunit of DNA gyrase (Gellert *et al.*, 1977; Sugino *et al.*, 1977; Higgins *et al.*, 1978) and was renamed *gyrB* (Hansen and von Meyenburg, 1979). Mizuuchi *et al.* (1978) found that the ATPase activity of bacterial gyrase was strongly inhibited by novobiocin and suggested that the drug inhibits bacterial gyrases by blocking the access of ATP to the B-subunit. Further work demonstrated that only the B-subunit of bacterial gyrase is required for catalysing the cleavage of ATP and this ATPase activity is competitively inhibited by novobiocin (Sugino

et al., 1978; Staudenbauer & Orr, 1981). These results indicated that the toxicity of novobiocin in bacteria is due to its effect on the ATP binding site of the B-subunit of DNA gyrase.

1.3 DNA gyrase

Bacterial DNA gyrase (a type II topoisomerase) was originally detected as a host factor required for site-specific integration of bacteriophage λ (Gellert *et al.*, 1976a). The active enzyme consists of two pairs of non-identical protomers: two A subunits each with a molecular mass of 97kDa, encoded by the *gyrA* gene (Swanberg and Wang, 1987; Bachmann and Low, 1980) and two B subunits each with a molecular mass of 90kDa, encoded by the *gyrB* gene (Adachi *et al.*, 1987). Although there is some conflicting evidence as to the relative abundance of subunits *in vivo*, the enzyme requires an equal number of protomers for its activity (Cozzarelli, 1980). DNA gyrase or prokaryotic topoisomerase II is the only topoisomerase isolated, so far, with the ability to introduce negative supercoil turns into relaxed, covalently closed, circular double-stranded DNA in an ATP-dependent reaction. A model of the process is presented in Fig. 1.3 and the essential features are described below.

The breakage-rejoining process, resulting from type II topoisomerase activity is initiated by binding of the enzyme to its nucleic acid substrate, forming a complex in which approximately 140bp of DNA is wrapped around the protein (Klevan and Wang, 1980). Although type II topoisomerase interacts with preferred sequences, the specificity is not as stringent as that of restriction endonucleases. This phenomenon, allowing the enzyme to act at numerous sites along the DNA strand may be critical to the physiological function of the enzyme (Vosberg, 1985).

The subsequent step in the enzyme activity is the establishment of a double-stranded DNA cleavage/religation equilibrium. This equilibrium highly favours the religation process (Osheroff, 1989). The breakage of each strand of the double-helix DNA is thought to be mediated by a single protomer (GyrA) of the enzyme. Following hydrolysis of the DNA, tyrosine 122 of subunit A becomes covalently bound to both newly

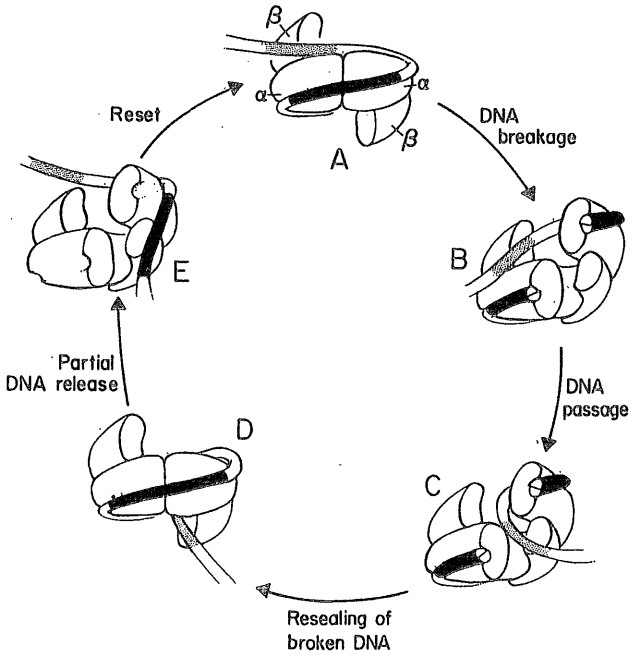


Figure 1.3 A model for negative supercoiling of DNA by gyrase

Step A, represents a gyrase molecule consisting of two GyrA and two GyrB monomers with a circular duplex DNA wrapped in a right-handed superhelix. Step B, the DNA is cleaved by the enzyme. Step C, the unbroken segment of the DNA is passed through the breakage. Step D, the broken DNA is resealed. Step E, the DNA strand is released from the enzyme (Morrison and Cozzarelli, 1981).

generated 5' ends via the O-phosphotyrosine bonds (Wang, 1987). Once the enzyme has created a double-strand break in the nucleic acid "backbone", a segment of DNA is passed through the break. The enzyme then re-establishes a DNA cleavage/religation equilibrium favouring rejoining/religation over breakage (Robinson and Osheroff, 1991). The introduction of negative supercoils requires the hydrolysis of ATP and the number of superhelical turns introduced into an initially relaxed, circular DNA molecule has been calculated to be approximately equal to the number of ATP molecules hydrolysed by DNA gyrase (Sugino and Cozzarelli, 1980). Therefore, two ATP molecules are hydrolysed per cycle of reaction by DNA gyrase, leading to the introduction of two negative superhelical turns. In the presence of the non-hydrolysable ATP analogue ADPNP (5'-adenylyl-β-γ-imidodiphosphate), limited supercoiling by gyrase is observed, suggesting that nucleotide binding can stimulate one round of supercoiling but that hydrolysis of ATP is required to return the gyrase to its original conformation, thereby permitting enzyme turnover (Sugino *et al.*, 1978).

In the absence of ATP, DNA gyrase is capable of relaxing negatively supercoiled DNA (Gellert *et al.*, 1977; Sugino *et al.*, 1977). The relaxing activity is much less efficient than the supercoiling activity, with about 20-40 times as much enzyme required for a comparable rate (Higgins *et al.*, 1978; Gellert *et al.*, 1979). Relaxation of negative supercoiled DNA by DNA gyrase is inhibited by quinolone drugs but not by coumarins (Gellert *et al.*, 1977; Sugino, *et al.*, 1977). It is possible that DNA relaxation is simply the reverse of the supercoiling reaction and that ATP hydrolysis is required to drive DNA strand passage in one direction only.

In addition to these activities, DNA gyrase can catenate and decatenate two duplex circles (Kreuzer and Cozzarelli, 1980; Mizuuchi *et al.*, 1980) and resolve topologically knotted duplex DNA (Liu *et al.*, 1980; Mizuuchi *et al.*, 1980). Experimental evidence supports a role for gyrase in the decatenation of daughter chromosomes. For example, nucleoids isolated from a *gyrB* temperature-sensitive mutant grown at the non-permissive temperature are found to be doublets (Steck and Drlica, 1984) and these doublets can be resolved by gyrase *in vitro*. Also, a number of mutants defective in chromosome

partitioning have been isolated and termed Par mutants. Several of these have now been linked to DNA gyrase. The ParA phenotype has been shown to be attributable to a mutation in the *gyrB* gene and thus is likely to be a consequence of a defect in decatenation (Kato *et al.*, 1989). The ParD phenotype has been shown to be a consequence of two mutations, one of which maps to *gyrA* and is thought to be primarily responsible for the partition defect (Hussain *et al.*, 1987a; Hussain *et al.*, 1987b). Again this defect may be attributable to impaired decatenation. Recently, the *E. coli parC* and *parE* genes have been sequenced (Kato *et al.*, 1990). The ParC protein shows homology to gyrase A subunit, while ParE shows homology with the B subunit. Preliminary experiments indicate that ParC and ParE in combination can relax negatively supercoiled DNA and may represent a new type II topoisomerase in *E. coli* , which has been named topoisomerase IV (Kato *et al.*, 1990). It is not yet clear whether gyrase and topoisomerase IV have distinct functions in the partition process. Further experiments on purified topoisomerase IV will be required to address this question.

As described above, coumarin antibiotics inhibit DNA replication and, to a lesser extent, transcription in bacteria (Smith and Davis, 1967; Staudenbauer, 1975). This inhibitory effect was thought to be due to the drug inhibiting the B subunit of bacterial DNA gyrase (Gellert *et al.*, 1976b; Mizuuchi *et al.*, 1978; Sugino *et al.*, 1978). For some time, negatively supercoiled DNA in bacteria appeared to be essential for almost every DNA-dependent biological activity and the role of DNA gyrase in DNA replication was attributed to a requirement for superhelicity in facilitating DNA strand separation and hence replication fork movement (Alberts and Sternglanz, 1977; Itoh and Tomizawa, 1977). As expected, temperature-sensitive DNA gyrase mutants of *E. coli* were found to be impaired in DNA replication at the non-permissive temperature (Orr *et al.*, 1979; Fairweather *et al.*, 1980). These gyrase mutants were found to inhibit the initiation of DNA replication, but not chain elongation (Orr and Staudenbauer, 1981; Orr *et al.*, 1984). Further evidence for the involvement of gyrase in the initiation of DNA replication has been supplied by the identification of a preferred gyrase binding site at the *E. coli* origin of replication (*oriC*), (Lother *et al.*, 1984). It is possible that the enzyme is required to maintain a highly negative

supercoiled domain at *oriC* , necessary for the binding of initiation proteins such as *DnaA*. However, the exact role of gyrase at this site has not been elucidated and the precise role of gyrase in the initiation of DNA replication remains unclear.

The study of temperature-sensitive DNA gyrase mutants indicated that inactivation of DNA gyrase by novobiocin produces a different phenotype to that observed following temperature inactivation of the enzyme. For example, in cells treated with novobiocin, DNA replication is inhibited almost instantly, indicating that chain elongation is disrupted; in contrast, temperature inactivation of gyrase mutants leads to a delayed inhibition of DNA replication with no substantial affect on chain elongation (Orr *et al.*, 1984).

As well as inhibiting DNA replication, novobiocin also inhibits RNA synthesis (Smith and Davis, 1967; Ryan, 1976; Orr *et al.*, 1979; Wahle and Mueller, 1980). This inhibition of transcription was assumed to be the result of the inhibitory effect of the drug on DNA gyrase. It has been shown that negatively supercoiled DNA is a better substrate for transcription than relaxed, nicked or linear DNA and that novobiocin and other DNA gyrase inhibitors affect the level of gene expression (Sanzey, 1979; Smith, 1981). This effect was usually attributed to an increased rate of transcription initiation on supercoiled DNA. Furthermore, Wahle and Mueller (1980) showed that the inhibition of gyrase by novobiocin resulted in a decrease in the rate of ribosomal RNA chain elongation and suggested that the elongation rate may be affected by the superhelical density of the template. A number of other groups observed promoter-specific effects following treatment with antibiotics directed against DNA gyrase (Shuman and Schwartz, 1975; Smith *et al.*, 1978; DeWyngaert and Hinkle, 1979; Sanzey, 1979). Wahle *et al.* (1984) later demonstrated that these effects were not related to the loss of supercoiling because, in several cases (Smith *et al.*, 1978; Sanzey, 1979), the concentrations of antibiotics used (especially those of nalidixic acid) were far below the concentration required *in vitro* to reduce the activity of DNA gyrase (Gellert *et al.*, 1977). Moreover, as in the case of DNA replication, the effects of novobiocin on gene expression were found to be different from temperature inactivation of gyrase mutants (Wahle *et al.*, 1984). In *gyrB (ts)* mutants,

temperature inactivation of DNA gyrase only reduced the initiation of transcription with no apparent effect on the rate of elongation (Wahle *et al.*, 1984).

Recently, evidence for the involvement of topoisomerases in the transcription process itself has accumulated. Transcription of an RNA polymerase complex along right-handed double-helical DNA presumably results in the relative rotation of the RNA polymerase complex around the DNA. It has been suggested that during coupled transcription and translation, the rotational resistance of the large RNA polymerase complex (and associated ribosomal proteins) will be great, resulting in the twisting of the DNA axis. This process would generate positive supercoiling ahead of the advancing polymerase complex and negative supercoiling behind it (Liu and Wang, 1987). The transcription of a plasmid containing two genes in opposite orientations (*e.g.* the *tet* and *bal* genes of pBR322) means that these two topological effects will reinforce each other and that positively and negatively supercoiled "domains" will arise in the DNA. It has, therefore, been suggested that the role of gyrase during the transcription process is to prevent the accumulation of positive supercoils ahead of the transcription complex, while topoisomerase I relaxes the negative supercoils behind it (Liu and Wang, 1987; Wang and Giaever, 1988).

Two previously observed phenomena have been used to support this transcriptional buffering theory. The first of these is the observation of Lockshon and Morris (1983) that after treatment of *E. coli* cells with inhibitors of DNA gyrase (oxolinic acid or novobiocin), positively supercoiled pBR322 DNA could be isolated. This observation has been used to support the idea that gyrase prevents the accumulation of positive supercoils generated as transcription proceeds. Secondly, pBR322 DNA isolated from topoisomerase I mutants (*topA*) is found to be highly negatively supercoiled (Pruss, 1985; Pruss and Drlica, 1986). This high degree of negative supercoiling was found to be dependent on the transcription of the *tet* gene of pBR322. Such results could be explained if the transcriptional buffering theory is correct. In this theory, transcription introduces both positive and negative supercoils, but in the absence of topoisomerase I, the accumulation of positive supercoils will be prevented by gyrase and the lack of a topoisomerase I activity will lead to a net

accumulation of negative supercoils. An *in vitro* investigation of the transcriptional buffering model has been performed by Tsao *et al.* (1989). Due to the magnitude of the supercoiling introduced into the template DNA in these experiments, Tsao *et al.* (1989) suggest that transcription may be one of the principal factors influencing intracellular DNA supercoiling.

Unfortunately, all the above groups have neglected to report that Lockshon and Morris (1983) also carried out experiments with a *gyrB* temperature-sensitive strain. When this strain was shifted to the restrictive temperature, a large degree of plasmid relaxation occurred. However, no positive supercoiled plasmid species were observed. This result suggests that the generation of positive supercoils is a drug-induced phenomenon and again highlights the discrepancy between novobiocin-induced effects and those observed following temperature inactivation of DNA gyrase.

The majority of the literature still indicates that novobiocin is a specific inhibitor of DNA gyrase. However, it is obvious from the data described above that temperature inactivation of DNA gyrase has different effects on DNA supercoiling and nucleic acid metabolism than treatment of cells with novobiocin. It would seem that, although novobiocin does inhibit DNA gyrase, it also has pleiotropic effects on bacterial cells.

1.4 Target of novobiocin in eukaryotes

In eukaryotes, novobiocin has been shown to inhibit DNA synthesis (Collins & Johnson, 1979; Lavin, 1981; Collins *et al.*, 1982; Mattern *et al.*, 1982; Clarkson & Mitchell, 1983), transcription (Mattern & Scudiero, 1981; Aller & Baserga, 1986), differentiation (Flickinger & Richman, 1984) and also affects chromatin structure both *in vivo* (Han *et al.*, 1985; Villeponteau *et al.*, 1984) and *in vitro* (Kmiec *et al.*, 1986; Almouzni and Méchali, 1988).

For some time, the target of novobiocin in eukaryotes was presumed to be topoisomerase II (Mattern & Painter, 1979). This theory was supported by several observations:

(i) The toxic effects of novobiocin on eukaryotes were found to be similar to those observed in bacteria (Mattern and Scudiero, 1981).

(ii) Sequence homology exists between eukaryotic topoisomerase II and bacterial DNA gyrase (Lynn *et al.*, 1986).

(iii) Novobiocin was shown to inhibit topoisomerase II, although at concentrations that are 1000 times greater than those required to inhibit bacterial gyrases (Liu *et al.*, 1980).

Little is known about torsional strain in eukaryote DNA and the role of DNA topoisomerases in maintaining it. Unlike bacterial gyrase, eukaryotic topoisomerase II does not appear to be capable of supercoiling DNA (Hsieh & Brutlag, 1980; Liu *et al.*, 1981; Goto & Wang, 1982). Moreover, in yeast, the analysis of temperature-sensitive mutants defective in *TOP2* (the structural gene for topoisomerase II) suggests that the major role of the yeast type II topoisomerase is to resolve the tangled chromosomes at the end of mitosis (DiNardo *et al.*, 1984; Uemura and Yanagida, 1984; Holm *et al.*, 1985, 1989). At the restrictive temperature, *top2(ts)* mutants do not undergo normal mitotic nuclear division; cells arrest with large buds and an elongated, somewhat diffuse nucleus is wedged in the neck of the mother cell. Inactivation of *top2* at other stages of the cell cycle does not cause an irreversible phenotype and lethality is prevented at the non-permissive temperature if cells are prevented from passing through the cell cycle (Holm *et al.*, 1985).

A recent study of the meiotic role of topoisomerase II in *S. cerevisiae* has indicated that it is only required at the time of cell division (Rose *et al.*, 1990). In cold-sensitive *top2* mutants induced to sporulate at the restrictive temperature, premeiotic DNA synthesis and commitment to meiotic levels of recombination were normal but the cells failed to complete the first meiotic nuclear division. Moreover, the presence of a mutation in the *RAD50* gene which blocks recombination (Game *et al.*, 1980) allowed *top2* mutants to pass through meiosis I, suggesting that topoisomerase II is required at meiosis I for the resolution of recombined chromosomes (Rose *et al.*, 1990). As yet, no temperature-sensitive alleles of *TOP2* defective in other functions have been described.

A type II topoisomerase has also been implicated in the segregation of daughter chromosomes of the SV40 virus (Sundin and Varshavsky, 1981; Yang *et al.*, 1987). It

seems that *in vivo* either topoisomerase I or topoisomerase II is sufficient to provide the unlinking activity, necessary for fork propagation during DNA replication in these simple eukaryotic systems. However, newly synthesised daughter chromosomes are normally multiply intertwined and their resolution into separate monomer circles can be accomplished only by topoisomerase II in cell-free systems (Yang *et al.*, 1987).

1.5 The target of novobiocin in yeast

Novobiocin does not have an inhibitory effect on the growth of wild-type yeast possibly because the drug cannot enter the cell. However, treatment of wild-type yeast cells with high concentrations of novobiocin causes a transient morphological effect; cells appear shmoo-like (Cross *et al.*, 1988) *i.e.* pear shaped (Dr. M. Pocklington, personal communication). Despite the normal novobiocin-resistant phenotype of wild-type yeast, the isolation of a number of permeability mutants which are sensitive to novobiocin has facilitated the use of *S. cerevisiae* as a model for the effect of novobiocin on eukaryotes.

Work on these permeability mutants has provided three independent methods of assessment, proving that the yeast type II topoisomerase is not the major target of novobiocin (Pocklington *et al.*, 1990a):

(i) The yeast *top2* temperature-sensitive mutants have a terminal phenotype which is different from the phenotype of novobiocin-sensitive cells treated with novobiocin. When drug sensitive yeast cells are incubated with the minimum concentrations of novobiocin that cause cessation of cellular growth, DNA and RNA synthesis are inhibited and most cells are arrested as shmoo-like, swollen, unbudded cells within two hours. Fluorescence microscopy using the DNA-specific dye DAPI reveals that within 90 minutes of the addition of novobiocin to novobiocin-sensitive cells, mitochondria accumulate as clusters of condensed particles, often situated close to the nucleus; upon further incubation, these particles become scattered throughout the swollen cell. The nucleus loses its spherical shape and its outline becomes generally diffuse. In contrast, at the restrictive temperature *top2(ts)* mutants do not undergo normal mitotic nuclear division; cells arrest with large buds and an elongated, somewhat diffuse nucleus is wedged in the neck of the mother cell

(DiNardo *et al.*, 1984; Holm *et al.*, 1985). The shift of *top2(ts)* mutants to the restrictive temperature has no effect on the mitochondria and DNA replication proceeds normally; cell arrest only occurs when the lack of a topoisomerase II activity prevents the segregation of the newly replicated chromosomes at the end of mitosis.

(ii) Novobiocin-resistant mutants are not topoisomerase II mutants. When cell-free extracts of novobiocin-resistant mutants are prepared and tested for their ability to unknot DNA, no novobiocin resistant, type II topoisomerase activity is detected.

(iii) Unlike bacterial DNA gyrase, topoisomerase II does not bind significantly to novobiocin. Affinity chromatography on novobiocin-Sepharose, a technique previously employed to purify DNA gyrase (Staudenbauer & Orr, 1981; Orr and Staudenbauer, 1982), reveals that topoisomerase II is eluted from the resin at 100-200mM KCl (see Fig. 1.4), whereas bacterial gyrase can only be eluted from the column in a 5M urea wash (see Fig. 1.5).

In addition to the evidence described above, there are a number of reports from higher eukaryotic systems which support the idea that topoisomerase II is not the specific target of novobiocin. For example, novobiocin has been shown to abrogate, almost completely, the cytotoxicity of the topoisomerase II inhibitor *m*AMSA (4'-(9-acridinylamino) methanesulphon-*m*-anisidide) due to its inhibitory effect on DNA synthesis (Utsumi *et al.*, 1990). Furthermore, inhibition of RNA polymerase I, II and III directed translation *in vitro* by novobiocin has been shown to be *via* a mechanism distinct from topoisomerase II (Gottesfeld, 1986; Webb *et al.*, 1987; Webb and Jacob, 1988). The data presented in these papers seem to suggest that novobiocin interrupts ATP-dependent DNA-protein or protein-protein interactions essential for the formation of stable class I, II and III preinitiation complexes and ultimately the initiation of transcription. This inhibition of transcription could account for the blocking of the *Drosophila* heat shock response by novobiocin, which was previously assumed to be due to the inhibition of topoisomerase II (Han *et al.*, 1985).

As described earlier, one of the remarkable properties of novobiocin is its differential effect on prokaryotic gene expression (Wahle *et al.*, 1984). A report by Aller

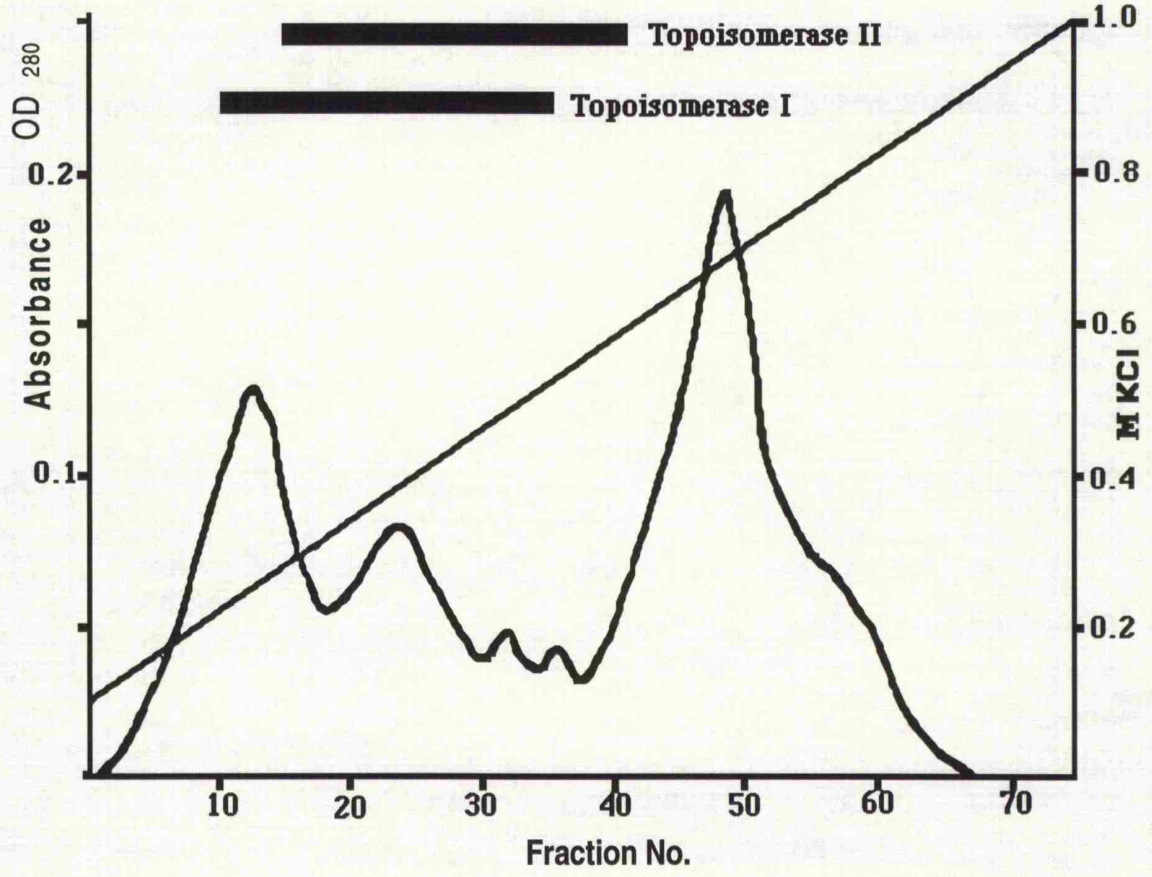


Figure 1.4 Binding of yeast topoisomerases to a novobiocin-Sepharose column

Crude yeast extract in buffer (25mM Hepes pH8.0; 1mM dithiothreitol; 1mM EDTA; 10% (v/v) ethylene glycol and 100mM KCl) was applied to a 20ml column. The column was washed extensively with buffer and the bound proteins were eluted with a linear gradient of 100mM to 1M KCl in buffer. Topoisomerase activities in the fractions were determined after dilution following the method of Liu *et al.*, (1981). The positions of the topoisomerase activities in the elution profile are shown by the heavy horizontal bars (after Pocklington *et al.*, 1990a).

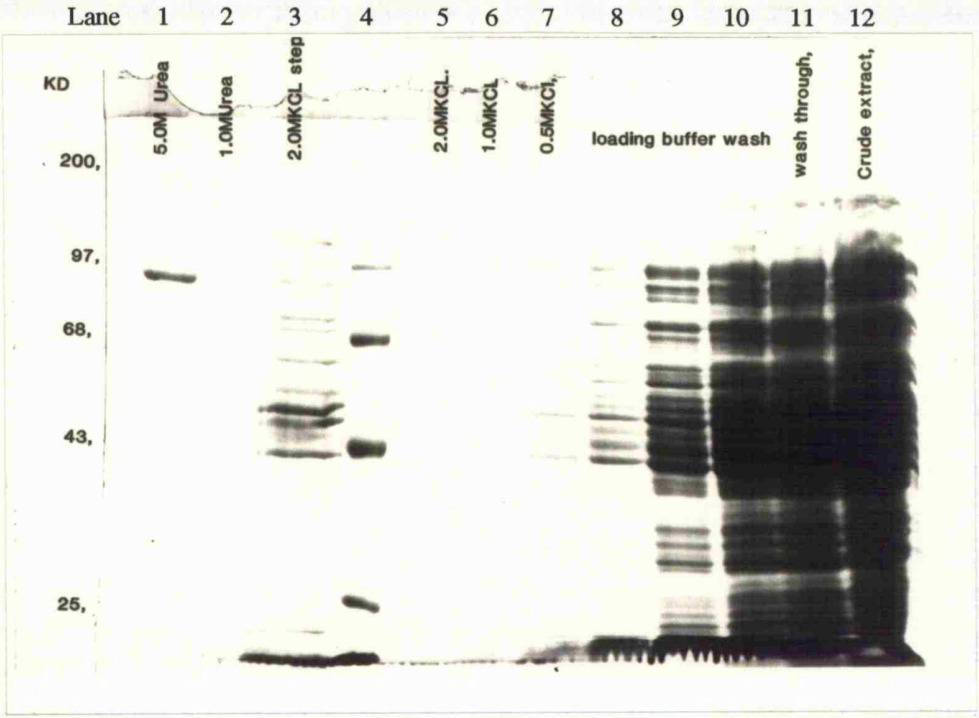


Figure 1.5 SDS polyacrylamide (10%) gel of E. coli proteins binding to the novobiocin-Sepharose column

E. coli proteins were applied to the column in buffer B (25mM Hepes pH8.0; 1mM dithiothreitol; 1mM EDTA; 10% (v/v) ethylene glycol and 100mM KCl) and eluted with buffer B containing KCl or urea. Lane 12, crude extract; lane 11, wash through; lanes 8-10, loading buffer wash; lane 7, 0.5M KCl wash; lane 6, 1.0M KCl wash; lane 5, 2.0M KCl; lane 3, 2.0M wash; lane 2, 1M urea wash; lane 1, 5.0M urea wash. Lane 4 shows molecular weight markers (kDa). Note that there is only one band in the 5M urea wash which runs just in front of the 97kDa marker; this is the B-subunit of bacterial DNA gyrase (photograph supplied by Dr. J.R. Jenkins).

and Baserga (1986) indicates that this effect may also occur in eukaryotic cells. In their experiments monitoring the levels of transcription of various fibroblast genes, it was found that novobiocin decreased the transcription of all genes tested with the exception of *c-myc* which appeared to be induced by novobiocin treatment. This observation is also supported by experiments which indicate that novobiocin-induced differentiation of cancer cells causes altered gene expression (Kaneko, 1990; Nordenberg *et al.*, 1992)

Experiments on SV40 DNA synthesis in mammalian cells (CV-1 cells) indicate that DNA polymerase α and γ are novobiocin sensitive (Edenberg, 1980). Independent experiments carried out by Nakayama and Sugino (1980) also indicate that purified DNA polymerase α from calf thymus and *Drosophila melanogaster*, as well as DNA polymerase γ from calf liver, are sensitive to concentrations of novobiocin shown to inactivate topoisomerase II (Goto and Wang, 1982). In addition, DNA polymerase I, DNA polymerase II and topoisomerase I from yeast are all inhibited by similar concentrations of novobiocin (Nakayama and Sugino, 1980).

These observations highlight the difficulties in assigning a particular enzyme as the target of a drug based strictly on drug sensitivity without the characterisation of specific drug-resistant mutants. Furthermore, these data suggest that the sensitivity of topoisomerase II and DNA polymerases to novobiocin in higher eukaryotes could reflect a non-specific secondary effect.

As described above, novobiocin has a global effect on cell viability, inhibiting DNA synthesis, transcription and affecting differentiation and chromatin structure. In the light of the results obtained in yeast and other eukaryotic systems, it would seem that these effects are due to a target(s) other than topoisomerase II. In order to identify such target(s) it was obvious that further investigation was necessary. To this end, both genetic and biochemical methods have been used in order to identify the cellular target(s) of novobiocin using yeast as a representative eukaryotic organism.

1.6 Identification of the target of novobiocin using genetic methods

In view of the effects of novobiocin on eukaryotic cells, the activity of the main target (whether or not it is topoisomerase II) must be of great importance to the cell. Initially work in our laboratory concentrated on the identification of the main genetic target(s) of novobiocin in yeast. A number of novobiocin-sensitive mutants were obtained from Dr. L Johnson (MRC, National Institute of Medical Research, Mill Hill) and these were characterised genetically. The novobiocin-sensitive mutants were found to fall within two complementation groups, which were termed *nbs1* (novobiocin sensitive 1) and *nbs5*. Characterisation of these complementation groups indicated that *nbs1* strains are not only sensitive to novobiocin but also to many other toxic agents, whereas *nbs5* strains are only sensitive to novobiocin.

An *nbs1* strain was used to isolate novobiocin-resistant mutants that were simultaneously temperature-sensitive. A number of mutants were obtained and were shown to be novobiocin-resistant but they retained their sensitivity to other drugs, suggesting that the resistance to novobiocin was not due to a suppressor of the *nbs* allele. The wild-type gene was cloned through complementation of the Ts phenotype and was identified as the *SUP45* gene (Pocklington *et al.*, 1990b). *SUP45* is known as an "omnipotent" suppressor and is readily isolated as an allo-suppressor in a wild-type background (Crouzet and Tuite, 1987). Its function is not fully understood but it may operate by allowing the misreading of nonsense codons, producing functional full length polypeptides.

At the restrictive temperature, the *sup45 (ts)* mutants arrest at G1; DNA and RNA synthesis are inhibited and mitochondrial distribution is affected. These effects are similar to those produced by novobiocin (see above). Indirect immunofluorescence studies suggest that the *SUP45* gene product is located in the yeast spindle plaque body (E. Orr, personal communication). It is therefore possible that the G1 arrest observed in *sup45 (ts)* mutants could be due to a direct or indirect effect on the proteins associated with the spindle plaque body. Evidence for the localisation of *CDC28* (p34 kinase) and cyclins (proteins involved in control of passage through cell cycle "start") in the centrosome

(Bailly *et al.*, 1989 and 1992; Gallant and Nigg, 1992; Pagno *et al.*, 1992) supports this possibility. If *SUP45* is associated with the spindle plaque body, its suppressor activity could be attributed to its effect on the microtubule network and hence ribosomal organisation. Alternatively, the *SUP45* gene product could be conferring novobiocin resistance by suppressing the *nbs1* mutation. In order to identify the wild-type gene responsible for novobiocin sensitivity in *nbs1*, the *nbs1* gene was cloned by direct complementation of the novobiocin sensitivity and is currently being analysed.

Similarly, the wild-type allele of *nbs5* was cloned and was later characterised as a previously described gene, *SST2*. Targeted integration of wild-type *SST2* to the *nbs5* locus restored novobiocin resistance. Furthermore, when a strain containing the wild-type *SST2* locus was crossed with *nbs5* no recombination between the loci was observed, confirming that the *nbs5* and *SST2* are allelic. The *SST2* gene is normally active in preventing an over-response to the yeast mating pheromone, rescuing the cells from the G1 arrest following the mating-type response, thus allowing cells to revert to the vegetative state.

Three cell types with different properties exist in *S. cerevisiae*: *MAT**a*** cells, *MAT*α cells and diploid cells (*MAT**a**/MAT*α cells). The *MAT**a*** cells mate with *MAT*α cells to produce diploid cells (*MAT**a**/MAT*α cells) and the latter undergo meiosis and sporulation to produce *MAT**a*** and *MAT*α cells. The different properties of the three cell types are determined by a single locus, the *MAT* locus (located on the right arm of chromosome III), which, in turn, controls gene transcription so that each cell type expresses a set of unique genes. The *MAT**a***-specific genes are expressed only in cells which carry the ***a*** allele at *MAT* (*MAT**a***); whereas *MAT*α-specific genes are expressed only in cells that carry the α allele at *MAT* (*MAT*α). As well as these mating type specific genes, a number of haploid specific genes are expressed only in mating type homozygous cells (*i.e.* in both *MAT**a*** and *MAT*α cells). Diploid specific genes are expressed only in *MAT**a**/MAT*α cells, (for reviews see Sprague *et al.*, 1983; Nasmyth and Shore, 1987). *MAT*α cells secrete a 13 amino acid polypeptide, α-factor pheromone, which interacts with an α-factor specific receptor on the surface of **a** cells. Conversely, *MAT**a*** cells secrete **a**-factor pheromone which is recognised by an **a**-factor specific receptor on the surface of α cells. It is thought

that when pheromone binds to its receptor on a cell of the opposite mating-type a signal transduction pathway is activated which mediates the mating response. These responses are necessary for conjugation and include agglutinin induction, cell division arrest in G1 phase, Ca^{2+} uptake, competence for nuclear fusion, changes in the transcription of genes involved in mating and the production of a distinct cellular morphology (shmoo)(Cross *et al.*, 1988).

The mode of action of the *SST2* gene product remains unclear, but it must interact either with the mating factor receptor, or with the G protein complex immediately downstream of the receptor (Dietzel and Kurjan, 1987). The ability of *SST2* to rescue cells from the mating response is very important as it ensures return to the normal cell cycle should conjugation not occur.

Since only *MAT**a*** mating type *nbs5* cells were found to be novobiocin sensitive and *SST2* ameliorates the response to α-factor, two mechanisms explaining the sensitivity of *nbs5* (*sst2*) mutants to novobiocin were proposed. Novobiocin may induce α-factor production in *MAT**a*** cells. This α-factor would then be secreted and could interact with the cells own α specific receptors, thus stimulating irreversible commitment to the mating-type response in the absence of a functional *SST2* gene product. Alternatively, novobiocin may interact directly with the α-factor receptor, again stimulating irreversible commitment to the mating-type response in *sst2* mutants. A number of experiments were carried out to determine which of these possibilities is true.

In order to test the first hypothesis, the *MFα1* and *KEX2* genes were deleted in an *nbs5* background. *MFα1* is one of two structural genes which encode α-factor (Kurjan and Herskowitz, 1982; Singh *et al.*, 1983); *MFα1* encodes four copies of the mature mating factor, where as *MFα2*, the second structural gene, encodes two copies. *KEX2* encodes an enzyme required for the maturation of α-factor encoded by *MFα1* and *MFα2* (Julius *et al.*, 1984; Fuller *et al.*, 1988). When both *MFα1* and *KEX2* are deleted the resultant mutant strain is unable to produce mature α-factor and is sterile. If the first hypothesis, *i.e.* that novobiocin activates the production of α-factor in *MAT**a*** cells is correct, deletion of the *MFα1*, *KEX2* genes would prevent novobiocin from causing

irreversible commitment to the mating-type response. Thus, *mfα1*, *kex2*, *nbs5* (*sst2*) mutant cells should be resistant to novobiocin. However, the *mfα1*, *kex2*, *nbs5* (*sst2*) mutant cells are still novobiocin sensitive, indicating that the novobiocin sensitivity of *nbs5* is not mediated by the production of α-factor.

In order to test the second hypothesis, *i.e.* that novobiocin interacts directly with the α-factor receptor stimulating irreversible commitment to the mating-type response in *sst2* mutants, the gene encoding the α-factor receptor (*STE2*) was deleted in an *nbs5* background. The *STE2* gene is one of a number of genes identified by the isolation of mutants defective in mating or insensitive to arrest by mating factors (MacKay and Manney, 1974; Hartwell, 1980). The wild-type genes are termed *STE* genes because mutants are sterile. The *STE2* gene is one of the *MATa* specific genes mentioned earlier and has been shown to encode the α-factor receptor (Jenness *et al.*, 1983; Nakayama *et al.*, 1985). When the *STE2* gene is deleted, no α-factor receptor is produced by the mutant strain and no mating type response is seen in the presence of α-factor. Therefore, if novobiocin exerts its effect on the *nbs5* strain by direct interaction with the α-factor receptor, then a *ste2, nbs5* (*sst2*) mutant should be novobiocin resistant. This was found to be the case because when the *ste2, nbs5* (*sst2*) mutant was produced and tested for novobiocin sensitivity the cells were found to be totally insensitive to the antibiotic (Dr. M. Pocklington, personal communication).

This genetic work suggests that the target of novobiocin is the α-factor receptor itself and that the effect of novobiocin on wild-type cells is normally masked by the activity of the wild-type *SST2* (*NBS5*) gene which ameliorates the mating signal (Dr. M. Pocklington, personal communication). The significance of these results with respect to the target of novobiocin in higher eukaryotes is difficult to interpret. No higher eukaryotic protein sequences with significant identity to that of α-factor receptor have been found and this suggests that a homologue is not the target of novobiocin in higher eukaryotes. Nevertheless, the α-factor receptor is structurally similar to a group of receptors that contain seven hydrophobic trans-membrane domains, including rhodopsin, the β-adrenergic receptor and the muscarinic acetylcholine receptor (Nakayama *et al.*, 1985;

Burkholder and Hartwell, 1985; Hagen *et al.*, 1986). The significance of these findings with regard to novobiocin is unclear and it is possible that some other target or targets are responsible for the effect of novobiocin in higher eukaryotes.

A mutant which confers novobiocin resistance has been isolated in higher eukaryotes (BHK cells) and these mutants have no apparent effect on topoisomerase II activity (Ishida *et al.*, 1987). Unfortunately, the gene(s) conferring this resistance has not yet been identified.

1.7 Identification of the target of novobiocin using biochemical methods

A biochemical approach has also been used in the search for the target(s) of novobiocin. Using the technique of affinity chromatography on novobiocin-Sepharose (Staudenbauer & Orr, 1981), six major novobiocin-binding proteins have been isolated from yeast. As mentioned above, both topoisomerase I and II bind to the novobiocin column but are eluted with the majority of cellular proteins in buffer containing less than 0.5M KCl. They do not therefore constitute major novobiocin-binding proteins (Pocklington *et al.*, 1990a). Two of the novobiocin-binding proteins are eluted in buffer containing high salt concentrations (2M KCl); these are a high molecular mass protein of approximately 200kDa, identified and characterised as the yeast heavy chain myosin (Watts *et al.*, 1985) and a protein of approximately 52kDa, identified as the *SUP45* gene product (Jenkins *et al.*, 1990). The remaining four novobiocin-binding proteins of molecular mass 200kDa, 52kDa, 35kDa and 20kDa are not eluted by high salt concentrations and can only be eluted in a 5M urea wash. Similar denaturing condition are required to elute the B-subunit of bacterial DNA gyrase from novobiocin-Sepharose.

Antibodies raised against the 52kDa protein eluted in the 5M urea fraction were used in indirect immunofluorescence microscopy and immuno-gold electron microscopy. The results demonstrate that the antigen is localised within the mitochondria in both yeast and higher eukaryotes (Jenkins *et al.*, 1990). The N-terminal amino acid sequence of the

52kDa protein was determined and the sequence obtained identified the protein as the β-subunit of the yeast mitochondrial F_1 ATP synthetase (Jenkins *et al.*, 1990).

The interference of novobiocin with the ATP-synthesising enzyme (F_1 ATP synthetase) should be sufficient to arrest cellular growth, if ATP production is halted. In fact, Downes *et al.* (1985) suggested that the toxicity of novobiocin might be mediated by its effect on mitochondrial structure and hence ATP metabolism. However, unlike other eukaryotes, yeast can grow on fermentative media without mitochondria. Under these conditions, novobiocin still inhibits cellular growth, indicating that its poisoning effects might be exerted *via* other cellular components perhaps through one of the as yet unidentified novobiocin-binding proteins.

The α-factor receptor could theoretically be one of the unidentified novobiocin-binding proteins. However, the cell extracts used in the affinity-purification procedure only contain soluble proteins. Therefore, many membrane proteins will not be present in the cell extracts and since the α-factor receptor is an integral membrane protein it is not surprising that it has not been identified by this method.

Finally, it is important to note that a biochemical target of a drug found by *in vitro* studies need not necessarily be a physiological target. For example, a protein-drug interaction *in vitro* may not be mirrored *in vivo* as the drug may not come into contact with the "target" *in vivo* due to cellular compartmentalisation. It is because of such possibilities that both genetic and biochemical studies have been undertaken in the search for the target(s) of novobiocin in yeast.

1.8 Novobiocin-binding site

There has been much speculation as to what primary protein sequence constitutes a novobiocin-binding domain. As yet, no such site has been identified in bacterial DNA gyrases. A comparison between the B-subunit of bacterial DNA gyrase (from *E. coli* and *Bacillus subtilis*) and the β-subunit of the F_1 ATP synthetase revealed very little homology at the amino acid level. A five amino acid motif YQPTL has been suggested as a putative novobiocin-binding domain (Jenkins *et al.*, 1990). However, this suggestion may be a little

simplistic, as a number of proteins have been shown to possess binding sites consisting of a number of very small but well-conserved amino acid motifs, positioned at variable distances from each other (Barton and Sternberg, 1990; Karplus et al., 1991). Since the ATPase activity of the B-subunit of bacterial gyrase is competitively inhibited by novobiocin, it has been speculated that novobiocin and ATP compete for the same binding domain (Mizuuchi *et al.*, 1978; Sugino *et al.*, 1978; Staudenbauer & Orr, 1981). In this context, it is interesting to note that the putative novobiocin binding motif suggested by Jenkins *et al.* (1990) is some distance from the nucleotide-binding site in the β-subunit of the ATP synthetase. Despite this, the β-subunit of ATP synthetase can be specifically eluted from a novobiocin-Sepharose with 10mM ATP, indicating that the binding of novobiocin and ATP to the protein is mutually exclusive.

Sugino *et al.* (1978) suggested that weak homology between the structure of the adenine-ribose moiety of ATP and the coumarin-novose moiety (Fig. 1.1) of novobiocin cannot account for the competitive inhibition. Furthermore, ATP binding seems to be highly specific in DNA gyrase since other nucleoside triphosphates such as GTP, CTP and UTP are poor substrates for and inhibitors of the ATPase reaction. Sugino *et al.* (1978) also showed that the K_i value for novobiocin is very low, more than four orders of magnitude less than the K_m for ATP. They speculate that novobiocin may block the access of ATP without sharing binding sites and suggest that novobiocin binding induces a conformational change, thus preventing ATP binding. This suggestion is consistent with the five amino acid motif YQPTL constituting a novobiocin binding domain despite being some distance from the nucleotide binding site in the β-subunit of ATP synthetase.

Recently, the crystal structure of the N-terminal fragment of the *E. coli* DNA gyrase B protein complexed with ADPNP has been resolved at 2.5Å resolution (Wigley *et al.*, 1991). This fragment is reported to hydrolyse ATP and bind coumarin drugs (Contreras and Maxwell, 1992). The ATP binding site of the DNA gyrase B protein has been identified by X-ray crystallography (Wigley *et al.*, 1991). Interestingly, none of the *gyrB* mutations which confer coumarin resistance have been mapped to the ATP binding site (Contreras and Maxwell, 1992). The substitution of Arg-136 with Cys, His or Ser in DNA

gyrase confers coumarin resistance and, although not localised within the ATP binding site, the mutations affect ATP hydrolysis. These mutants show a reduction in ATP hydrolysis with $GyrB_{Cys\text{-}136}$ being the most active and $GyrB_{Ser\text{-}136}$ the least active. Furthermore, a correlation between the ATPase activity and the level of DNA supercoiling has been observed (Contreras and Maxwell, 1992). From these and from steady-state kinetics data (A. Maxwell, personal communication) it has been suggested that the ATP and novobiocin do not share a common binding site. However, this conclusion must be viewed with some caution as there is a formal possibility that no mutations which confer novobiocin resistance have been identified in the ATP binding site because they are lethal. As mentioned above, the $GyrB_{Cys\text{-}136}$ $GyrB_{His\text{-}136}$ $GyrB_{Ser\text{-}136}$ mutants show a slight reduction in ATP hydrolysis, presumably due to conformational changes at the ATP binding site. It is therefore possible that these slight conformational changes in the ATP binding site may prevent the binding of novobiocin but only slightly reduce the affinity of the enzyme to ATP, thus explaining the novobiocin resistance conferred by these mutations. Such conformational changes in the ATP binding site can easily be envisaged because in the *E. coli* DNA gyrase B subunit the guanidinium group of Arg-136 forms a hydrogen-bond with the main chain carbonyl group of Tyr-5, a residue in the proposed ATP-binding site (Wigley *et al.*, 1991). Therefore, substitution of Arg-136 with another amino acid is likely to disrupt this hydrogen-bond formation.

As yet, none of the data regarding the binding site of novobiocin is conclusive; some data indicate that novobiocin binds to the ATP binding site, whereas other data indicate that the binding site is elsewhere. It seems that the binding site of novobiocin may not be fully resolved until X-ray crystallography is carried out on the B subunit of DNA gyrase complexed with novobiocin.

1.9 Pharmacological studies of novobiocin

A number of studies to determine the structural moiety or moieties responsible for the biological activity of novobiocin have been carried out. As described in section 1.1 and shown in Fig. 1.1, novobiocin can be subdivided chemically into three distinct components;

a benzoic acid moiety (A ring), a coumarin moiety (B ring) and the sugar, novose (C ring). It has been found that novenamine (B ring & C ring, see Fig. 1.1), novobiocic acid (A ring & B ring, see Fig. 1.1), or any of the individual sub-entities are essentially devoid of any antibacterial activity against whole cells (Reusser and Dolak, 1986). However, when assayed for their ability to inhibit DNA replication in toluenised *E. coli* cells or their inhibition of supercoiling by *M. luteus* DNA gyrase *in vitro*, novenamine was found to be as potent as novobiocin. This result implies that the B and C rings are the minimal elements required for the interaction with gyrase (Reusser and Dolak, 1986). Later experiments have shown that both chlorobiocic acid, an analogue of novobiocic acid (A ring & B ring) and 3 (carbobenzoxyamino)-4, 7-dihydroxy-8-methoxy-coumarin, an analogue of coumarin (B ring), inhibit DNA supercoiling by *M. luteus* DNA gyrase *in vitro* (Althaus *et al.*, 1988). These data point to the importance of the coumarin portion (B ring) in the interaction with gyrase, but suggest that appropriate side-chain substitutions to the coumarin moiety may be required for biological activity (Althaus *et al.*, 1988). Such initial attempts at the systematic dissection of the structural elements of coumarins and assessment of their anti-microbial and anti-gyrase properties may help to improve the pharmacological prospects of coumarin antibiotics. Nevertheless, the effects of coumarins on eukaryotic cells cannot be ignored. Particularly the seemingly at odds cytotoxic effects of novobiocin described in the literature; Eder *et al.* (1989 and 1991) having shown that novobiocin increases the cytotoxic effect of alkylating agents whereas, Wood and Stansfield (1992) have shown that it inhibits T-cell mediated cytotoxicity. Further study of the effects of novobiocin in yeast may help to resolve these problems and indicate potentially useful applications for the antibiotic.

1.10 Yeast as an experimental organism

The yeast *Saccharomyces cerevisiae* is a simple and rewarding system for understanding eukaryotic biology at the cellular and molecular levels. It is a unicellular microorganism that grows almost as fast as bacteria (its growth rate is approximately half that of *E. coli*) on relatively inexpensive media. Consequently it is suitable for the

preparation of large quantities of protein for biochemical analyses. *S. cerevisiae* has a well-defined genetic system consisting of 16 chromosomes that are extensively marked with known genes. It can grow as a haploid and a diploid organism making it possible to isolate conditional lethal recessive mutations and study their effect on the cell. Yeast have a transformation system that enables gene cloning and manipulation, including the preparation of mutants *in vitro*. Moreover, being eukaryotes, yeast share many fundamental properties of cell biology with multicellular organisms (such as cytoskeletal organisation, subcellular organelles, secretion systems, receptor and second messenger arrangements, metabolic regulation and some chromosome functions).

The cell cycle of *S. cerevisiae* (Fig. 1.6) permits the phenotypical analysis of cell cycle division (*cdc*) mutants, *i.e.* a microscopic demonstration of the particular phase of the cell cycle at which a mutation arrests cell growth. Moreover, this growth arrest can be correlated to the failure of a certain physiological process. For example, if the cell cycle halts when the majority of cells have small buds, this indicates that cells have been arrested at the S phase, the DNA synthesis period (Pringle and Hartwell, 1981).

Since the widespread application of recombinant DNA technology to the entire spectrum of organisms, it has been shown that most eukaryotic proteins are well conserved at the amino acid level. This conservation extends to *S. cerevisiae*, showing that despite the fact that it is a unicellular organism *S. cerevisiae* is a surprisingly typical eukaryote (Botstein and Fink, 1988). The greatest conservation is observed in ubiquitin and cytoskeletal elements, but is still substantial (about 60% identity) for a greater variety of enzymes and regulatory proteins (See Table 1.1).

These high levels of conservation at the amino acid level indicate that there has been functional conservation between yeast and higher eukaryotes. This argument has been further supported in several cases by the demonstration that corresponding mammalian genes can function in yeast cells. For example, mammalian *ras* genes complement yeast *ras* mutants (Kataoka *et al.*, 1984; Tatchell *et al.*, 1984; Tamanoi *et al.*, 1984). In some cases, yeast proteins have been shown to function in mammalian cells; for example,

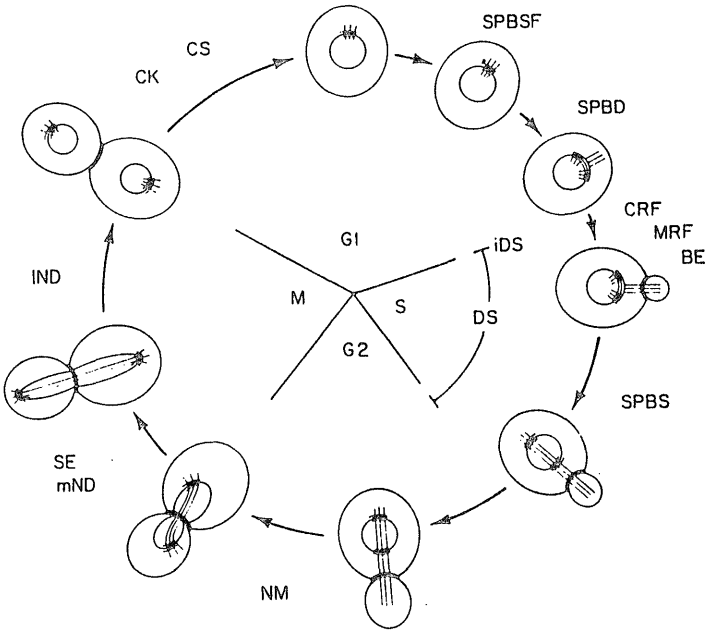


Figure 1.6 The cell cycle of *Saccharomyces cerevisiae*

The yeast cell cycle is divided into a G1 phase, which precedes the initiation of chromosomal DNA replication; an S phase, in which chromosomal DNA is replicated; a G2 phase and an M phase, during which mitosis and nuclear division occur.

Abbreviations: SPBSF, spindle-pole-body satellite formation; SPBD, spindle-pole-body duplication; CFR, formation of chitin ring; MRF, formation of the microfilament ring; BE, bud emergence; iDS, initiation of chromosomal DNA synthesis; DS, chromosomal DNA synthesis; SPBS, spindle-pole-body separation; NM, nuclear migration; mND medial stage of nuclear division; SE, spindle elongation; lND, late stage of nuclear division; CK, cytokinesis; CS, cell separation (Pringle and Hartwell, 1981).

Protein	Identity (%) in amino acid sequence
Ubiquitin	96
Actin	89
ß-tubulin	75
HMGCoA reductase	66
Cytochrome c	63
Citrate synthetase	62
RAS1/N-ras; RAS2/K-ras	60
Glucose transporter	25

Table 1.1 Conservation between yeast and human proteins at the amino acid level

Degree of identity in amino acid sequence between corresponding proteins of yeast (*Saccharomyces cerevisiae*) and humans (*Homo sapiens*). The data was generated from sequences in Genbank and other published sources. (After Botstein & Fink, 1988).

chicken-yeast chimeric tubulin is incorporated into mouse microtubules (Bond *et al.*, 1986).

Thus, functional conservation highlights the power of yeast genetics in the investigation of mammalian gene function and makes *S. cerevisiae* an ideal organism to study the function of eukaryotic proteins which have universal significance.

1.11 Aims of this study

The ultimate goal of this work is to identify the exact method by which novobiocin affects eukaryotic cells.

To this end, the aim of this study was to use affinity chromatography on novobiocin-Sepharose to purify the as yet unidentified 200kDa, 35kDa and 20kDa novobiocin-binding proteins and to use the purified proteins to isolate, identify and characterise the corresponding genes, by:

(i) using the protein as immunogens to raise antibodies.

Antibodies should allow the screening of expression libraries thereby facilitating the cloning of the genes. Antibodies are often used to indicate possible function *in vivo* by localisation of the antigens within the cells.

(ii) obtaining N-terminal sequence data.

This data should allow the screening of data bases to determine if a corresponding gene has been cloned. It should also allow the production of a degenerate oligonucleotide which can be used to screen genomic libraries, thereby facilitating the cloning of the genes.

Chapter 2

Materials and methods

2.1 Sources of reagents and miscellaneous materials

The sources of reagents, materials and equipment are mentioned here or in the relevant methods section only if regarded as important. All other chemicals were bought from reputable biological chemical companies and were of analytical grade. Reagents were purchased as follows: β-mercaptoethanol, Freund's complete and incomplete adjuvent from Sigma Chemical Company Ltd., Poole, Dorset; restriction enzymes and buffers from New England Biolabs Inc., CP Laboratories (distributors), Bishop's Stortford, Hertfordshire; DNA polymerase I (Klenow fragment), bovine serum albumin (RNase/DNase free), random hexadeoxyribo-nucleotides, deoxyNTPs, dideoxyNTPs and T7 polymerase from Pharmacia Biosystems Ltd., Milton Keynes; phenol from Fisons plc., Loughborough; restriction enzymes and buffers, T4 ligase and buffer, λ/*Hin*dIII and ΦX174/*Hae*III DNA fragments and RNA size markers from Gibco-BRL Life Technologies Inc., Paisley, Scotland; calf intestinal phosphatase, restriction enzymes and buffers from Boehringer and Mannheim , GmbH Mannheim, Germany; agarose from ICN Biomedicals Ltd., High Wycombe, Buckinghamshire; Accugel 40™ (40% (19:1) acrylamide: bisacrylamide stock solution) and Protogel™ (30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide stock solution) from National Diagnostics, Flowgen Instruments Ltd. (distributors), Sittingbourne, Kent; Sepharose CL-6B,TEMED and APS from Bio-Rad Laboratories Ltd., Hemel Hempsted, Hertfordshire; Hybond-N, radioactive [α-^{35}S]ATP and [α-^{32}P]CTP were obtained from Amersham International, Aylesbury, Buckinghamshire.

2.2 Strains

Strains of *S. cerevisiae* and *E. coli* used in this study are listed in Table 2.1.

Strain	**Genotype**	**Source**
S. cerevisiae		
Zn1d	*MATa, ade2, trp1, LEU2/leu2, his3, ura3, myo1*	Watts *et al.*, 1987
483/1a	*MATa, SUQ5, ade2-1, his5-2,lys1-1, can1-100, ura3-1, PNM1ψ*	Mick Tuite (Kent)
842	*MATα/MATa, leu2/leu2, ade1/ade1,his 3/his3, ura3/ura3, trp1-1/trp1-1*	K. Nasmyth
8HA	*MATa, trp1, ura3, ade1, leu2*	M. Pocklington (Leicester)
S150-2B/hem13Δ	*MATa, leu2, his3, trp1, ura3*	R. Labbe-Bois

E.coli		
RR1 lambda cI_{857}	F-, *hsd*(r_B-,m_B-), *ara14, proA2, LacY1, galK2, rspL20*(Sm^R), *xyl*-5, *mtl*-1, *supE44*, lambda cI_{857}	Bolivar *et al.*, 1977
DH5α	*supE44, ΔlacU169*, (ϕ80 *lacZ*ΔM15), *hsdR17, recA1, endA1, gyrA69, thi*-1, *relA1*	Hanahan, 1983
C600	*supE44, hsdR, thi*-1, *leuB6, lacY1, tonA21*	Young & Davis, 1983a
Y1090	*supF, hsdR, araD139, Δlon, ΔlacU169, rpsL, trpC22*::Tn10(*tet r*) pMC9	Young & Davis, 1983b
WL87	*supE, supF, hsdR, hsdM+, trpR, metB*	Loenen, 1982

Table 2.1 -Strains used in this study.

2.2.1 Bacterial culture media

Luria Broth (LUB) contained 1% (w/v) Tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, pH7.0. Luria agar (LUA) was prepared by the addition of 1.5% (w/v) agar to the liquid medium. Ampicillin was added at 100μg/ml (final) to select for cells containing plasmids encoding β-lactamase.

2.2.2 Yeast culture media

Yeast were routinely grown in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose). For solid medium 2% (w/v) agar was added to the liquid medium.

Synthetic minimal medium contained 0.67% (w/v) Bacto-yeast nitrogen base, 2% (w/v) glucose; 2% (w/v) agar and amino acid supplements were added as and when appropriate (see Table 2.1).

The strain S150-2B/hem13Δ (Table 2.1) was grown in YPD supplemented with 0.2% (v/v) Tween 80 and 30mg l^{-1} ergosterol or 15mg l^{-1} hemin.

Diploid yeast strains were induced to sporulate by plating out on sporulating medium (0.1M potassium acetate, 0.1% (w/v) yeast extract, 0.5% (w/v) glucose, 2% (w/v) agar); nutritional supplements were added as required.

2.3 Vectors

Vectors used in this study are listed in Table 2.2

2.4 General Methods

2.4.1 Restriction endonuclease digestion of DNA

DNA was digested at a final concentration of 2-200 ng μl^{-1} with at least 2 units μg^{-1} DNA of the required enzyme in a buffer recommended by the manufacturer. Unless otherwise stated, reactions were incubated at 37°C for 1 hour.

Vector	Genetic markers	Source
pUC 18	*amp*	Yanisch-Perron *et al.*, 1985
pUC 19	*amp*	Yanisch-Perron *et al.*, 1985
pIC19H	*amp*	Marsh *et al.*, 1984
pIC20H	*amp*	Marsh *et al.*, 1984
pIC20R	*amp*	Marsh *et al.*, 1984
pEX1-3	*amp*	Stanley & Luzio, 1984
YDp-L	*amp*, *LEU2*	Berben *et al.*, 1991

Table 2.2-Plasmids used in this study.

2.4.2 Quantitation of DNA and RNA

The concentration of small quantities of DNA or RNA was determined by comparison of intensity of ethidium bromide fluorescence under ultraviolet light with that of a known amount of DNA or RNA after agarose gel electrophoresis (see section 2.5). For higher accuracy, the absorbance of ultraviolet (UV) light was measured at 260nm on a U-200 Double-Beam Spectrophotometer (Hitachi Ltd., Tokyo, Japan) and DNA concentration determined using an optical density of 1 unit corresponding to a concentration of 50μg ml^{-1} for double-stranded DNA and 40μg ml^{-1} for single stranded DNA and RNA

2.4.3 Centrifugation

Centrifugation of solutions up to 1.5 ml was performed in Eppendorf tubes in a MSE Microcentaur. Larger volumes were centrifuged using either a Sorvall RC5B Refrigerated Superspeed or an Omnispin Sorvall centrifuge. Ultracentrifugations were carried out in either a Beckman TL-100, Beckman L5-65B or a Sorvall OTD65B ultracentrifuge.

2.4.4 Ethanol precipitation of DNA

To concentrate DNA or for recovery from unsuitable buffers, DNA was precipitated by the addition of 0.1x volume 3M sodium acetate pH4.5 and 2x volumes 100% ethanol to the solution. This was incubated at -70°C for 10-20 minutes, then centrifuged for 5-10 minutes at 12,000g in a microcentrifuge. The supernatant was removed and the pellet washed with 200-500μl of 80% ethanol and recentrifuged at 12,000g for 5 minutes. The supernatant was again removed and the pellet was air-dried and resuspended in distilled water or appropriate buffer, at the desired concentration.

2.4.5 Phenol/chloroform extraction of DNA

Phenol, chloroform and isoamyl alcohol were mixed in the ratio 25:24:1 (w/v/v). The solution was saturated with 1M Tris-HCl pH8.0 and stored at 4°C under 10mM Tris-HCl pH8.0, after the addition of 0.1% (w/v) 8-hydroxyquinoline.

Standard extractions were carried out by the addition of an equal volume of phenol/chloroform/isoamyl alcohol to the DNA solution, followed by thorough mixing and separation of the phases by centrifugation at 12,000g in a microcentrifuge. The DNA-containing aqueous layer was carefully removed, transferred to a clean tube and ethanol precipitated.

2.4.6 Autoradiography of samples labelled with ^{32}P-dCTP

A sheet of Fuji X-ray film was placed over the filter to be autoradiographed in a tightly-closing cassette containing an intensifying screen (Genetic Research Instrumentation Ltd., Dunmow, Essex) and the exposure carried out at -70°C for the length of time required to give suitable band intensity. The use of an intensifying screen amplifies the signal but also reduces the definition of bands. Therefore, if maximum band sharpness was required, exposure was carried out at room temperature.

The X-ray film was processed using LX 24 X-ray developer, FX-40 X-ray liquid fixer and HX-40 X-ray liquid hardener (all from Eastman Kodak Company, Rochester, New York, USA).

2.4.7 Photography of gels

Gels stained with ethidium bromide were photographed on a UV transilluminator using a Polaroid MP-4 Land Camera and Kodak T max 100 professional film, which was processed using LX 24 X-ray developer and Kodak 3000 film fixer and hardener.

2.5 Agarose gel electrophoresis

2.5.1 Standard analytical agarose gels

The percentage agarose (w/v) used was chosen to separate efficiently molecules of known or anticipated size (Table 2.3). Electrophoresis buffer used was either TBE (90mM Tris-Borate, 2mM EDTA) or TAE (40mM Tris-acetate, 1mM EDTA). Agarose was dissolved in the appropriate buffer by heating in a microwave oven. The agarose was allowed to cool to 60°C and EtBr added to a final concentration of 0.2μg ml^{-1} before being poured into a horizontal mould. The gel was allowed to set at room temperature. A 0.1x

Agarose in gel %(w/v)	Size range of DNA Molecules (Kb)
0.3	60-5
0.6	20-1
0.9	10-0.8
1.2	7-0.5
2.0	3-0.1

Table 2.3- Percentage agarose (w/v) used was chosen to separate efficiently molecules of known or anticipated size.

volume of sample buffer (20% (w/v) ficoll 400, 100mM EDTA pH8.0, 0.25% (w/v) bromophenol blue) was added to DNA samples before loading and to allow sizing of DNA λ/*Hin*dIII and ΦX174/*Hae*III were used as molecular weight markers.

Electrophoresis was carried out in TBE or TAE buffer containing 0.2μg ml^{-1} EtBr, at a constant voltage of 2-12Vcm^{-1} of gel depending on the speed and resolution required. DNA was visualised by the fluorescence of EtBr on a UV transilluminator.

2.5.2 Purification of DNA from low-gelling-temperature agarose

The method used was described by Crouse *et al.* (1983).

A 0.6% gel was prepared as described in section 2.5.1 except that SeaPlaque low-gelling-temperature agarose was used and the gel was cooled to 40°C before pouring. Electrophoresis was carried out at a maximum of 7Vcm^{-1} of gel to avoid heating of the buffer or gel. When DNA fragments were adequately separated, the required fragment was excised from the gel in as small a volume as possible. The gel slice was placed in a pre-weighed microcentrifuge tube and diluted, with distilled water, to an appropriate concentration (assuming a gel density of 1mg μl^{-1}). Sufficient DNA was loaded to allow dilution by at least 2-fold. The gel containing the DNA was melted at 65°C before use.

2.5.3. Electroelution of DNA onto dialysis membrane

This method is simplified from that of Yang *et al.* (1979).

Dialysis membrane was prepared by boiling in 2% (w/v) $NaHCO_3$, 1mM EDTA for 20 minutes followed by several washes in distilled water; the dialysis membrane was stored in 50% ethanol untill required. When required, dialysis membrane was washed several times in distilled water and cut into pieces 0.5cm deeper than the gel and 0.5cm wider than the wells being used. Samples were electrophoresed through 0.8% (w/v) agarose until fragments were adequately separated. DNA was viewed using a hand-held UV lamp, ensuring that exposure to UV light was kept to a minimum. The power supply to the gel was disconnected and a single piece of dialysis membrane inserted into a vertical incision made in the gel just below the desired fragment. If necessary, another piece of membrane was inserted above the fragment to block any unwanted DNA. The power was

reconnected at 20 Vcm^{-1} of gel and the DNA electrophoresed onto the membrane. When all the DNA had migrated onto the membrane, the incision was extended and a gap of about 2mm was opened between the gel and the face of the membrane. With the power supply still connected, the membrane was quickly transferred to a microcentrifuge tube, trapping a corner of the membrane in the lid. To collect the DNA from the membrane, the tube was centrifuged briefly and 15µl of distilled water was applied to each side of the membrane and the tube centrifuged again. The membrane was disposed of and the DNA phenol/chloroform extracted and ethanol precipitated.

2.6 Subcloning of DNA fragments

2.6.1 Ligation of DNA fragments (Crouse *et al.* 1983)

Enzymes which gave cohesive ends compatible with those of enzyme sites in the multiple-cloning-site of pUC or pIC were chosen. Vector and insert DNA were digested with the appropriate restriction enzyme, phosphatase-treated if necessary (see section 2.6.2), recovered by the method described in section 2.5.2 and diluted with water to 10ng μl^{-1}.

Ligation reactions contained equimolar amounts of vector and insert DNA to a final concentration of approximately 1ng μl^{-1} in 1x ligation buffer (50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 5% (w/v) polyethylene glycol 6000). When DNA purified from low gelling temperature agarose was used in ligations it was ensured that the final concentration of agarose was less than 0.1%. T4 ligase (0.01 unit μl^{-1}) was added to the mixtures and the reactions incubated at room temperature for at least 12 hours.

2.6.2 Phosphatase treatment

If DNA was to be inserted into a single enzyme site in a vector, the vector was treated with phosphatase to prevent self-ligation. The vector was digested as in section 2.4.1 and after digestion the buffer was altered to that of 1x REact 3 (Gibco-BRL; 50mM Tris-HCl pH8.0, 10mM $MgCl_2$, 100mM NaCl) if possible. If not, DNA was ethanol

precipitated and resuspended in 1x REact 3. To this 0.1 unit of calf intestinal phosphatase was added and incubation was carried out at 37°C for 30 minutes.

2.6.3 *E. coli* transformation

Different methods of transformation were carried out depending upon the efficiency required. In general, the chemical transformation method was used for subcloning and electroporation for preparation of libraries.

2.6.4 Chemical transformation

Method based on that described by Hanahan (1983).

E. coli DH5α were grown to an OD_{600} of 0.5-0.6 in LB containing 1% (w/v) glucose, 10mM $MgCl_2$, 1mM $CaCl_2$ and 100μg ml^{-1} ampicillin. Cells were harvested by centrifugation at 4000rpm for 5mins at room temperature in a Sorvall SS34 rotor. The cells were resuspended in 0.4x the original volume of ice-cold TfbI (30mM potassium acetate, 100mM $RuCl_2$, 10mM $CaCl_2$, $MnCl_2$ and 15% (v/v) ethanediol adjusted to pH5.8 with 0.2M acetic acid). The cells were centrifuged as above but at 4°C and resuspended in 0.04x original volume of ice-cold TfbII (10mM MOPS, 75mM $CaCl_2$, 10mM $RuCl_2$ and 15% (v/v) ethanediol adjusted to pH6.5 with NaOH). Microcentrifuge tubes containing cells in 100μl aliquots were snap frozen using dry ice/IMS and stored at -80°C.

For transformation, one 100μl aliquot of cells was removed from the -80°C freezer and placed on ice for 5 minutes to thaw. DNA (1-10ng) was added to the cells which were further incubated on ice for 30-60 minutes, heat shocked at 50°C for 45 seconds and incubated on ice a futher 2 minutes before being equilibrated to room temperature. One ml of SOC medium (2% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgSO_4$ and 20mM glucose) was added and the cells incubated at 37°C for 60 minutes. The cell suspension was plated on LB plates containing 100μg ml^{-1} ampicillin 25μg ml^{-1}, IPTG and 40 μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies. Plates were inverted and incubated overnight at 37°C and transformants were identified as white colonies against a background of blue colonies.

2.6.5 Electroporation of *E. coli*

Electroporation was done following the method of Dower *et al.* (1988) using a Bio-Rad Gene Pulser.

2.6.6 Analysis of *E. coli* transformants

Aliquots of 3ml LB containing 100μg ml^{-1} ampicillin were inoculated from selected white colonies and the cultures shaken overnight at 37°C. Plasmid DNA was isolated as described in section 2.7.2 and was checked for the presence of insert by restriction enzyme digestion followed by agarose gel electrophoresis.

2.7 Isolation of plasmid DNA from *E. coli*

Different methods of preparation were carried out depending upon the amount and quality of DNA required.

2.7.1 Large-scale preparation

Large-scale preparation of DNA was carried out using the alkaline lysis method (Birnboim and Doly, 1979).

Plasmid containing *E. coli* was inoculated into 400ml of nutrient broth containing 0.2% (w/v) glucose, 20ml of minimal salt solution (50mM NaCl, 10mM Tris-HCl pH7.5, 10mM $MgCl_2$, and 1 mM DTT) and 100μg ml^{-1} ampicillin, at 37°C overnight. The cells were harvested by centrifugation in a GS3 rotor at 6,000rpm for 5 minutes at 4°C. The supernatant was discarded leaving the pellet as dry as possible.

The pellet was resuspended in 50ml ice-cold lysis buffer (50mM glucose, 25mM Tris-HCl pH8.0, and 10mM EDTA) and incubated on ice for 10 minutes. One hundred ml of freshly prepared 0.2M NaOH, 1% (w/v) SDS solution was added to the suspension, mixed thoroughly and the tubes incubated on ice for a further 5 minutes. To each tube 75ml of ice-cold potassium acetate pH4.8 was added and the tubes were left for 15 minutes on ice. The solution was centrifuged at 9,000rpm in a GS3 rotor at 4°C for 20 minutes. The supernatant was transferred to a fresh tube and mixed with 0.6x volume of propan-2-

ol and incubated on ice for 1 hour. Precipitated nucleic acid was pelleted at 9,000rpm in an SS34 rotor for 30 minutes and the pellet dried and dissolved in distilled water at 65°C.

The plasmid was purified by equilibrium centrifugation in caesium chloride-ethidium bromide gradients (final density, ρ= 1,3860) in a TLA 100 rotor using a Beckman TL-100 Ultracentrifuge at 100,000rpm. Ethidium bromide was removed by CsCl saturated propan-2-ol and the plasmid was ethanol precipitated followed by overnight dialysis against TE buffer. The plasmid was again ethanol precipitated, washed and the pellet resuspended in distilled water.

2.7.2 Small-scale preparation

In order to obtain 2-4µg quantities of DNA the method of Serghini *et al.* (1989) was used. DNA prepared by this method was used to analyse plasmid DNA for size of insert and for mapping sites within insert DNA when analysing transformants.

2.8 Isolation of λ DNA from *E. coli.*

The isolation of λ DNA is based on the method of Blattner *et al.* (1977).

Using the top of a Pasteur pipette, one isolated plaque and surrounding cells were transferred from agar plates to 50ml LB containing 0.1M $MgSO_4$, 2% (w/v) maltose and incubated at 37°C overnight. One ml of chloroform was added to the resulting lysate which was incubated for a further 15 minutes. Cell debris were pelleted by centrifugation in a GSA rotor at 3,700rpm for 15 minutes at 4°C. The supernatant was transferred to a new tube and incubated with 350 units of DNase I, 50µg RNase A at 37°C for 15-30 minutes. To precipitate phage particles, an equal volume of 20% (w/v) PEG 6,000/ 2M NaCl in λ buffer was added and the solution incubated on ice for 1 hour. Precipitated phage particles were pelleted by centrifugation in a GSA rotor at 10,000rpm for 10 minutes at 4°C. The pellet was resuspended in 1ml of λ buffer, transferred to an Eppendorf tube and centrifuged in a microfuge for 2 minutes. The clear supernatant was transferred to fresh tubes and the phage lysed by the addition of 10µl of 10% (w/v) SDS and 10µl of 0.5M EDTA pH 8.0 followed by incubation at 68°C for 10 minutes. The DNA was

phenol/ chloroform extracted twice, ethanol-precipitated, washed and the pellet resuspended in distilled water.

2.9 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Proteins were separated on 5-15% polyacrylamide gels, (5-15% (v/v) acrylamide (Protogel™), 375mM Tris-HCl pH8.8, 0.1% (w/v) SDS, 0.025% (w/v) APS and 0.002% (v/v) TEMED) with a 3.5% polyacrylamide stacking gel (5% (v/v) acrylamide (Protogel™), 125mM Tris-HCl pH6.8, 0.1% (w/v) SDS, 0.3% (w/v) APS and 0.002% (v/v) TEMED). The gel running buffer was 1x Tris-glycine (250mM glycine, 25mM Tris-HCl pH8.8) with 0.15% (w/v) SDS. Samples were mixed with SDS-loading buffer (0.5M Tris-HCl pH6.8, 25% (v/v) glycerol, 6% (w/v) SDS, 0.14M β-mercaptoethanol, 0.025% (w/v) bromophenol blue) and boiled for 3 minutes before loading. The loaded mini-gels were run at 275V and Protean II gels (Bio-Rad) were run at 40mA or at 65V overnight. To visualise the protein bands, the gel was stained in 0.25% (w/v) Coomassie Blue R, 50% (v/v) methanol and 10% (v/v) glacial acetic acid for 10-15 minutes followed by destaining with 10% (v/v) glacial acetic acid and 20% (v/v) methanol until the background staining was removed.

2.10 Preparation of novobiocin-Sepharose

Novobiocin was coupled to Sepharose following the procedure described in Staudenbauer and Orr (1981). Ten grams of epoxy-activated Sepharose CL-6B (Pharmacia) was swollen in 1.0 litre distilled water for 60 minutes at room temperature and washed on a scintered glass filter with 1 litre of 0.3M Na_2CO_3 pH9.5 (buffer A). The gel was then mixed with a solution of 1g novobiocin (Sigma) in 30ml buffer A and gently shaken for 16 hours at 37°C. Excess epoxy groups were blocked by the addition of ethanolamine (final concentration 1M) and shaking was continued for a further 4 hours at 37°C. The product was then washed sequentially with 1 litre each of NaCl in buffer A,

distilled water, 0.5M NaCl in 0.1M sodium acetate pH4.0 and distilled water. Care was taken to protect the novobiocin from strong light at all times.

2.11 Fractionation of yeast extract

Yeast cells were grown in YPD medium until middle to late logarithmic (fermentative) phase, harvested by centrifugation and the pellet was frozen at -20°C. The cells were broken using a Hughes press pre-cooled to -20°C. The press is comprised of a storage chamber connected to a collection chamber by a very fine aperture. Cell breakage occurs by applying pressure to the barrel of the apparatus, forcing the frozen cell pellet through the fine aperture into the collection chamber. The resulting cell lysate was made to 5mM $MgCl_2$ and treated with deoxyribonuclease I and protease inhibitors; trypsin inhibitor (10μg ml^{-1}), PMSF (0.5mM) and benzamide (1mM) then incubated on ice for 15 minutes. An equal volume of 2M KCl was added and the mixture was centrifuged at 185,000 x g for 60 minutes at 4°C. The clear fraction of the supernatant was carefully removed and loaded onto a novobiocin-Sepharose affinity column. An elution protocol of 1M KCl, 2M KCl, and 5M urea in buffer B (25mM Hepes pH8.0; 1mM dithiothreitol; 1mM EDTA; 10% (v/v) ethylene glycol and 100mM KCl) at 4°C was used. Proteins in solution were precipitated overnight at 4°C by the addition of TCA to a final concentration of 15% (w/v) (final concentration) and pelleted by centrifugation in a Microcentaur benchtop centrifuge (13500rpm) at room temperature for 10 minutes. The proteins were resuspended in SDS-sample buffer (0.5M Tris-HCl pH6.8, 25% (v/v) glycerol, 6% (w/v) SDS, 0.14M β-mercaptoethanol, 0.025% (w/v) bromophenol blue). Samples were neutralised by the addition of a minimum volume of Tris-saturated water and stored at -20°C. If required the 50kDa ATPase protein could be specifically eluted after the 2M KCl wash by buffer B containing 15mM ATP and 5mM Mg^{2+}.

2.12 Preparation of crude total yeast protein extract

A 20ml culture of yeast cells was grown at 30°C to an OD_{600} of 1.0 in YPD. The cells were pelleted and washed twice in 5ml of buffer A (1.2M sorbitol, 50mM Tris-HCl

pH7.5, 20mM EDTA and 1% (v/v) β–mercaptoethanol). The pellet was resuspended in 150µl of the same buffer containing 100µg ml^{-1} Zymolyase 60,000 and incubated at 37°C for 15 minutes to form spheroplasts. The spheroplasts were lysed by the addition of 0.5ml of SDS-sample buffer (0.5M Tris-HCl pH6.8, 25% (v/v) glycerol, 6% (w/v) SDS, 0.14M β-mercaptoethanol, 0.025% (w/v) bromophenol blue) and incubated at 100°C for 5 minutes. The resulting protein solution was sonicated briefly in order to shear high molecular weight DNA.

2.13 Amino acid sequencing

2.13.1 PVDF method

Samples from the 5M urea fraction were electrophoresed on a 10% SDS-PAGE gel and electroblotted onto polyvinyline difluoride (PVDF) membrane using 50mM glycine, 50mM Tris-HCl pH10 transfer buffer and stained with Coomassie blue R-250. The stained 35kDa and 200kDa bands were excised and loaded separately onto an Applied Biosystem 470A gas phase sequencer.

2.13.2 Protein in solution method

The proteins contained in the 5M urea fraction from the novobiocin affinity column were separated by SDS-PAGE. Gel-slices containing the 200kDa and the 20kDa proteins were cut out of the unstained gel by estimating their position using slices, stained with Coomassie blue, cut from either side of the gel. The proteins were electroeluted separately from the unstained gel slices using an electroelution chamber at 100 volts for 4-5 hours in 1xTris-glycine buffer(25mM Tris, 190mM glycine) 0.2% (w/v) SDS. The protein was run through a BT2 nitrocellulase filter (Schleicher & Schuell) onto a dialysis membrane, the current was then reversed for 45 seconds to elute the protein from the membrane and the collection chamber washed with 1x Tris-glycine buffer. The Tris-glycine was removed by passing the sample through a Sephadex G25 size fractionation column (Pharmacia LKB Biotechnology). The protein samples were then loaded onto an Applied Biosystems 470A gas phase sequencer.

2.13.3 Cyanogen bromide cleavage

The sample used for cyanogen bromide cleavage of the 20kDa protein was purified using the method described in section 2.13.2. The cyanogen bromide cleavage reaction was carried out by Dr. K. Lilley (Dept. of Biochemistry, University of Leicester). The polypeptide products were separated by SDS-PAGE, electroblotted onto polyvinyline difluoride (PVDF) membrane and stained with Coomassie blue R-250. The stained polypeptide bands were excised and loaded separately onto an Applied Biosystem 470A gas phase sequencer.

2.14 Recovery of antigen and the raising of antibodies

Antibodies were raised in rabbits (New Zealand Whites) by multiple injections of a protein/adjuvent mixture (30-50μg of protein per injection).

The proteins contained in the 5M urea fraction from the Novobiocin affinity column were separated by SDS-PAGE. Slices containing the 200kDa or the 35kDa protein were cut from the gel which had been stained, destained and soaked in sterile distilled water for several hours to remove residual acid. The gel-slices were mixed, separately, with an equal volume of sterile distilled water and sonicated until liquid. An equal volume of Freund's Complete Adjuvent (1mg ml^{-1} *Mycobacterium tuberculosis* heat killed and dried, suspended in 85% (v/v) paraffin oil and 15% (v/v) mannide monooleate) was added and an emulsion was formed by sonication of the suspension which was expressed twice through a 18G needle before being injected. After the primary injection of the rabbits, antigen was suspended in Freund's Incomplete Adjuvent (85% (v/v) paraffin oil and 15% (v/v) mannide monooleate) for subsequent injections.

2.15 Western transfer

Following electrophoresis using SDS-PAGE, proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) at 300mA for 20-30 minutes (Towbin *et al.*, 1979). Transferred proteins were visualised with Ponceau S (BDH) and the stain was removed by washing with TBS. The membrane was then processed by incubation, in the

following order: 3% BSA (w/v) in TBS (10mM Tris-HCl pH7·4, 150mM NaCl) overnight at 4°C; TBS twice for 5 minutes each at 25°C; primary IgG [1:500 diluted in TBS + 1% (w/v) BSA] at 37°C for 2 hours; TBS four times for 5 minutes each at 25°C; secondary IgG (goat anti-rabbit) diluted as for primary IgG at 37°C for 1 hour; TBS four times for 5 minutes each at 25°C; tertiary IgG [rabbit peroxidase anti-peroxidas (PAP), 1:1000 diluted in TBS] at 25°C for 1 hour. The complex was detected by a colour reaction as follows; ten ml of methanol containing 30mg of 4-chloro-1-napthol was added to 30ml of TBS. Thirty µl of H_2O_2 was added to the mixture which was added to the nitrocellulose membrane. The membrane was incubated at room temperature with shaking until the colour development was satisfactory. Further colour development was prevented by washing the filter in water and drying.

2.16 Affinity purification of antibodies

The antigen for affinity purification was electroblotted onto nitrocellulose (see above). The band of interest (visualised by Ponseau S) was cut out as a strip. The strip was washed several times in TBS and blocked overnight in 20% (v/v) foetal calf serum (FCS), 10% (w/v) dried milk powder (Marvel milk™) at 4°C. After blocking, the strip was washed five times for 5 minutes each in TBS at room temperature and then incubated overnight with antiserum containing 10mM sodium azide at 4°C. Excess antiserum was removed and the strip washed 5 times for 5 minutes each in TBS at room temperature. The antibodies were eluted with 0.2M glycine-HCl pH2.2 at 4°C for 5 minutes, the strip was removed and the eluant was brought to pH7.5 by the addition of a predetermined volume of 2M Tris-base pH9.0. BSA was then added to a final concentration of 1% (w/v) and the mixture dialysed against 100 volumes TBS, 10mM sodium azide for 4 hours at 4°C. Affinity purified antibodies were stored at 4°C in solution with 10mM sodium azide.

2.17 Indirect immunofluorescence microscopy

2.17.1 Fixation of cells

S. cerevisiae cells were fixed by the addition of a final concentration of 3.7% (v/v) formaldehyde to a growing culture, which was then left incubating for a further 30 minutes. The cells were harvested and resuspended in 1.2M sorbitol containing 50mM potassium phosphate pH6.5 and 3.7% (v/v) formaldehyde, then left at room temperature for a further 1-2 hours. The cells were washed twice in 1.2M sorbitol containing 50mM Tris-HCl pH7.5 and 10mM β-mercaptoethanol. Zymolyase 60,000 was added to a final concentration of 100μg ml^{-1} and the mixture incubated at 30°C for approximately 30-60 minutes until spheroplasts were formed. Spheroplasts were washed three times in TBS, resuspended in TBS containing 0.2% (v/v) Triton X-100 (Sigma) and left for 10 minutes on ice. The cells were washed three times in TBS and were ready for use in immunofluorescence studies or stored in 1.2M sorbitol at -20°C.

2.17.2 Fluorescent staining

For indirect immunofluorescence microscopy, fixed cells were incubated with the affinity purified antibody at a dilution of 1:20 in TBS for 2 hours at 37°C, then washed three times in TBS. This process was repeated with either rhodamine-linked goat anti-rabbit IgG (Jackson ImmunoResearch; diluted 1:50) or fluorescine isothyocyanate(FITC)-linked goat anti-mouse IgG (Jackson ImmunoResearch; diluted 1:50).

When staining with 4,6-diamino 2-phenylindole (DAPI), fixed cells were incubated with DAPI at a final concentration of 20ng ml^{-1} for 1 hour at 37°C, then washed three times in TBS.

Following the staining procedure, cells were spread out on a microscope slide and allowed to dry. A drop of antifade (10mg p-phenylenediamine in 1ml TBS buffer and 9ml glycerol) was added to the dry cells, covered with a coverslip and pressed gently together with a piece of tissue to absorb excess antifade. The coverslip was then sealed with nail polish to prevent the cells from desiccating. Indirect fluorescence microscopy was performed using a Zeiss Axiophot photomicroscope (Williamson & Fennel, 1975)

2.18 Expression library construction and screening

S. cerevisiae chromosomal DNA from strain 483/1a was partially digested with *Sau*3AI. DNA fragments in the size range 1-4kb were isolated from a agarose gel and ligated into expression vectors pEX1, pEX2 & pEX3 digested with *Bam*HI and dephosphorylated. The resultant recombinant plasmids were transformed by electroporation into the *E. coli* strain RR1 lambda cI_{857} and the transformants selected on LB plates containing 100μg ml^{-1} ampicillin at 30°C. After approximately 16 hours, transformant colonies were lifted onto nitrocellulose filters (Schleicher & Schuell) which were placed, colonies facing upward, onto new LB plates containing 100μg ml^{-1} ampicillin for a further 4 hour incubation at 30°C. Expression of recombinant plasmid protein was induced by incubation of the plates at 45°C for 2 hours. Filters were transferred to 3MM paper (Whatman) saturated with 0.1% (w/v) SDS for 15 minutes and then suspended in chloroform tanks for 20 minutes. The filters were then processed by incubation with gentle shaking in the following solutions in the order: blocking solution [2% (w/v) Marvel milk™, 3% BSA (w/v) in TBS] at 4°C overnight ; TBS four times for 5 minutes each at 25°C; primary IGg, diluted 1:500 in TBS, 1% (w/v) BSA at 25°C for 3 hours; TBS four times for 5 minutes each at 25°C; secondary IgG (goat anti-rabbit), diluted as for primary IgG at 25°C for 2 hours; TBS four times for 5 minutes each at 25°C; tertiary IgG (rabbit PAP), diluted 1:1000 in TBS at 25°C for 1 hour. The complex was detected using a colour reaction as described in section 2.15.

2.19 Genomic library screening

The recombinant phage (NM728) containing *Hin*dIII digested chromosomal DNA from *S. cerevisiae* strain 483/1a, were plated out to give approximately 3-4000 plaques per plate. The plates were chilled at 4°C for at least 1 hour. Dry nitrocellulose filters (Schleicher & Schuell) were placed onto the plates for 30-60 seconds, then immersed DNA side up in denaturing solution (1.5M NaCl, 0.5M NaOH) for 30-60 seconds. Filters were then transferred to neutralising solution (1.5M NaCl, 0.5M Tris-HCl pH8.0)

for 5 minutes, rinsed in 2x SSC (SSC is 0.15M NaCl, 0.015M sodium citrate, pH7.4) and placed on 3MM paper to dry. Once dry, filters were baked for 2 hours at 80°C.

Ten ng of probe DNA was labelled using the random priming method (see section 2.21.1).

Prehybridisation and hybridisation were carried out in 0.5M Na_3HPO_4 pH7.4, 1mM EDTA, 7% (w/v) SDS, 50μg ml^{-1} tRNA. Prehybridisation was carried out in 40ml hybridisation solution at 65°C for 1 hour. Filters were then transferred to a perspex hybridisation box containing 20ml of fresh hybridisation solution preheated to 65°C. Denatured radiolabelled DNA was added to the solution and hybridisation allowed to proceed at 65°C, overnight. Filters were washed at 65°C for 10 minutes with each wash as follows: twice in 0.2M Na_3HPO_4 pH7.4, 1% (w/v) SDS, twice in 0.1M Na_3HPO_4 pH7.4, 1% (w/v) SDS and twice in 40mM Na_3HPO_4 pH7.4, 0.1% (w/v) SDS. Filters were blotted to remove excess liquid, wrapped in foil and autoradiographed using Fuji RX film.

2.20 Southern blotting

Southern blotting method, after Southern (1975).

Gels to be blotted were photographed, then denatured in 0.5M NaOH, 1M NaCl for 30 minutes with gentle shaking and neutralised in 0.5M Tris-HCl pH7.5, 3M NaCl for 30 minutes.

Gels were then transferred onto a wick of 3MM (Whatman) paper soaked in 20x SSC on a glass plate over a reservoir of 20x SSC. Hybond-N was cut to cover the gel, was pre-soaked in 3x SSC and placed on the gel avoiding the creation of air bubbles. Two similarly cut sheets of 3MM were soaked in 3x SSC and placed on top of the filter, again, avoiding the creation of air bubbles. A stack of paper towels was placed on top of the 3MM paper to draw the SSC upwards. A glass plate and a 500g weight were placed on top to maintain contact between the gel and the filter. The towels were changed regularly during the first hour of the blotting to avoid patchy transfer. The transfer was allowed to proceed for at least 2 hours or overnight for transfer of genomic DNA.

After blotting, the position of the wells was marked with a Biro pen, the filter rinsed in 3x SSC for 1 minute and blotted dry on 3MM paper. DNA was fixed onto the filter by a 45 second exposure to UV light on the transilluminator (through a layer of Saran-Wrap™ (Dow Inc., USA)).

2.21.1 Radiolabelling probe DNA by random priming

Radiolabelling method, after Feinberg & Vogelstein (1983, 1984)

Probe DNA was recovered from agarose gels by electroelution onto dialysis membrane see section 2.5.3. DNA was denatured by boiling at 100°C for 3-5 minutes, then 10-20ng of probe DNA was immediately added to the labelling mix containing 30% (v/v) OLB (100mM dATP, 100mM dTTP, 100mM dGTP, 250mM Tris-HCl pH6.6, 540μg ml^{-1} hexadeoxyribonucleotides (Pharmacia), 0.5mg ml^{-1} DNase-free BSA, 10μCi [α–^{32}P] dCTP (Amersham) and 0.5 units Klenow fragment of DNA polymerase I. The reaction was incubated for 5-16 hours at room temperature and the labelled DNA denatured, as before, prior to its incubation with the filter.

2.21.2 Prehybridisation and hybridisation of filters

Phosphate/SDS method, after Church & Gilbert (1984)

Prehybridisation and hybridisation were carried out in 0.5M Na_3HPO_4 pH7.5, 1mM EDTA, 7% (w/v) SDS. Filters were prehydridised in a 200ml hybridisation bottle containing 20ml hybridisation buffer at 65°C for at least 60 minutes in a Bachofer Rotisserie oven. The prehybridisation solution was replaced by 10ml of fresh hybridisation buffer preheated to 65°C. Denatured radiolabelled DNA was added to the solution and the hybridisation carried out at 65°C overnight.

Filters were washed at 65°C, for 10 minutes with each wash as follows: two times in 0.2M Na_3HPO_4 pH7.4, 1% (w/v) SDS, two times in 0.1M Na_3HPO_4 pH7.4, 1% (w/v) SDS and two times in 40mM Na_3HPO_4 pH7.4, 0.1% (w/v) SDS. Filters were blotted to remove excess liquid, wrapped in Saran-Wrap™ and autoradiographed using Fuji RX film.

Denhardt's method, after Denhardt (1966)

Denhardt's solution (50x concentration) is 1% (w/v) Ficoll 400, 1% (w/v) polyvinyl pyrrlidone and 1% (w/v) bovine serum albumin fraction V. Prehybridisation and hybridisation were carried out in 6x SSC, 5x Denhardt's solution, 0.5% SDS (w/v) and 5μg ml^{-1} *E. coli* tRNA. Filters were prehydridised in a 200ml hybridisation bottle containing 20ml hybridisation buffer at 65°C for at least 60 minutes in a Bachofer Rotisserie oven. The prehybridisation solution was replaced by 10ml fresh hybridisation buffer preheated to 65°C. Denatured radiolabelled probe DNA was added to the solution and hybridisation carried out at 65°C overnight.

Filters were washed at 65°C, for 10 minutes with each wash as follows: two times in 1x SSC, 0.5% (w/v) SDS, two times in 0.5x SSC 0.5% (w/v) SDS and two times 0.1x SSC 0.01% (w/v) SDS. Filters were blotted to remove excess liquid wrapped in Saran-Wrap™ and autoradiographed using Fuji RX film.

2.22 DNA sequencing

2.22.1 Creation of deletion clones

Sequential deletions were introduced into insert DNA cloned into pUC and pIC vectors by the method of Henikoff (1984). Two enzymes which do not cut within the insert but have recognition sites in the polylinker were used to linearise the plasmid. One of the enzymes was chosen to generate a 3' overhang which is resistant to the activity of exonuclease III, protecting the vector sequence. The other enzyme was chosen to generate a blunt end or 5' overhang which are both exonuclease III sensitive, allowing the enzyme to digest insert sequence. The linear plasmid was incubated with a molar excess of exonuclease III and aliquots were removed from the reaction at timed intervals. Following the digestion of a single strand, the second strand was digested by incubation with S1 nuclease at room temperature for 30 minutes and the reaction stopped by incubation at 65°C for 10 minutes. Each timepoint aliquot was divided in two; half of the reaction was analysed on an agarose gel to assess the course of the deletions and the other half was

blunt-end ligated. DNA from each timepoint was transformed into *E. coli* and clones analysed as described in section 2.6.6.

2.22.2 Preparation of DNA for sequencing

The method used for the preparation of plasmid DNA from cultures and its subsequent denaturation and neutralisation was based on that of Kraft *et al.* (1988). However, better results were obtained by substituting a centrifuged through Sepharose CL-6B for the polyethylene glycol precipitation to remove degraded RNA.

Sepharose CL-6B mini-columns were prepared as follows: a small hole was made in the bottom of a 0.5ml microcentrifuge tube. To this tube 25µl of a slurry of 200µm glass beads (Ballotini number 11; Jencons (Scientific) Ltd., Leighton Buzzard) was added, followed by 300µl of Sepharose CL-6B equilibrated in 10mM Tris-HCl pH8.0, 0.1mM EDTA (with a packed gel to buffer ratio of 2:1). The 0.5ml tube was left open and placed inside a 1.5ml microcentrifuge tube, which was placed in a 15ml plastic centrifuge tube. This apparatus was centrifuged for 4 minutes at 1000rpm in a Sorvall Omnispin. The liquid collected in the 1.5ml tube was discarded, the apparatus reassembled and centrifuged as above for a further 2 minutes. At this stage the mini-column was ready for the sample to be loaded and to prevent drying was used within 20 minutes.

A sample in a 20µl volume was applied to the top of the column without disturbing the Sepharose. The 0.5ml tube was placed in a clean 1.5ml tube and centrifuged as above for 4 minutes. The column was discarded and the sample in the 1.5ml tube was now ready for further manipulation.

2.22.3 Sequencing reactions

Double stranded DNA sequencing (Chen & Seeburg, 1985) was performed by the dideoxy chain termination method (Sanger, 1977).

The primers used for the sequencing reactions were the universal M13 "forward" and "reverse" primers: 5'-GATAAAACGACGGCCAGT-3' and 5'-AACAGCTATGACCATG-3', respectively. Double-stranded plasmid DNA prepared as described in section 2.22.2 was denatured by incubation with 0.2M NaOH and 200µM

EDTA at room temperature for 5 minutes. The mixture was neutralised with 8µl 1M Tris-HCl pH4.5 and the sample ethanol precipitated, washed and dried. Dried pellets of denatured and neutralised DNA were resuspended in 10µl distilled water. Two µl annealing buffer (280mM Tris-HCl pH7.5, 100mM $MgCl_2$, 350mM NaCl) and 2µl of primer (2-5ng μl^{-1}) was added, the samples mixed and incubated at 37°C for 20 minutes, then at room temperature for 10-20 minutes. Three µl of labelling mix (2µM dCTP, 2µM dGTP, 2µmM dTTP), 1µl [α-^{35}S]dATP (10µCi μl^{-1}, >400Ci $mmol^{-1}$) and 2µl (3 units) T7 DNA polymerase were added and the samples incubated at room temperature for 5 minutes. The reaction was stopped by transferring 4.5µl of this reaction mixture into each of four 2.5µl termination mixes prewarmed at 37°C. Each termination mix contained three of the dNTPs at a concentration of 150mM and a limiting concentration (15mM) of a polymerisation inhibiting dideoxyribonucleotide. The tube containing ddGTP was labelled 'G' and the tube containing ddATP was called 'A' etc. The termination reactions were incubated at 37°C for 5 minutes , then 1 unit of DNA polymerase I (Klenow fragment) was added to each reaction mix and a futher 5 minute incubation carried out at 37°C. The termination reaction was stopped by the addition of 5µl of loading dye (95% (w/v) deionised formamide, 20mM EDTA, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue) and the reactions electrophoresed or stored at -20°C.

2.22.4 Electrophoresis of single stranded DNA from sequencing reactions

Electrophoresis of sequencing reactions were carried out using BRL Life Technologies Inc. Sequencing kit, model S2, with 0.4mm spacers and sharkstooth combs. The glass plates were thoroughly cleaned and one side of the smaller plate was siliconised using 3ml of 2% (w/v) dimethyldichlorosilane dissolved in 1,1,1 trichloroethane. The mould was prepared by placing one glass plate on top of the other, separated by spacers and securely taping the bottom and both sides of the plates.

Sequencing gels were 6% polyacrylamide; [from Accugel 40™,40% (19:1) acrylamide: bisacrylamide stock solution] in 1x TBE, containing 50% (w/v) urea. The urea was dissolved in the gel mixture by heating, the solution was allowed to equilibrate to room temperature and was then filtered under suction through Whatman No.1 filter paper. The

gel was cast following the addition of 0.07% (w/v) APS and 0.048% (v/v) TEMED. Inverted sharkstooth combs were inserted at the top of the gel to ensure a level air-gel interphase and the gel allowed to set for 1 hour. The tape was removed from the bottom of the mould, the gel placed into the apparatus and the top and bottom chambers filled with 1x TBE buffer. The sharkstooth combs were removed, the well area washed with the buffer and the combs inserted to form the wells.

The gel was pre-run at 75W for 20 minutes. Samples were heat-denatured at 80°C for 2 minutes and wells rinsed out before loading samples. Electrophoresis was carried out at 65W to maintain the gel temperature at 50°C throughout the run. After sufficient length of time (judged by the migration of marker dyes), the plates were dismantled and the gel, supported on the larger plate, was submerged in fixing solution [10% (v/v) glacial acetic acid, 10% (v/v) methanol] for 20 minutes at room temperature. The gel was transferred onto Whatman 3MM filter paper, dried under vacuum at 80°C for 40-60 minutes and autoradiographed at room temperature.

2.22.5 Analysis of sequence data

All DNA sequence data, nucleotide and peptide data manipulations were carried out on a VAX cluster using the Genetics Computer Group sequence analysis software package (Devereux *et al.*, 1984).

2.23 Total yeast DNA preparation

A 20ml culture of yeast cells was grown at 30°C to an OD_{600} of 1.0 in YPD. The cells were pelleted and washed twice in 5ml of buffer A (1.2M sorbitol, 50mM Tris-HCl pH7.5, 20mM EDTA and 1% (v/v) β–mercaptoethanol). The pellet was resuspended in 150µl of the same buffer containing 100µg ml^{-1} Zymolyase 60,000 and incubated at 37°C for 15 minutes to form spheroplasts. The spheroplasts were lysed by the addition of 0.5ml of 100mM NaCl, 50mM Tris-HCl pH8.0, 20mM EDTA, 1% (w/v) SDS and 10 µg ml^{-1} proteinase K and the mixture incubated at 65°C for 90 minutes.

The lysate was extracted with phenol/chloroform and ethanol precipitated, washed with 80% (v/v) ethanol and dried. The dried pellet was resuspended in 0.5ml of distilled

water and incubated at 65°C with 10 μg ml^{-1} RNaseA. Finally, 10μg ml^{-1} proteinase K and 1% (w/v) SDS (final concentration) were added to the mixture, which was incubated for a further 60 minutes. The proteins were removed by phenol/chloroform extraction and the DNA was ethanol precipitated and the pellet resuspended in distilled water.

2.24 Isolation and separation of yeast chromosomes

Wild-type yeast cells were grown at 30°C to stationary phase in YPD media. The cells were centrifuged at 3000rpm in Omnispin centrifuge for 20 minutes at 4°C. The pellet was resuspended in 50mM EDTA pH8.0, at a ratio of 6 volumes EDTA solution to 4 volumes cells; 100μl of 2mg ml^{-1} Zymolyase 60,000 was added and incubated at 37°C for 20 minutes to allow the formation of spheroplasts.

A 1.0% (w/v) low melting temperature agarose solution in 125mM EDTA pH7.5, was melted and cooled to 50°C. Approximately 0.9ml melted agarose was mixed with 0.3ml cell/enzyme suspension, pipetted into a mould chamber and allowed to cool at 4°C for 20 minutes. The agarose samples were then removed from the mould using a clean spatula, placed into a petri dish containing LET buffer (0.5M EDTA, 10mM Tris-HCl pH7.5 and 7.5 % (v/v) β-mercaptoethanol) and incubated at 37°C overnight.

The LET buffer was removed, replaced with NDS buffer (10mM Tris-HCl pH7.5, 0.5M EDTA pH8.0, 1% (w/v) N-laurylsarcosine and 1mg ml^{-1} proteinase K) and the samples were further incubated at 50°C overnight. Subsequently, the NDS buffer was removed; the agarose samples were washed with 50mM EDTA pH8.0 for 15 minutes at room temperature and then washed again overnight with the same solution.

The agarose samples were cut to fit approximately 75% of the height of the gels sample wells. Once agarose samples were placed into the sample wells; 0.5% (w/v) low-melting-temperature agarose was poured into each well and allowed to harden at room temperature for 30 minutes.

The pulsed field electrophoresis was performed using a CHEF-DR™II (Bio-Rad). The parameters were set as follows: switch time was 60 seconds for 15 hours, followed by

a 90 second switch time for 8 hours; the voltage was 200 volts at 4°C. The electrophoresis was carried out through a 1% (w/v) agarose gel in 0.5 x TBE.

When electrophoresis was completed, the gel was stained with 0.5μg ml^{-1} ethidium bromide for 15 minutes and destained by two 15 minute washes in distilled water. DNA fragments were visualised using an UV transilluminator. Finally, the DNA fragments were blotted on to Hybond-N™ and processed as for Southern blots (see section 2.20).

2.25 Analysis of yeast RNA

2.25.1 Preparation of RNA from yeast

A 500ml culture of yeast cells was grown at 30°C to an OD_{600} of 1.0 in YPD. The cells were centrifuged at 7,000rpm in a GS3 rotor for 10 minutes at 4°C. The pellet was resuspended in denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate pH6.5, 0.5% (w/v) sodium sarcosyl and 100mM β-mercaptoethanol) and frozen at -20°C. The cells were broken in a Hughes press pre-cooled to -20°C. The resulting cell lysate was placed on ice and 0.1x volume of 3M sodium acetate pH4.0 was added followed by an equal volume of water-saturated-phenol and a 0.1x volume of chloroform/isoamyl alcohol (mixed in the ratio 24:1 v/v). This was incubated on ice, then at 60°C for 10 minutes each and centrifuged at 9,000rpm in a HB4 rotor. The aqueous phase was removed and phenol extracted as above once more. An equal volume of propan-2-ol was added to the aqueous phase and the mixture incubated on ice for 1 hour then centrifuged at 10,000 rpm in a SS34 rotor for 10 minutes. The resulting pellet was washed with 80% (v/v) ethanol, air dried and resuspended in DEPC-treated water. The concentration of RNA was determined by measuring its absorbance as described in section 2.4.2. The integrity of the RNA was assessed as described in the next section.

2.25.2 Analysis of RNA by agarose gel electrophoresis

The RNA was denatured prior to electrophoresis by incubation at 55°C in 1-3 volumes of GFM buffer (1.1M deionised glyoxal, 78% (v/v) deionised formamide, 0.6x MOPS buffer) for 15 minutes. Denatured RNA samples with 0.1x volume of RNA

sample buffer [40% (v/v) deionised formamide, 50% (v/v) glycerol, 1x MOPS, 0.05% (w/v) xylene cyanol and 0.05% (w/v) bromophenol blue] were loaded onto a 1.5% (w/v) agarose, 1x MOPS gel and electophoresed for 2.5-3 hours in 1x MOPS buffer at 60mA. If RNA was to be directly visualised to examine the integrity of the RNA preperation, 0.5μg ml^{-1} ethidium bromide was added to the GFM buffer prior to incubation with the RNA.

2.25.3 Northern blotting

Following electrophoresis, RNA was transferred onto Hybond-N™ as described in section 2.20. After blotting overnight, the filter was washed in 3x SSC pH7.0 and dried at 37°C for 15 minutes. The RNA was fixed to the filter by a 30 second exposure to UV light using a transilluminator (through a layer of Saran-Wrap™). The filter was baked for 1 hour at 80°C to remove residual glyoxal and the filter stored at room temperature.

2.25.4 Prehybridisation and hybridisation of RNA filters

Prehybridisation and the hybridisation was carried out in 6x SSC pH7.0, 5x Denhardt's solution, 50% (v/v) deionised formamide, 0.5% (w/v) SDS and 100μg ml^{-1} sheared single-stranded herring sperm DNA. Prehybridisation was carried out in 30ml of prehybridisation solution at 42°C for 5 hours in a Bachofer Rotisserie oven. The prehybridisation solution was replaced by 10ml fresh hybridisation buffer preheated to 42°C. Denatured radiolabelled probe DNA was added to the solution and the hybridisation carried out at 42°C overnight. The filter was washed, 10 minutes each wash as follows: twice in 2x SSC pH 7.0, 1% (w/v) SDS at 42°C; twice in 2x SSC pH7.0, 1% (w/v) SDS at 65°C; twice in 0.5x SSC pH7.0, 0.1% (w/v) SDS at 65°C. Filters were blotted to remove excess liquid wrapped in Saran-Wrap™ and autoradiographed using Fuji RX film.

2.26 Gene disruptions in *S. cerevisiae*

Cloned gene fragments were disrupted and used in gene replacement experiments according to the method of Rothstein (1983).

The cloned fragment containing the gene of interest was cut with a restriction enzyme that cleaved within the open reading frame. A fragment with compatible ends containing a selectable marker was ligated into this site. The fragment used for the disruption was cloned into the gene at a site that allowed sufficient flanking sequence to permit homologous pairing with both sides of the chromosomal target. A linear fragment containing the selectable marker and the flanking sequence homologous to the target DNA, was excised from the plasmid.

A diploid yeast strain with the appropriate auxotrophic marker was transformed with the linear fragment and plated out on selective medium. Transformants were picked, sporulated and tetrad analysis was carried out. DNA was extracted from cells expressing the auxotrophic marker and Southern blot analysis was performed to verify the gene disruption.

2.27 Transformation of yeast by electroporation.

A 100ml culture of yeast was grown at 30°C to an OD_{600} of 0.6 in YPD. The cells were centrifuged at 3000rpm in an Omnispin centrifuge at room temperature for 4 minutes. The pellet was resuspended in an equal volume of sterile distilled water and centrifuged as above. This process was repeated 7-8 times. The cells were then resuspended in 1ml sterile distilled water for electroporation.

DNA (1-10μg) was added to 200μl of the cell-suspension in an electroporation chamber. The instrument was set up at 5 kV cm^{-1} and a capacitance of 25mF. YPD (800μl) was immediately added to the mixture after the electrical pulse and the cells transferred to an Eppendorf tube. The cells were allowed to recover at 30°C for 30-60 minutes before being pelleted in a microcentrifuge at 6500rpm for 40 seconds. The pellet was resuspended in 100μl of YPD, plated on selection media and incubated at 30°C until colonies appeared.

2.28 Tetrad analysis

2.28.1 Induction of sporulation

Diploid strains of *Saccharomyces cerevisiae* were spread onto sporulation medium, the plates were sealed with "nescofilm" to prevent desiccation and incubation was carried out for several days at 26°C. Under such conditions of nutrient limitation, cells arrest vegetative growth at the G1 phase. They undergo meiosis and produce four haploid ascospores. Tetrads can be visualised microscopically within an individual ascus.

2.28.2 Dissection of ascospores

A loopful of spores were resuspended in 100µl of dH_2O containing 10% (v/v) glusulase and incubated at room temperature. Digestion of the spore sac was monitored microscopically and stopped when the spores were loosely packed but still recognisable as a tetrad.

A 20µl aliquot of spore solution was spotted onto a YPD plate and the plate held at an angle to allow the spores to spread out in a line along the plate. A micromanipulator (Allen Benjamin Inc. Tempe Arizona) designed according to the specifications of Sherman (1973) was used to dissect the tetrads. Plates were incubated at 30°C for 24-48 hours.

Chapter 3

Isolation of novobiocin-binding proteins

3.1 Introduction

In prokaryotes, novobiocin was identified as a specific inhibitor of bacterial DNA gyrase, a type II topoisomerase that introduces negative supercoils into relaxed, covalently closed, double-stranded DNA molecules (Gellert *et al.*, 1976b). However, later work has indicated that some of the effects of novobiocin may be due to secondary targets (Fairweather *et al.*, 1980; Orr *et al.*, 1984). The effects of novobiocin on eukaryotic cells, including inhibition of DNA synthesis, transcription, modulation of chromatin structure and differentiation, are similar to those observed in prokaryotes. Because of these observations, the sequence homology between bacterial DNA gyrase and eukaryotic topoisomerase II (Lynn *et al.*, 1986) and reports that novobiocin inhibits some purified eukaryotic topoisomerase II (Goto and Wang, 1982), the antibiotic has frequently been referred to as a specific inhibitor of eukaryotic topoisomerase II activities.

However, recent work has unambiguously demonstrated that topoisomerase II is not the main target of novobiocin in yeast. Unlike bacterial gyrases, the yeast enzyme does not bind novobiocin *in vitro* and the terminal phenotype of yeast *top2 (ts)* mutants is different from the terminal phenotype of drug-sensitive yeast strains treated with novobiocin. Furthermore, novobiocin-resistant mutants are not *top2* mutants (Pocklington *et al.*, 1990a). These conclusions are further supported by analysis of BHK cells which indicates that topoisomerase II activity is not altered in cells which are resistant to novobiocin (Ishida *et al.*, 1987). These observations called for further study of the mode of action of novobiocin in order to fully understand the mechanism by which the drug exerts its toxic effects.

Antibiotics often have a highly selective action upon biological processes. Nevertheless, the basis of such selectivity can vary from one antibiotic to another and an antibiotic may interfere directly or indirectly with single or multiple targets within a cell.

In studying the mode of action of an antibiotic, genetic studies of drug-resistant or drug-sensitive mutants have often been beneficial. In the case of novobiocin, such studies are in progress and have been described in chapter 1. Alternatively, the mode of action of an antibiotic can be identified using a biochemical approach. This involves the isolation of the target(s) of a drug through a purification procedure known as affinity chromatography.

Affinity chromatography is one of the most powerful methods that can be applied to protein purification. Numerous books and reviews covering the theory of affinity chromatography have been published (see Lowe and Dean, 1974; Turkova, 1978; Dean *et al.*, 1985). The procedure takes advantage of one or more biological properties of the molecule(s) being purified, through the utilisation of specific and reversible interactions which occur between biomolecules. These interactions are not due to the general properties of the molecule such as isoelectric point (pI), hydrophobicity or size. The concept is realised by binding the ligand to an insoluble support and packing the support into a chromatography column. In principle, only enzymes or proteins with appreciable affinity for the ligand will be retained on such a column; others will pass through unretarded. Specifically adsorbed protein can then be eluted by altering the composition of the solvent to favour dissociation. Thus, affinity chromatography can be applied when any particular ligand interacts specifically with other molecules. For example, specific adsorbents have been used to purify enzymes, antibodies, nucleic acids and cofactors, vitamins, repressors, drug or hormone binding receptors and transport proteins (see Ostrove, 1990). In addition, this technique has often been used for concentrating protein solutions and for separating denatured from biologically active forms of proteins.

In this study, a biochemical approach was used in an attempt to identify the target(s) of novobiocin in eukaryotic cells. This approach utilises a protein purification method which previously made possible the isolation of the novobiocin target in bacteria, the B-subunit of DNA gyrase (Staudenbauer and Orr, 1981). In this procedure, isolation of the

target was achieved through affinity chromatography of crude cell extract using novobiocin coupled to Sepharose.

As described in chapter 1, initial studies have shown that six proteins from yeast cell extracts bind novobiocin-Sepharose. Two of the proteins were shown to have low affinity to the immobilised drug, as they were eluted from novobiocin-Sepharose in high salt buffer. These proteins are a high molecular mass protein of approximately 200kDa which has been identified as the yeast heavy chain myosin (Watts *et al.*, 1985; Watts *et al.*, 1987; Sweeney *et al.*, 1991) and a protein of approximately 52kDa, identified as the *SUP45* gene product. The remaining four novobiocin-binding proteins of molecular mass 200kDa, 52kDa, 35kDa and 20kDa display high affinity for novobiocin. These proteins cannot be eluted from the resin by high salt buffer but only through the use of denaturing conditions (*e.g.* 5M urea wash). Similar denaturing conditions were required to elute the B-subunit of DNA gyrase from novobiocin-Sepharose (Staudenbauer and Orr, 1981; Orr and Staudenbauer, 1982).

The 52kDa protein eluted in the 5M urea fraction has been previously identified as the β-subunit of the yeast mitochondrial F_1 ATP synthetase through N-terminal amino acid sequencing (Jenkins *et al.*, 1990). The remaining three proteins with high affinity for novobiocin have not yet been characterised. Hence, the aim of this study was to purify and identify these proteins, either by N-terminal protein sequencing or through reverse genetics, by cloning their respective genes from expression libraries, screened with antibodies raised against them.

The preparation of novobiocin-Sepharose and its use as an affinity absorbent for the isolation of novobiocin-binding proteins is described in this chapter. Also described is the method used to raise antibodies against novobiocin-binding proteins, the use of these antibodies in indirect immunofluorescence microscopy and initial attempts to identify the proteins through the amino acid sequencing of their N-termini.

3.2 Preparation of novobiocin-Sepharose

In order to produce a novobiocin-Sepharose column, novobiocin had to be coupled to epoxy-activated Sepharose using a slight modification of a previously described method (Staudenbauer & Orr, 1981). The coupling gel contains reactive oxirane groups that can form stable ether bonds with nucleophilic hydroxy and amino groups of the ligand (Sundberg and Porath, 1974). Under weak alkaline conditions, coupling occurs primarily at the phenolic hydroxy group of the 4-hydroxy-3-(3-methylbut-2-enyl) benzoic acid moiety of novobiocin (see Fig. 3.1). Various structural modifications are possible in this part of the molecule without loss of its biological activity (Godfrey and Price, 1972). Furthermore, the 1,4-butandiol-diglyceryl ether residue, connecting the ligand to the matrix, provides a hydrophilic spacer arm which minimises any interference of the matrix with the binding reaction between the immobilised drug and the target proteins.

When the product of the first coupling between novobiocin and epoxy-activated Sepharose was used in affinity chromatography, no proteins were found in the 5M urea wash, indicating that the coupling reaction had not been successful. When a new stock of novobiocin was used and the coupling reaction repeated, a protein elution profile similar to that obtained by Jenkins *et al.* (1990) was observed. Since the other chemicals used in both coupling reactions were from the same source, it appears that the novobiocin used in the first coupling reaction was unsuitable and had perhaps been degraded.

3.3 Protein extraction and purification

The *S. cerevisiae* strain *Zn1d* (Table 2.1) which contains a truncated myosin heavy chain gene, *myo1*, (Watts *et al.*, 1987) was used to prepare protein extracts for affinity-chromatography. This *myo1* strain was used to avoid cross-contamination of the 200kDa protein which displays high affinity to novobiocin with the myosin heavy chain, also 200kDa, which has previously been shown to bind to novobiocin although with lower affinity. *Zn1d* was grown in batch cultures to middle or late log phase, pelleted and the cells broken in a Hughes press (chapter 2). This method of breaking yeast cells provides

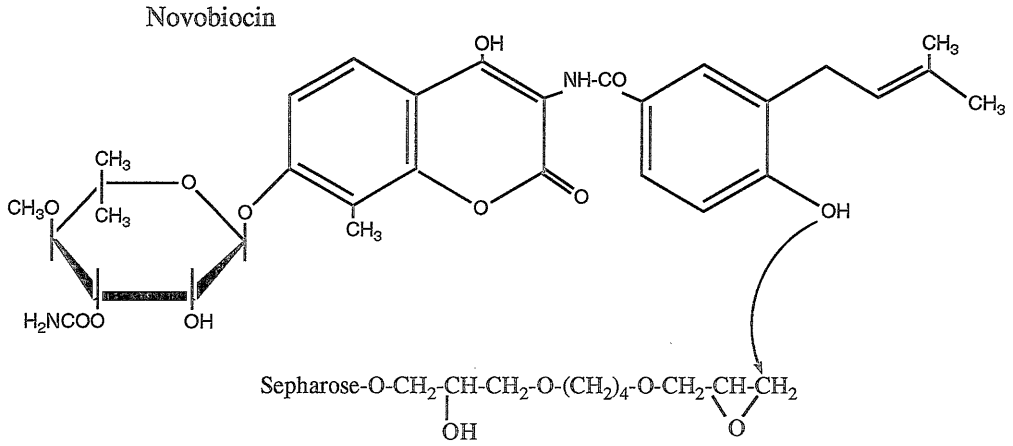


Figure 3.1 Preparation of novobiocin-Sepharose

Proposed model of coupling between novobiocin and Epoxy-activated Sepharose (after Staudenbauer and Orr, 1981).

good yields of protein and is quick and simple when compared to other methods such as sonication or enzymatic lysis. The cell lysate was treated with DNase I, in the presence of protease inhibitors and centrifuged in an ultra-centrifuge (see chapter 2). The clear supernatant was applied to the novobiocin-Sepharose column in the presence of 0.1M KCl. The bulk of the proteins were not retained by the resin and either passed through the column without binding or were eluted in a high salt (2M KCl) buffer. Under denaturing conditions (5M urea wash) four discrete proteins of 200kDa, 52kDa, 35kDa and 20kDa were eluted from the column (Fig. 3.2).

Initially, protease inhibitors were not added to the cell extracts. Later, proteolytic breakdown problems were encountered, particularly with the 200kDa protein and therefore protease inhibitors were routinely included in the preparations.

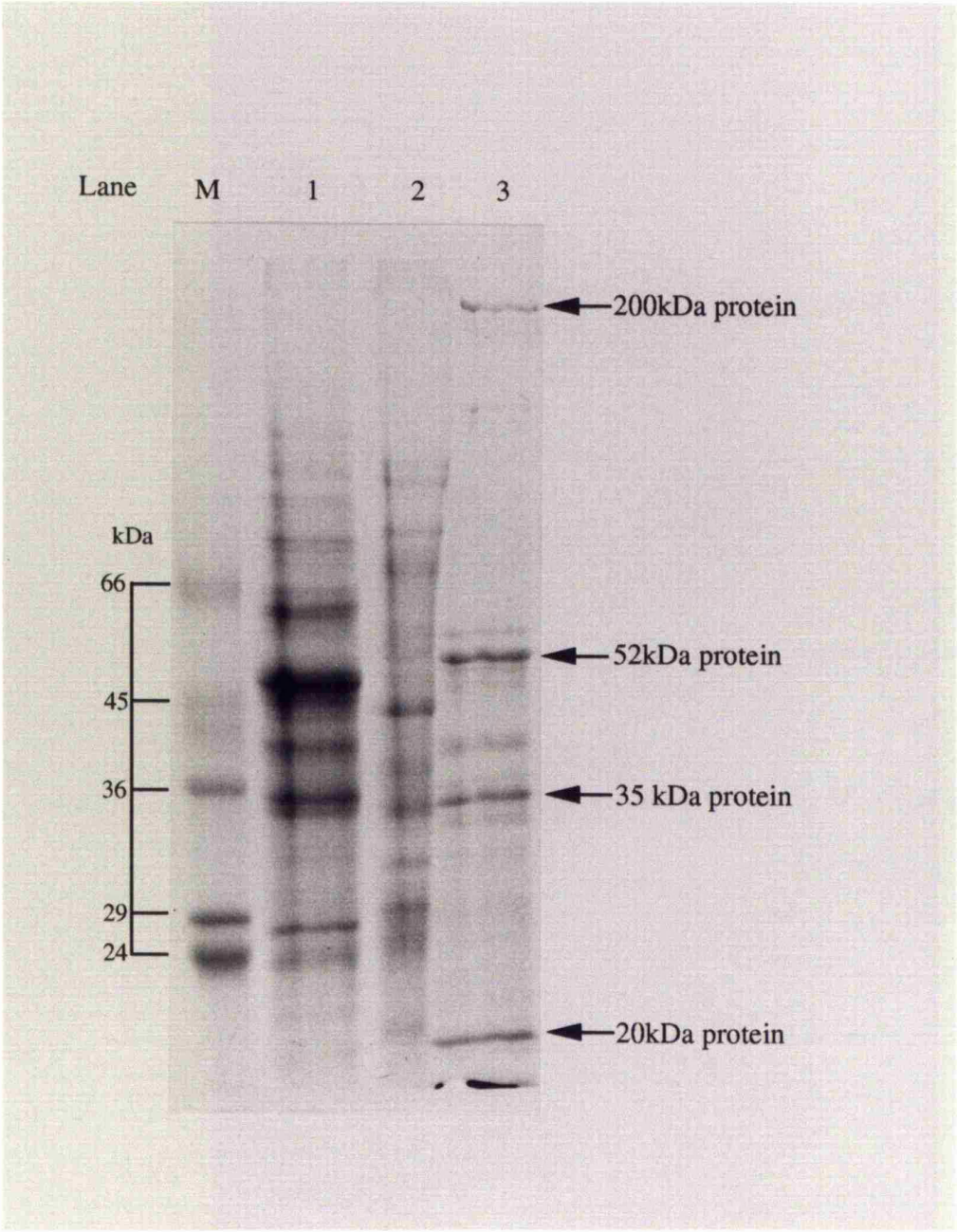
3.4 Raising antibodies to novobiocin-binding proteins

Relatively large amounts of purified novobiocin-binding proteins were required to immunise rabbits. The necessary quantities of proteins were obtained from large preparative protein gels (see Fig. 3.3). It is evident from Fig. 3.3 that the 5M urea wash applied to the preparative gel displays a higher background of proteins than the 5M urea fraction from a previous affinity purification analysed on a non-preparative gel (Fig. 3.2). This background was probably due to non-specific binding of proteins to the novobiocin column, as a consequence of overloading the column with crude cell extract in order to obtain the maximum amount of protein possible. Bands containing the 200kDa, 35kDa and 20kDa proteins were excised from the preparative gels and prepared, as described in chapter 2, for immunising rabbits. If required, the 52kDa protein (F_1 ATP synthetase β–subunit) could be specifically eluted from the column in buffer containing 15mM ATP and 5mM Mg^{2+}. Elution of the F_1 ATP synthetase β–subunit was often carried out to allow the overloading of polyacrylamide gels so that sufficient amounts of the 200kDa protein could be obtained either for injection or amino acid sequencing.

Figure 3.2 SDS-PAGE of proteins eluted from a novobiocin-Sepharose column

Yeast proteins prepared from the *myo1* strain *Zn1d* were applied to the column and eluted with buffer containing KCl or urea. Proteins were separated by electrophoresis on 15% SDS polyacrylamide gels and stained with Coomassie blue. Lane 1, crude extract; Lane 2, 2M KCl; Lane 3, 5M urea.

The 35kDa protein was eluted in the 5M urea fraction along with a 52kDa protein (the β-subunit of the mitochondrial F_1 ATP synthetase), a 200kDa protein and a 20kDa protein.


Lane
M
1
2
3
200kDa protein
kDa
66
45
36
29
24
52kDa protein
35 kDa protein
20kDa protein

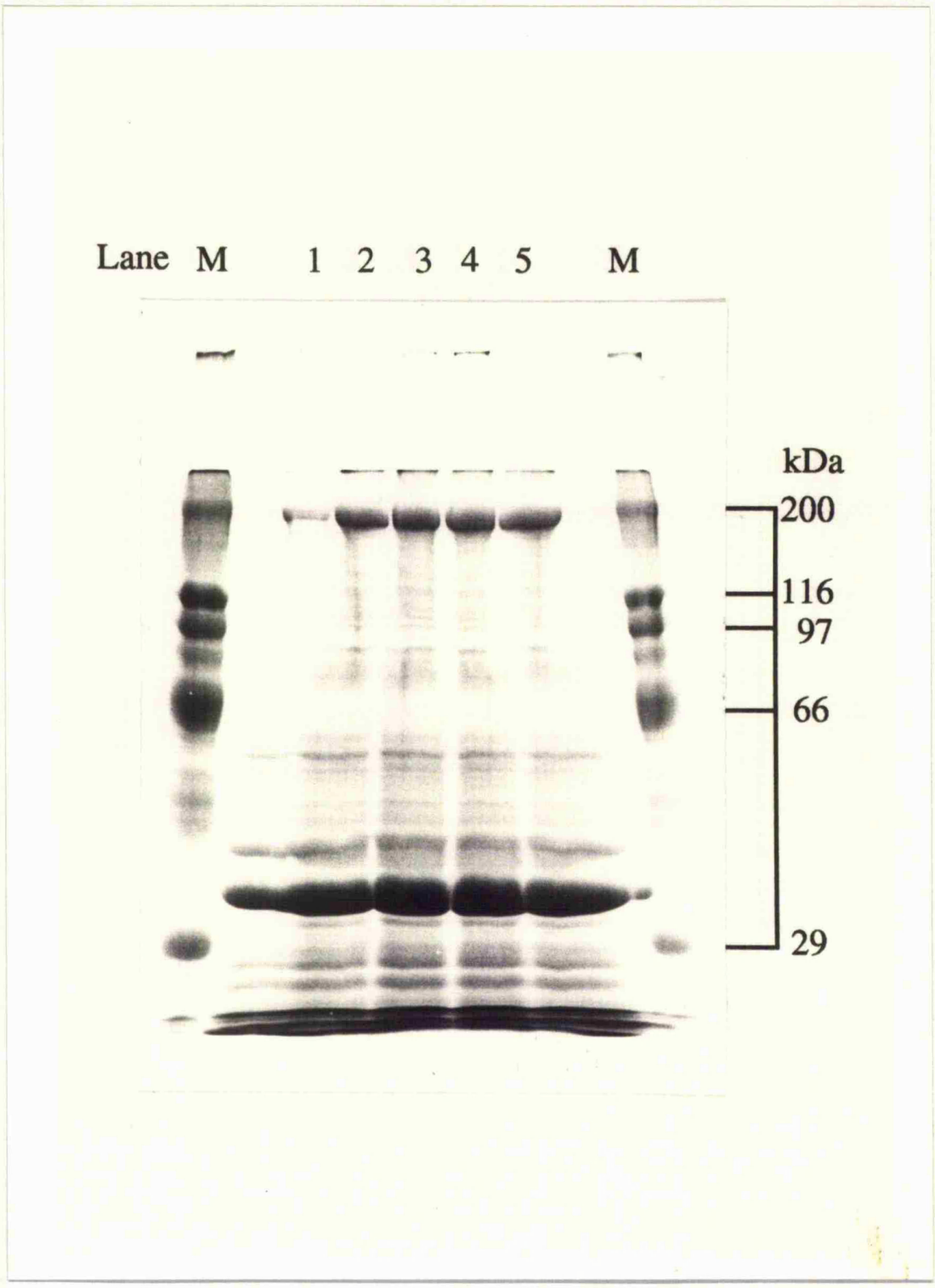


Figure 3.3 Preparative SDS-PAGE of novobiocin-binding proteins

Lanes 1-5, the proteins eluted from the novobiocin-Sepharose column by the 5M urea wash. Proteins were separated by electrophoresis on 10% SDS polyacrylamide gels and stained with Coomassie blue. Lanes M contain molecular mass markers (kDa). Note that because of the polyacrylamide concentration this gel does not show the 20kDa protein.

Rabbits were injected at four week intervals and a 5ml bleed was taken two weeks after each injection (see chapter 2). The antibody titre was estimated by Western blot analysis.

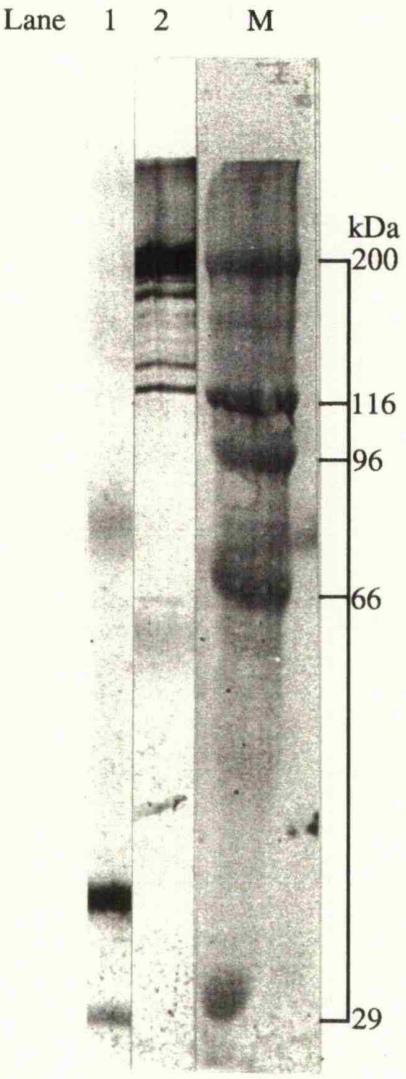
3.5 Western blot analysis of rabbit sera

Western blot analysis was carried out with sera from the immunised rabbits, as described in chapter 2. This analysis was carried out at regular intervals to determine if antibodies against a particular antigen were produced by the rabbits and, if so, to indicate their titre. Fig. 3.4 shows the Western blot results for the serum obtained from rabbits GEN 7J and GEN 7K which had been immunised with the 200kDa and 35kDa novobiocin-binding proteins, respectively. A 200kDa band can be clearly seen in lane 2, which has been incubated with serum from GEN 7J, indicating that anti-p200 antibodies have indeed been raised in this rabbit. A 35kDa band can be observed in lane 1 which was incubated with serum from GEN 7K which indicates that anti-p35 antibodies have been raised in this rabbit. It is clear that an additional band of approximately 29kDa is present in this lane. Initially, this band was thought to represent a break-down product of the 35kDa protein. Later, following the preparation of affinity purified p35 antibodies, this presumption was shown not to be the case. When the affinity purified p35 antibodies were used in Western blots, only the p35 protein was detected and not the lower band, indicating that antibodies from the crude serum recognise more than one protein. This result demonstrates that the 35kDa protein preparation used to raise the antibodies was contaminated with a low abundance 29kDa protein which either binds novobiocin with high affinity, or may be associated with the 35kDa protein.

Unfortunately, due to the limited time available for this study, antibodies against the 20kDa novobiocin-binding protein (Fig. 3.2) have not yet been raised.

Figure 3.4 Western blot of the 5M urea fraction using the serum from GEN 7J and GEN 7K.

Proteins from the strain *Zn1d* eluted from the novobiocin-Sepharose column in the 5M urea fraction were separated by electrophoresis on a 10% SDS polyacrylamide gel and the gel blotted onto nitrocellulose (see chapter 2). Blotted proteins were incubated with the GEN 7K serum (lane 1) and GEN 7J serum (lane 2), followed by goat anti-rabbit IgG and rabbit PAP, then developed (see chapter 2). In lane 1, a 35kDa protein band indicates the presence of anti-p35 antibodies in the serum. Note that, a 29kDa protein also cross-reacts with antibodies in the serum. In lane 2, a 200kDa protein band indicates the presence of anti-p200 antibodies in the serum. Lane M contains molecular mass markers stained with Ponceau S.


Lane 1 2 M
kDa
200
116
96
66
29

3.6 Immunofluorescence studies

Antibodies are an indispensable tool in localising antigens within the cell, thereby indicating their possible function(s) *in vivo*. Such localisation can be achieved through the use of indirect immunofluorescence microscopy. In this method, wild-type yeast cells are fixed in 3.7% (final concentration) formaldehyde, the cell walls removed by zymolyase digestion and the cells permeablised with 0.2% (v/v) Triton X-100 (see chapter 2). Prepared cells were incubated with primary affinity-purified antibodies, followed by an incubation with a fluorescent chromagen-linked secondary antibody (see chapter 2). The cells were mounted on a slide and observed using a Zeiss Axiophot photomicroscope.

Indirect immunofluorescence microscopy experiments using affinity purified anti-p35 antibodies detect very small spots throughout the cell, suggesting that the antigen is diffuse within the cell (data not shown). In contrast, affinity purified anti-p200 antibodies appear to recognise what may be filamentous structures within the cell (see Fig. 3.5). Using a double staining procedure, cells were stained with DAPI (which specifically binds to DNA) and the anti-p200 antibodies. The results obtained in this experiment (see Fig. 3.6) indicate that the structures recognised by the anti-p200 antibodies are not associated with the nucleus or mitochondria.

Since the anti-p200 antibodies recognise filamentous structures within the cell, it is possible that the antigen is a cytoskeletal or a cytoskeletal-associated protein. However, these structures were not stained with anti-tubulin IgG when the two antibodies were used together, indicating that the structure recognised by the anti-p200 antibodies is not tubulin. Similarly, double staining with anti-p200 antibodies and phalloidin, which binds specifically to actin filaments, ruled out an association of the p200 protein with the actin microfilaments. The suggestion that the p200 protein is a cytoskeletal or a cytoskeletal-associated protein was further supported by indirect immunofluorescence studies carried out on chick fibroblasts by Dr J.R. Jenkins (Fig. 6.7A). The anti-p200 antibodies recognise filamentous structures and the staining is remarkably similar to that obtained using anti-fibronectin antibodies (Fig. 6.7B).

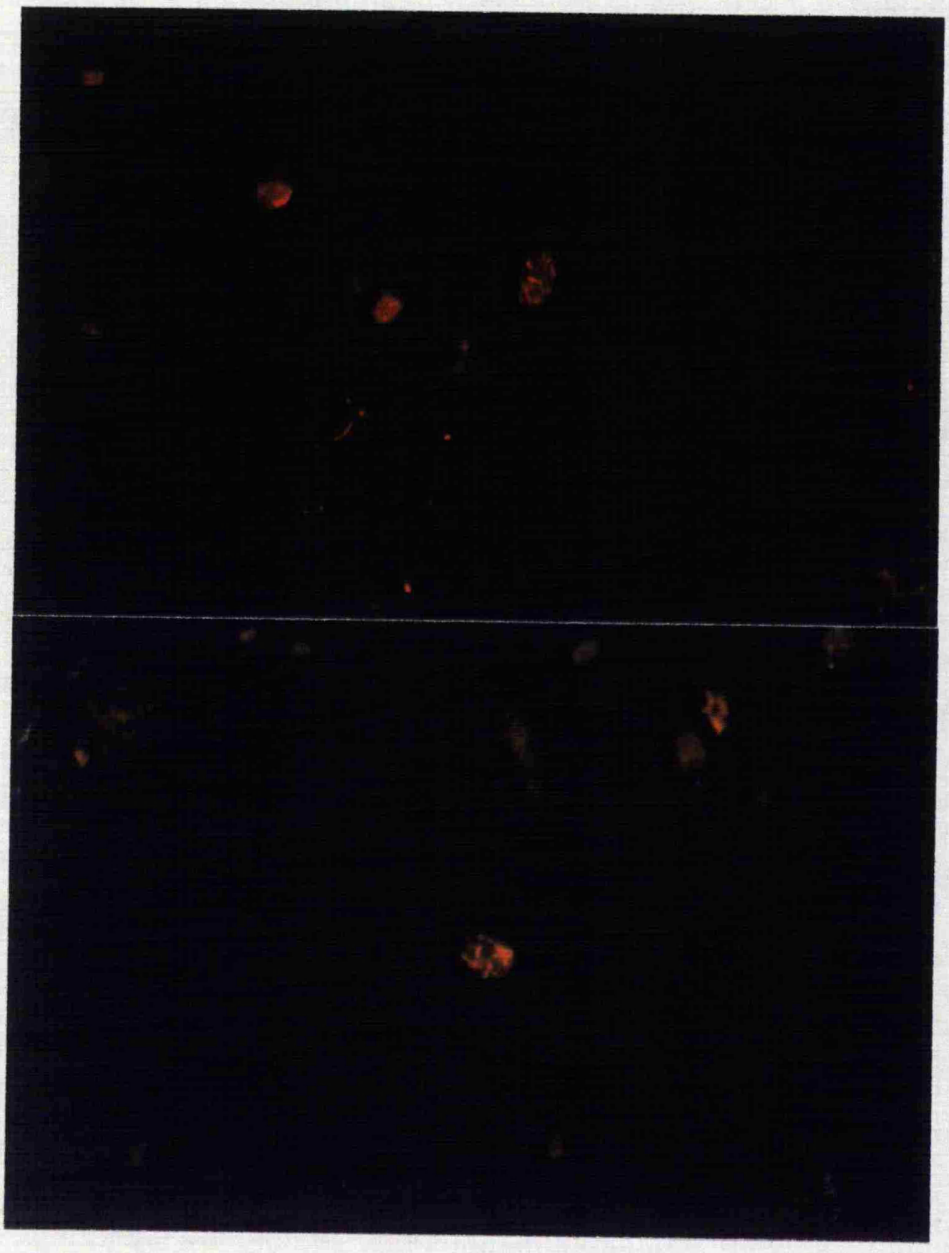


Figure 3.5 Indirect-immunofluorescence microscopy of *S. cerevisiae* using the anti-p200 antibodies.

Indirect-immunofluorescence microscopy of fixed *S. cerevisiae* cells following incubation with the affinity purified anti-p200 antibodies and rhodamine-linked goat anti-rabbit IgG. Cells were fixed and stained as described in chapter 2

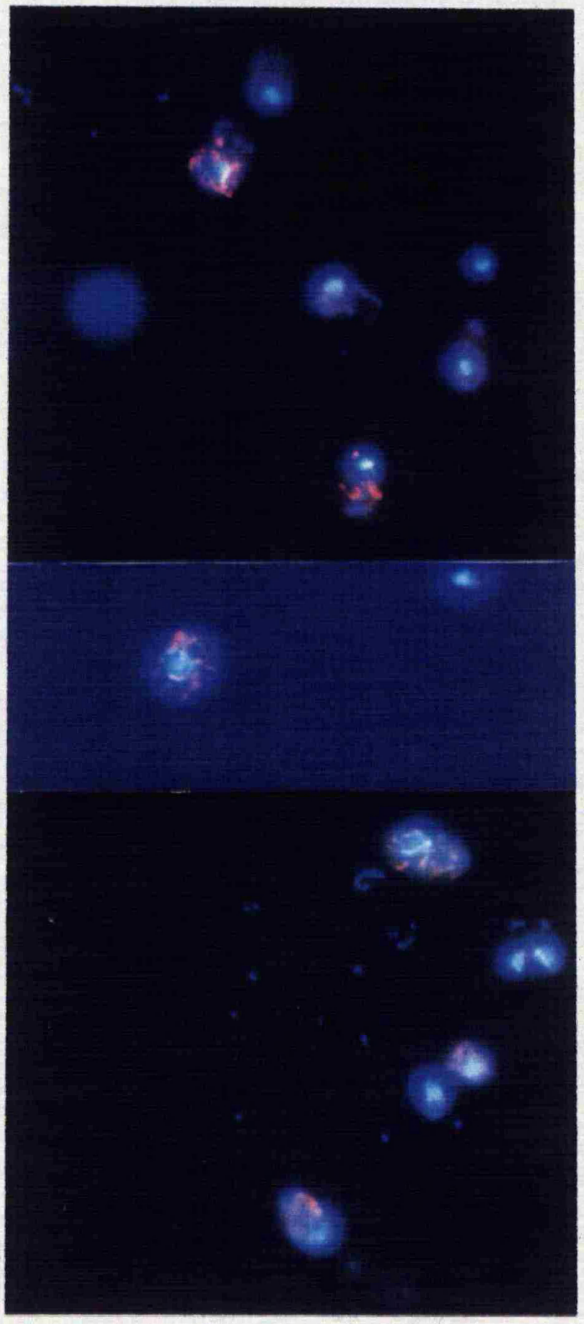


Figure 3.6 Indirect-immunofluorescence microscopy of *S. cerevisiae* using the anti-p200 antibodies and DAPI

Indirect-immunofluorescence microscopy of fixed *S. cerevisiae* cells following incubation with affinity purified anti-p200 antibodies followed by rhodamine-linked goat anti-rabbit IgG and DAPI. Cells were fixed and stained as described in chapter 2. Note that the DAPI (which stains DNA in the mitochondria and nucleus) and the anti-p200 antibodies do not co-localise, showing that the structure bound by the anti-p200 antibodies is not associated with the nucleus or mitochondria.

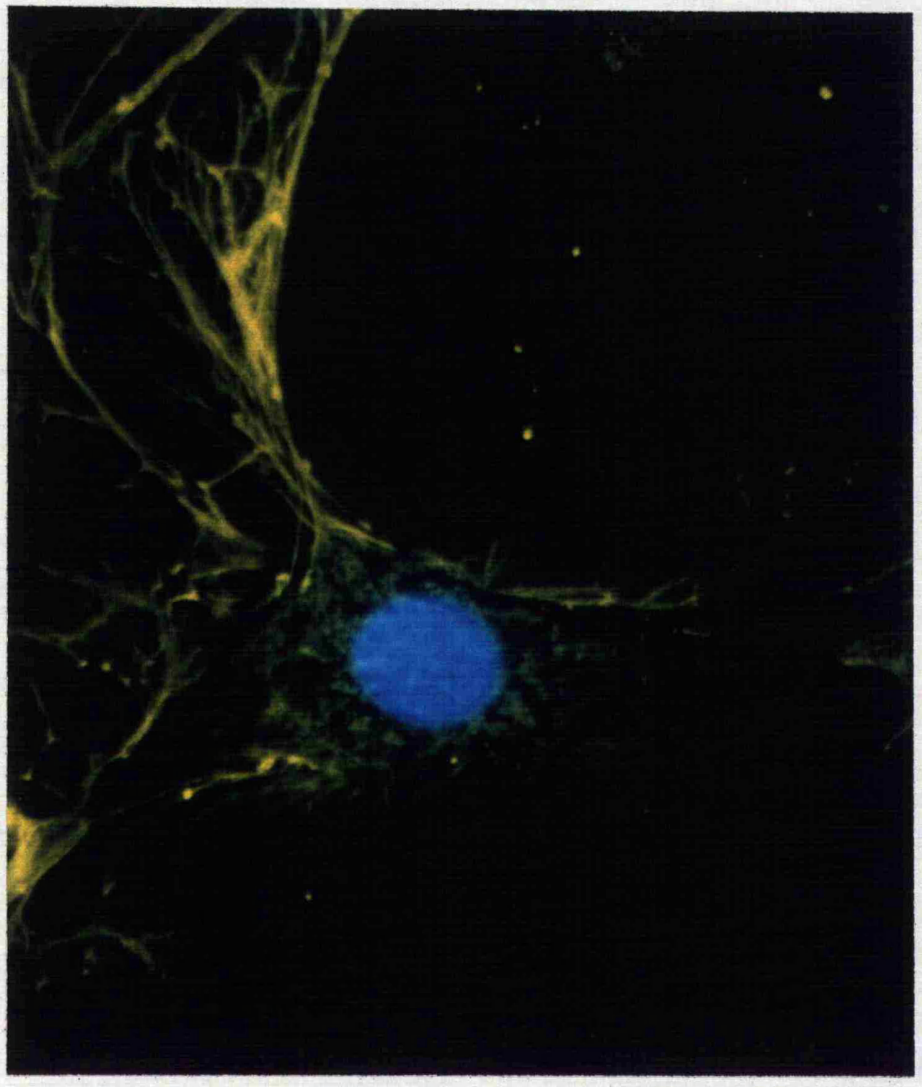


Figure 3.7A Indirect-immunofluorescence microscopy of chick fibroblasts using the anti-p200 antibodies and DAPI

Chick fibroblasts, grown on coverslips, were fixed in 3.8% formaldehyde at 37°C, washed 3 times in TBS, placed in acetone at -20°C and washed 3 times in TBS before blocking in TBS containing BSA for 3 hours. The fibroblasts cells were incubated with affinity purified anti-p200 antibodies followed by FITC-linked goat anti-rabbit IgG. Finally the cells were incubated with DAPI which stains DNA in the mitochondria and nucleus.

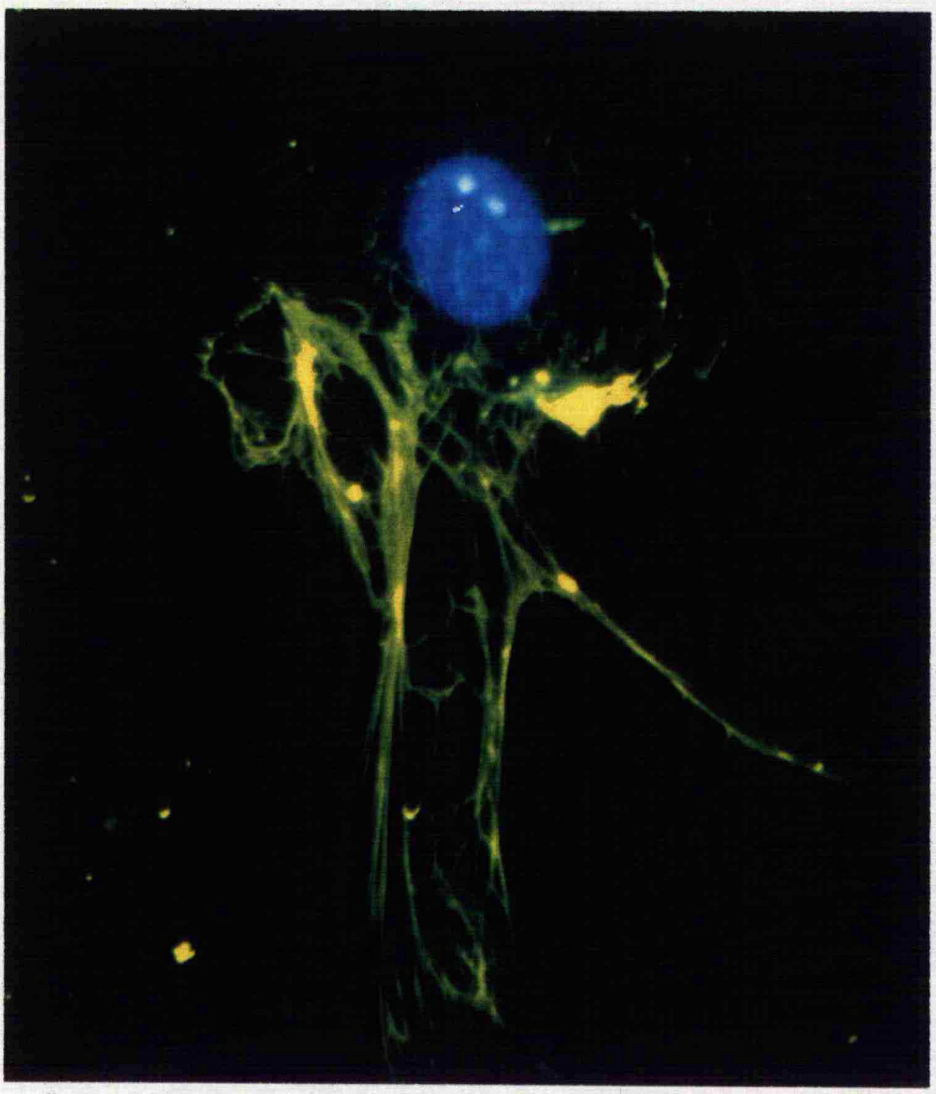


Figure 3.7B Indirect-immunofluorescence microscopy of chick fibroblasts using anti-fibronectin antibodies and DAPI

Chick fibroblasts, grown on coverslips, were fixed in 3.8% formaldehyde at 37°C, washed 3 times in TBS, placed in acetone at -20°C and washed 3 times in TBS before blocking in TBS containing BSA for 3 hours. The fibroblasts cells were incubated with with rabbit anti-human fibronectin antibodies followed by FITC-linked goat anti-rabbit IgG. Finally the cells were incubated with DAPI which stains DNA in the mitochondria and nucleus. (Fixing and staining of the cells was performed by Dr. J.R. Jenkins)

The 200kDa protein cannot be the yeast myosin heavy chain previously isolated on novobiocin-Sepharose (Watts *et al.*, 1985) as the 200kDa protein was isolated from *Zn1d* a strain which contains a truncated myosin gene (Watts *et al.*, 1987). Furthermore, the protein is not detected by antibodies raised against the yeast myosin heavy chain protein (S. Saville, personal communication).

3.7 Protein sequencing

A useful strategy to identify purified proteins is to obtain N-terminal amino acid sequence. This approach was successfully used to identify the 52kDa novobiocin-binding protein as the β-subunit of F_1 ATP synthetase and was consequently attempted for the 200kDa, 35kDa and 20kDa proteins.

3.7.1 N-terminal sequencing of the 200kDa protein

An initial attempt at sequencing the N-terminal of the 200kDa protein was made using the PVDF method (see chapter 2). Only two amino acid residues could be detected in this experiment. The difficulties in sequencing the 200kDa protein seem to be due to a general problem associated with the sequencing of high molecular mass proteins. Apparently, it is difficult to bind the required molar concentration of high molecular mass protein to PVDF membrane (Mathew Davidson, personal communication). Although this sequencing attempt was unsuccessful, it indicated that the protein is not blocked at the N-terminus. A protein is described as being blocked if N-terminal modification *e.g.*, myristoylation, prevents protein sequencing.

An alternative method to sequencing protein blotted on PVDF membrane makes use of a protein in free solution. In order to obtain a free solution of the 200kDa protein, the protein was electroeluted from an unstained polyacrylamide gel using a biotrap apparatus (chapter 2). Unfortunately, the electroelution was unsuccessful on two occasions, with no protein detected in the eluent by SDS-PAGE. When the gel slice was stained with Coomassie blue following the electroelution, the majority of the 200kDa protein was still present in the gel slice, indicating that the protein was not efficiently eluted

from the gel under the experimental conditions. The problem in eluting the 200kDa protein from polyacrylamide gels is probably due to its size because the 35kDa protein was successfully eluted from gels under the same conditions.

Following these attempts, another method of isolating the p200 was carried out. The concept behind this procedure was to reduce the volume of the 5M urea wash so that the protein could be isolated from the solution by size fractionation, using chromatography on a Superose 12 gel filtration column (Pharmacia LKB Biotechnology). This type of column allows protein separation according to molecular mass in the range 10-1000kDa. The 5M urea fraction, containing the novobiocin-binding proteins, was first dialysed against buffer B (25mM Hepes pH8.0; 1mM dithiothreitol; 1mM EDTA; 10% (v/v) ethylene glycol and 100mM KCl) at 4°C overnight to reduce the urea concentration in the solution. The sample was then dialysed against fresh buffer B without ethylene glycol; at this point protein in the dialysed sample precipitated. It was thought that the protein precipitated as the urea was required to keep the protein in solution. The procedure was repeated with 0.2M urea in the buffer (the maximum concentration of urea which is suitable for use in chromatography on a Superose 12 column). Under these conditions, the protein did not precipitate. However, reducing the sample volume by dialysis against buffer B containing 50% w/v PEG 20,000 and 0.2M urea, resulted in the precipitation of the high molecular mass proteins, leaving only proteins smaller than 43kDa in solution (see Fig. 3.8).

Due to time constraints, no further attempts were made to purify the protein. However, antibodies raised against the p200 were later successfully used in the isolation of the gene encoding the 200kDa protein (see chapter 4).

3.7.2 N-terminal sequencing of the 35kDa protein

A solution of the 35kDa protein for amino acid sequencing was produced by electoeluting the protein from an unstained polyacrylamide gel (Fig. 3.9). As is often the case, ambiguities occur at several amino acid residues in the sequence. The sequence does show a region (residues 11-15) which has limited degeneracy. This region is potentially

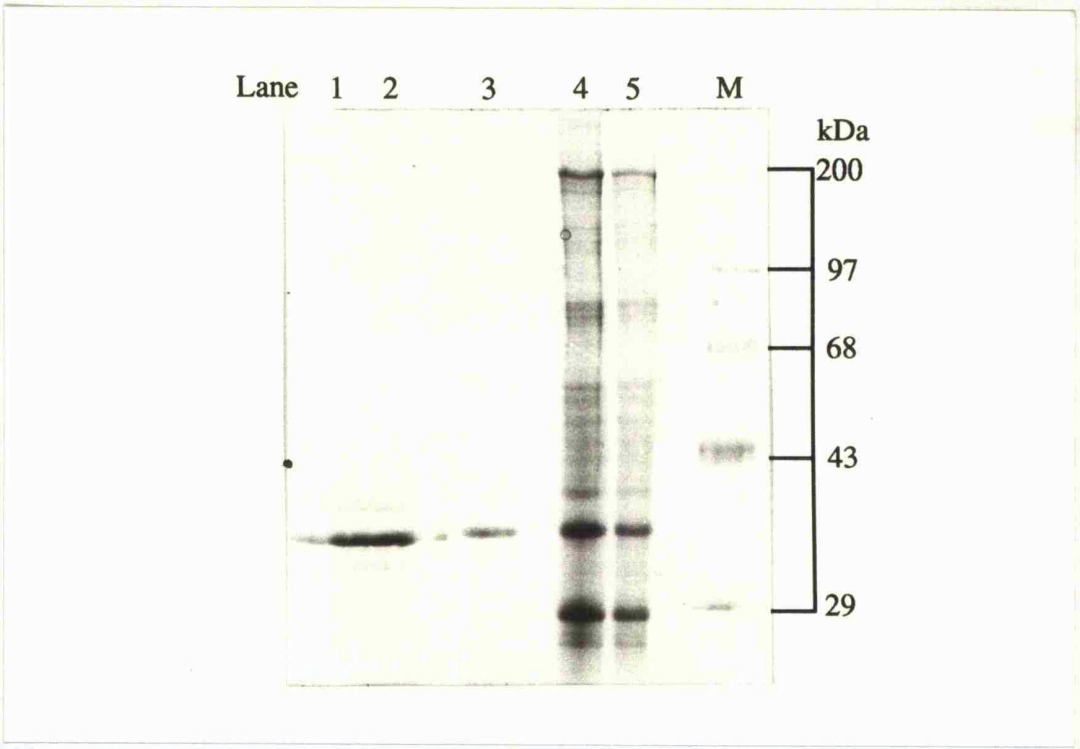


Figure 3.8 SDS-PAGE of proteins following PEG concentration

Proteins were separated by SDS-PAGE (10%) and stained with Coomassie blue. Lanes 4 and 5, protein which precipitated from solution following the concentration step in buffer B (25mM Hepes pH8.0; 1mM dithiothreitol; 1mM EDTA and 100mM KCl) containing PEG 20,000 and 0.2M urea. Lane 3, protein which remained in solution following the concentration step. Lanes 1 and 2, proteins which had remained in solution following the concentration step precipitated with 10% TCA. M, molecular weight markers (kDa).

amino acid No.	N-terminus	
1	Gly	
2	Gly	
3	Pro	
4	Gln	
5	Asp	
6	Pro	?
7	Gly	?
8	Asn	
9	Leu	
10	Thr	?
11	Ilu	
12	Asp	
13	Gln	
14	Gln	
15	Met	

Figure 3.9 N-terminal amino acid sequence of the 35kDa protein obtained by sequencing the protein in solution

The 35kDa protein was isolated and prepared for sequencing as described in chapter 2. Sequencing was carried out using an Applied Biosystems 470A gas-phase sequencer.

suitable for the design of degenerate oligonucleotides for the screening of yeast genomic libraries. Nevertheless, the sequence ambiguities meant that the data could not be relied upon for the design of degenerate oligonucleotides. In order to solve this problem, the N-terminal amino acid sequencing of the 35kDa protein was repeated using the PVDF method.

The 35kDa protein was blotted onto a PVDF filter (see Fig. 3.10) and sequenced. The N-terminal sequence data obtained using this protein sequencing method is shown in Fig. 3.11. The data obtained from this experiment resolved the ambiguities present in the initial sequencing data (see above). It also shows that the first two amino acids were not Gly, but Ser and Ala. This discrepancy is probably due to contamination of the protein solution used to obtain the first sequence with glycine present in the running buffer (see chapter 2). It would seem that a Sephadex G25 column used to remove the glycine from the running buffer during the sample preparation failed to remove all the glycine, resulting in incorrect identification of the first two amino acids.

The N-terminal amino acid sequence was used to screen the Swissprot protein data base but no significant match was found at the time. A region in the second amino acid sequence was identified as suitable for the preparation of a degenerate oligonucleotide probe which was to be used to clone the corresponding gene through the screening of a yeast genomic library (see Fig. 3.12 for the sequence of the oligonucleotide). Before using the degenerate oligonucleotide in library screening, it was decided to determine if the oligonucleotide would hybridise to yeast chromosomal DNA. The synthetic oligonucleotide was end-labelled with ^{32}P using T4 kinase and used in Southern blot analysis of yeast genomic DNA. Although the hybridisation and washes were carried out at low stringency, no hybridising fragment could be detected, indicating that the oligonucleotide failed to hybridise to the genomic DNA under the experimental conditions (data not shown). A positive control indicated that the oligonucleotide probe was efficiently labelled. The two most likely explanations for this failure to hybridise to genomic DNA are: *a*, the oligonucleotide did not match any yeast genomic sequence as it was produced from an incorrect peptide sequence; *b*, the degeneracy of the oligonucleotide was too high

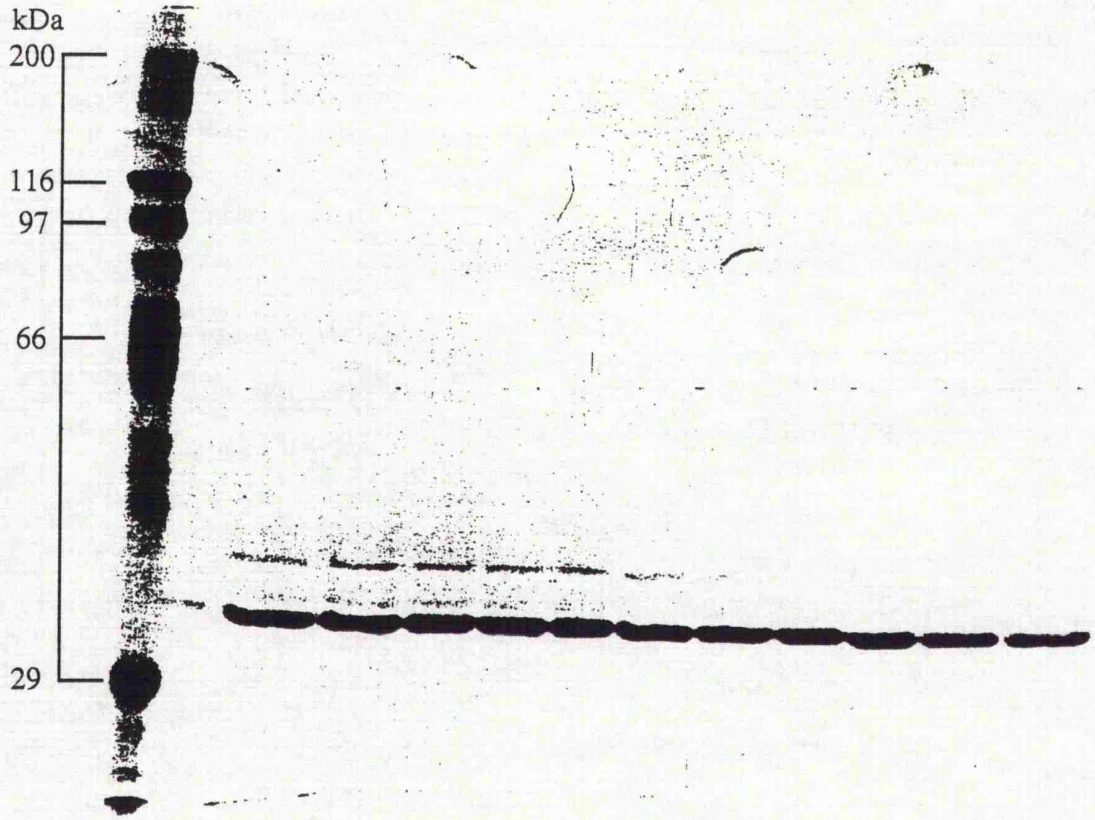


Figure 3.10 PVDF blot used to obtain N-terminal amino acid sequence of the 35kDa protein

Protein which had remained in solution following attempts to concentrate the 5M urea fraction from the novobiocin-Sepharose column (fig 3.7) was separated by SDS-PAGE (10%) and blotted onto the PVDF membrane at 250mA for 2 hours. Left, molecular mass markers (kDa). Right, 11 lanes with the 35kDa band predominant.

amino acid No.	N-terminus	Degeneracy	Possible amino acid codons
1	Ser	(6)	
2	Ala	(4)	
3	Pro	(4)	CCU CCC CCA CCG
4	Gln	(2)	CAA CAG
5	Asp	(2)	GAU GAC
6	Pro	(4)	CCU CCC CCA CCG
7	Val	(4)	GUU GUC GUA GUG
8	Asn	(2)	AAU AAC
9	Leu	(6)	
10	Pro	(4)	

Figure 3.11 N-terminal amino acid sequence of the 35kDa protein obtained using the PVDF membrane method

The 35kDa protein was isolated and prepared for sequencing as described in chapter 2. Sequencing was carried out using an Applied Biosystems 470A gas-phase sequencer. A possible site for the production of a degenerate probe is shown (amino acids 3-8), as well as the possible codons which code for this region.

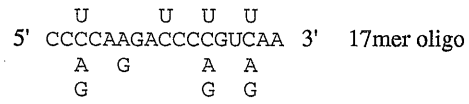


Figure 3.12 The 17mer degenerate oligonucleotide designed using N-terminal sequence data from the 35kDa protein

This oligonucleotide could be used to screen a yeast genomic library for the gene which codes for the 35kDa protein. The oligonucleotide displays a degeneracy of 256 *i.e.* the oligonucleotide solution contains 256 different sequence combinations.

to produce a detectable signal in the Southern hybridisation. Because of this result the oligonucleotide was not used in any further work.

3.7.3 N-terminal sequencing of the 20kDa protein

Sequencing the 20kDa protein *via* the PVDF method demonstrated that the protein is blocked at its N-terminus. In order to overcome this problem, the protein was isolated in solution and internally cleaved using cyanogen bromide (Gross, 1967). The polypeptides produced by this cleavage were separated by SDS-PAGE and blotted onto a PVDF filter. One of these polypeptides was sequenced (Fig. 3.13). Unfortunately, the sequence was of very poor quality with a number of ambiguities and was therefore of little use. The sequencing of cyanogen bromide cleavage products has not been repeated. Nevertheless, future sequencing of these polypeptides may yield interesting data.

3.8 Discussion

Three novel novobiocin-binding proteins of molecular mass 200kDa, 35kDa and 20kDa were isolated from yeast through affinity chromatography on novobiocin-Sepharose, a method described by Staudenbauer and Orr (1981). These proteins were isolated in addition to the three previously identified novobiocin-binding proteins: the yeast heavy chain myosin (Watts *et al.*, 1985), the *SUP45* gene product (Pocklington *et al.*, 1990b) and the β-subunit of the yeast mitochondrial F_1 ATP synthetase (Jenkins *et al.*, 1990).

The novel novobiocin-binding proteins were purified in order to identify them through N-terminal amino acid sequencing or through the cloning of their respective genes.

Antibodies were raised to the p200 and p35 proteins, making indirect immunofluorescence microscopy and the screening of yeast genomic expression libraries possible.

The anti-p200 antibodies identified novel structures in yeast when used in indirect immunofluorescence microscopy. These structures do not appear to be associated with the known yeast cytoskeletal networks, the nucleus or mitochondria. The anti-p200 antibodies

Amino acid No.	N-terminus
1	Ala
2	Ala
3	Leu
4	Trp/Val
5	?
6	Ala
7	Lys
8	Phe

Figure 3.13 N-terminal amino acid sequence obtained from a cyanogen bromide cleavage product of the 20kDa protein

The cyanogen bromide cleavage products were isolated and prepared for sequencing as described in chapter 2. Sequencing was carried out using an Applied Biosystems 470A gas-phase sequencer.

may be staining as yet unidentified organelles or components of bodies such as peroxisomes, which are small organelles recently discovered in yeast (Veenhius *et al.*, 1987; Veenhius and Harder, 1991).

Remarkably, the anti-p200 antibodies recognise an extracellular matrix component similar to fibronectin in indirect immunofluorescence microscopy of fibroblasts. In addition, the antibodies recognise human fibronectin in Western blots (Dr J.R. Jenkins, personal communication), suggesting that the p200 and fibronectin proteins share a common epitope(s). Despite these observations, DNA sequence data recently obtained for the p200 has shown that the two proteins do not display any homology (Dr. M. Murray, personal communication). However, both proteins are thought to be ubiquitinated and the common immunological recognition site could therefore be the ubiquitin portion of the proteins (Dr. M. Murray, personal communication).

In contrast to the anti-p200 antibodies, no distinct staining pattern was observed when anti-p35 antibodies were used in indirect immunofluorescence microscopy. The failure to obtain a distinct staining pattern may indicate that the antigen is diffuse within the cell or that the antigen is present at an extremely low level. Alternatively, the antibodies may not be suitable for immunofluorescence studies, or the antigen in the cell may be inaccessible to the antibodies.

The N-terminal sequence of the 35kDa novobiocin-binding protein was successfully determined and screening of the Swissprot protein data base indicated that the protein was novel. Although a degenerate oligonucleotide was produced using this protein sequence, it did not detect any yeast sequences in genomic Southern blot analysis; it was therefore, not used for genomic library screening. The p35 protein was sequenced for a third time later in this study and the results obtained are presented in chapter 7.

Difficulties were encountered in obtaining N-terminal sequence from the p200 and p20 proteins. In the case of the p200 protein, the problems were probably due to the large size of the protein, which causes inherent difficulties in protein sequencing. However, the limited sequence obtained indicated that the protein was not N-terminally blocked. The 20kDa protein was found to be blocked at its N-terminus, making sequencing of this

region impossible. Attempts to overcome this problem by sequencing peptides produced by cyanogen bromide cleavage yielded unreliable sequence data.

In conclusion, protein sequencing of the novobiocin-binding proteins being studied did not assist in their identification. However, antibodies raised against two of these proteins (p200 and p35) have been used to clone the corresponding genes, as described in chapter 4.

Chapter 4

Cloning the p35 gene

4.1 Introduction

As described in the previous chapter, N-terminal amino acid sequence data was of little use in the attempts to clone the genes encoding the novobiocin-binding proteins. However, antibodies to both the 200kDa and the 35kDa novobiocin-binding proteins have been raised successfully (see chapter 3). Antibodies have been widely used as immunological probes to screen expression libraries (Young and Davis, 1983a; Young and Davis, 1983b; Stanley and Luzio, 1984). This chapter describes the use of the anti-p200 and anti-p35 antibodies in screening yeast expression libraries and the cloning of the p35 gene. Yeast genomic DNA can be used to construct expression libraries because, unlike higher eukaryotes, yeast has a relatively small genome which contains comparatively few introns. The use of genomic DNA in the construction of expression libraries avoids the technical complications of constructing fully representative cDNA libraries.

Screening of expression libraries provides a means to isolate specific recombinant DNA sequences by virtue of the antigenicity of the expressed gene product. Thus, expression library screening relies on the ability to express foreign polypeptides from recombinant DNA in *E. coli*. There are three major problems associated with obtaining expression of foreign DNA as a stable polypeptide. The first is that most foreign DNA does not contain promoter sequences required for expression in *E. coli*. Therefore, a foreign gene must be placed under the control of an *E. coli* promoter that is efficiently recognised by *E. coli* RNA polymerase.

The second problem is that foreign polypeptides are often rapidly degraded in *E. coli*. The severity of this problem differs from antigen to antigen; some foreign proteins are very stable, whereas others are highly unstable. The instability of foreign proteins can be reduced, in many cases, by fusing the antigen to a stable host protein such as the N-

terminus of β-galactosidase and by using host mutants defective in proteolysis; for example, *lon* mutants of *E. coli* which are defective in one of the ATP-dependent proteases responsible for the destruction of abnormal proteins (Young and Davis, 1983a).

The third major problem associated with expression of foreign proteins in *E. coli* is that the presence of these unusual proteins can often be harmful or even lethal to the cell. The use of vectors which allow only transient expression of the foreign protein often solves this problem. Thus, the expression of the DNA encoding the foreign protein is repressed during early log-phase growth of the host. Toward the end of this period, while the transcription and translation apparatus is still fully active, the expression of the foreign protein can be induced so that satisfactory levels of potentially toxic proteins are produced before cells become inviable.

These concepts, designed to maximise the levels to which foreign proteins accumulate in *E. coli*, have been incorporated into a number of vector/host systems such as the λ*gt*11 (Young and Davis, 1983a) or pEX expression systems (Stanley and Luzio, 1984).

4.2 The choice of expression vector

A number of expression vectors are now available for the production of expression libraries. In this study, the expression vector chosen was the pEX1-3 family of plasmids (Fig. 4.1) which were originally developed for the construction of cDNA libraries (Stanley and Luzio, 1984). These vectors contain a *cro-LacZ* gene fusion which is under the control of the P_R promoter of bacteriophage λ. A polylinker has been engineered into the 3' end of the *LacZ* gene in all three translational reading frames and stop signals for transcription and translation inserted, so that any open reading frame in a DNA fragment cloned at the polylinker may be expressed as a hybrid cro-β-galactosidase protein. The pEX vectors are used in strains containing the cI_{857} temperature sensitive repressor of the bacteriophage λ. Thus, recombinant plasmids can be amplified at 30°C and transient expression is induced by shifting the temperature to 42°C.

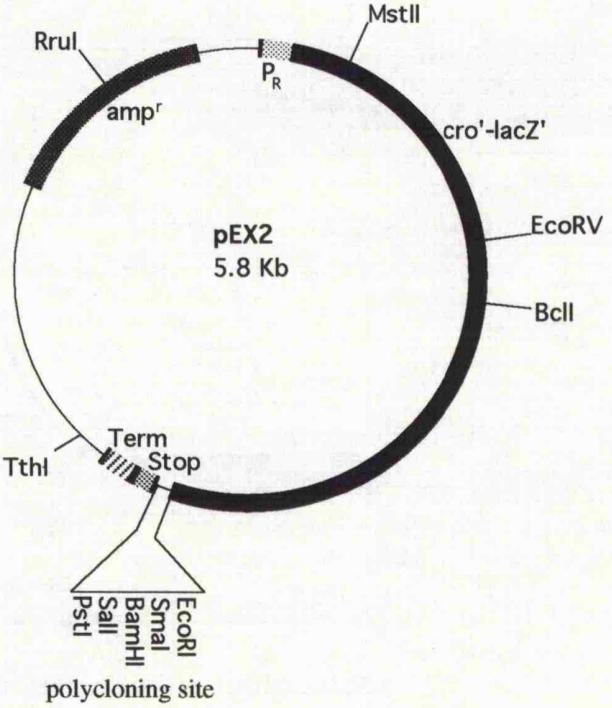


Figure 4.1 Map of pEX2

A polycloning site is present at the 3' terminus of the *LacZ* gene and is followed by translation stop codons (Stop) and a transcription terminator (Term) from the bacteriophage Tfd (after Stanley and Luzio, 1984).

The maps of pEX1 and pEX3 are essentially the same as that of pEX2 (shown above) except that, in altering the reading frame position of the cloning sites, the *Eco*RI site has been deleted in both cases.

4.3 Construction of yeast expression libraries

The construction of yeast expression libraries and the screening procedure is described in detail in chapter 2 and outlined below.

In order to generate genomic DNA fragments of random size, genomic DNA from a ρ^o 483/1a strain (Table 2.1) was partially digested with the restriction endonuclease *Sau*3AI. Previous experiments had shown that all the novobiocin-binding proteins are present in ρ^o strains (Dr. J.R. Jenkins, personal communication). Therefore, a ρ^o strain was used so that mitochondrial DNA sequences would not be represented in the library. This reduces the number of clones which need to be screened to represent the whole yeast genome. A number of partial digests were carried out and the best results were obtained through the digestion of 20μg of DNA with 0.1 units of *Sau*3AI at 37°C for one hour.

The partially digested DNA was ligated, in three separate reactions, with each of the three pEX vectors which had been digested with *Bam*HI and dephosphorylated. Each ligated vector was transformed separately, by electroporation, into *E. coli* RR1 λcI_{857} (Table 2.1) and transformants selected on ampicillin plates. The highest transformation frequency was obtained using ligation mixtures containing a 2:1 ratio of genomic DNA fragments to vector DNA. The electroporation method of transformation was used in preference to chemical transformation because the transformation efficiency of pEX using the chemical method was relatively poor.

The average pEX insert size was found to be approximately 1kb; this value was determined by analysing the insert sizes of several randomly picked transformants. The size of the yeast genome is approximately 16Mb. Therefore, to represent the whole yeast genome required the screening of at least 1.6×10^4 colonies for each pEX vector.

4.4 Screening of yeast expression libraries

The procedure for screening yeast expression libraries is summarised below (see chapter 2 for a detailed description).

Transformants from each of the three pEX vectors were plated out separately onto ampicillin plates, to give two to three thousand colonies per plate. After approximately 16 hours incubation at 30°C, colonies were lifted onto nitrocellulose filters which were placed onto new ampicillin plates for a further 4 hours at 30°C. The expression of the hybrid β-galactosidase protein was induced by a temperature shift to 42°C for 2 hours. The nitrocellulose filters were removed, the colonies lysed with 0.1% (w/v) SDS and treated with chloroform. The filters were then ready for use in immuno-screening.

Either the anti-p200 or the anti-p35 antibodies were used as the primary antibodies. These antibodies bind to colonies which produce a p200 β-galactosidase hybrid protein or a p35 β-galactosidase hybrid protein. Primary antibodies were detected using secondary (goat anti-rabbit IgG) and tertiary antibodies (rabbit PAP IgG). The final complexes were visualised by a colour reaction described in chapter 2.

Positive colonies were obtained with both the anti-p200 and the anti-p35 antibodies. Only the clones isolated by screening with the anti-p35 antibodies will be described further because the characterisation of positive clones detected by the anti-p200 antibodies has been carried out in our laboratory by Dr J.R. Jenkins and Dr. M. Murray.

Six colonies were detected using the anti-p35 antibodies and each was isolated as a single colony following the primary screen. These colonies were rescreened by Western blot analysis after induction of the pEX plasmids to determine if the β-galactosidase fusion proteins were in fact recognised by the anti-p35 antibodies. Of the six initial positives, only four expressed fusion proteins which were recognised in Western blot analysis; clones 1e1 and 1e2 produced a good response, whereas clones 3f1 and 3f3 produced a very weak response (data not shown). The insert sizes of these four positives were determined from the isolated plasmids. Plasmid DNA was digested with the restriction endonucleases *Sma*I and *Pst*I which cut at sites flanking the cloning site and the resulting fragments were separated by electrophoresis on a 2% (w/v) agarose gel in TBE buffer (see chapter 2). Clones 1e1 and 1e2 were found to have inserts of 1.5kb and 240bp, respectively, whereas the inserts of clones 3f1 and 3f3 were less than 100bp in length.

As mentioned earlier, the rabbit serum containing anti-p35 antibodies also recognised a protein of 29kDa in Western blot analysis. Therefore, before further work could be carried out, it was necessary to identify clones which expressed part of the p35 gene rather than part of the p29 gene. In order to achieve this, the 35kDa protein was used to affinity purify anti-p35 antibodies from the GEN7K rabbit serum, using the method described in chapter 2. The affinity purified antibodies were subsequently used in Western blot analysis of clones 1e1 and 1e2 (Fig. 4.2). Clones 3f1 and 3f3 were abandoned as their small inserts would have made their further manipulation difficult even if they proved to be positive.

The results of the Western blot analysis confirmed that the GEN7K serum detects both the 35kDa and 29kDa proteins in the 5M urea fraction eluted from novobiocin-Sepharose. However, when the affinity purified anti-p35 antibodies were used on the same fraction, only the 35kDa protein was detected, indicating that the affinity purification process was successful. The analysis also shows that β-galactosidase fusion proteins from both clones 1e1 and 1e2 are detected by the GEN7K serum whereas only the fusion protein produced by clone 1e2 was recognised by the affinity purified anti-p35 antibodies. This result indicated that clone 1e2 contained part of the 35kDa gene whereas clone 1e1 did not. Presumably, the fusion protein in the latter was recognised by anti-p29 antibodies, present in the crude serum.

4.5 Subcloning of the pEX clone 1e2

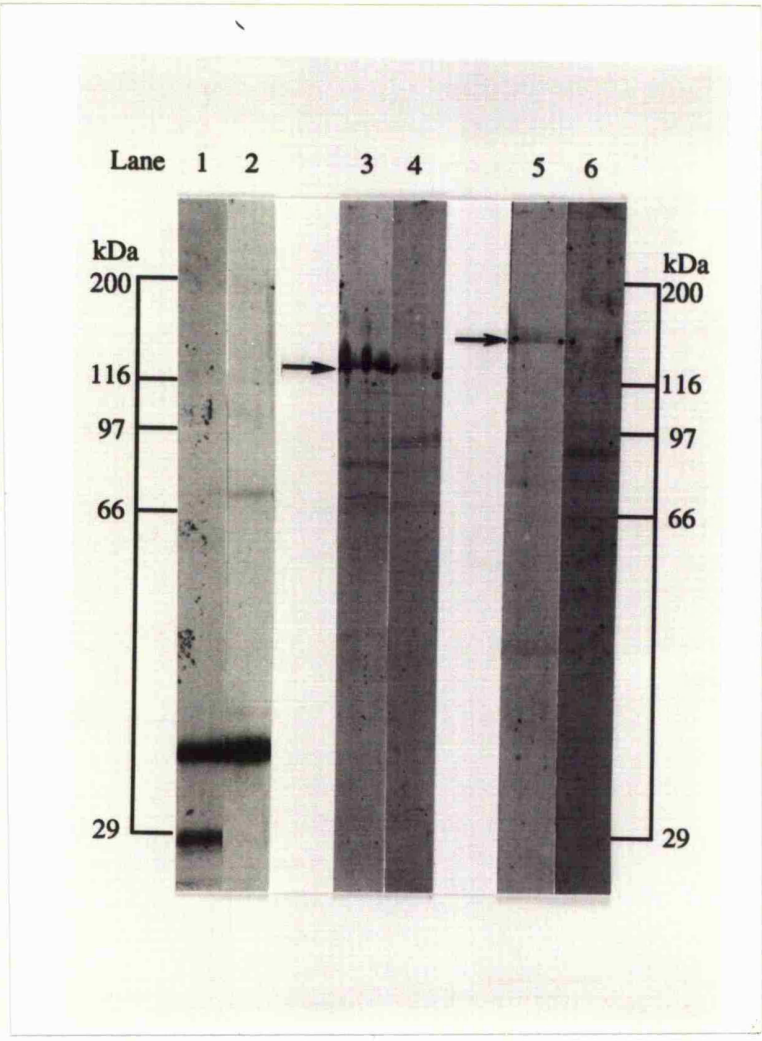
The insert in the pEX clone, 1e2, which encodes part of the fusion protein detected by the affinity purified anti-p35 antibodies, was subcloned into the pUC19 vector (Table 2.2) for subsequent DNA sequence analysis.

The pUC19 vector contains the β-lactamase gene (ampicillin resistance, Ap) for selection and *LacZα* gene fragment for the detection of recombinant plasmids in *E. coli*. This vector also contains multiple cloning sites at the polylinker, located downstream of the *LacZ* promoter. When pUC19 is transformed into the *E. coli* strain DH5α (Table 2.1), which lacks the *LacZα* gene fragment, in the presence of both the inducer IPTG and the

Figure 4.2 Immunoblot analysis of positive pEX clones

Antibodies were raised against the 35kDa protein and used to screen yeast genomic expression libraries constructed in pEX plasmids. Anti-p35 antibodies were affinity purified using the antigen immobilised to a solid phase (nitrocellulose). Western blot analysis of positive clones showed that the *lacZ*-fusion product of one clone was detected by both the serum and affinity purified antibodies.

Lane 1, yeast proteins eluted in the 5M urea fraction from the novobiocin-Sepharose column, probed with the anti-p35 serum.
Lane 2, yeast proteins eluted in the 5M urea fraction from the novobiocin-Sepharose column, probed with affinity purified anti-p35 antibodies.
Lane 3, crude *E. coli* extract containing the pEX positive, 1e2, probed with the anti-p35 serum.
Lane 4, crude *E. coli* extract containing the pEX positive, 1e2, probed with affinity purified anti-p35 antibodies.
Lane 5, crude *E. coli* extract containing the pEX positive, 1e1, probed with the anti-p35 serum.
Lane 6, crude *E. coli* extract containing the pEX positive, 1e1, probed with affinity purified 35kDa antibodies.


Lane 1 2 3 4 5 6
kDa
200
116
97
66
29
kDa
200
116
97
66
29

chromophore X-Gal, plasmid-containing colonies become blue instead of their normal white colour. Successful insertion of a DNA fragment into the cloning sites of the polylinker disrupts the *LacZα* gene fragment. Consequently X-Gal is not cleaved and the *E. coli* colonies remain white. The change in colour can therefore be used to identify recombinant plasmids.

The pEX clone, 1e2, was digested with the restriction endonucleases *Sma*I and *Pst*I. The resulting fragments were separated on a 2% (w/v) agarose gel run in TBE buffer. The lower band, corresponding to the 240bp *Sma*I-*Pst*I fragment, was recovered from the gel by electroelution onto dialysis membrane as described in chapter 2. The fragment was ligated into pUC19 plasmid double-digested with *Sma*I and *Pst*I. The ligated plasmids were transformed into competent *E. coli* DH5α cells and plated on nutrient agar containing ampicillin. White colonies were randomly picked and restriction analysis carried out on their plasmid DNA to confirm construction of the correct plasmids. The correct construct in the pUC19 vector contained the *Sma*I-*Pst*I insert and was named pNov35 (Fig. 4.3).

4.6 Sequencing of the pNov35 insert

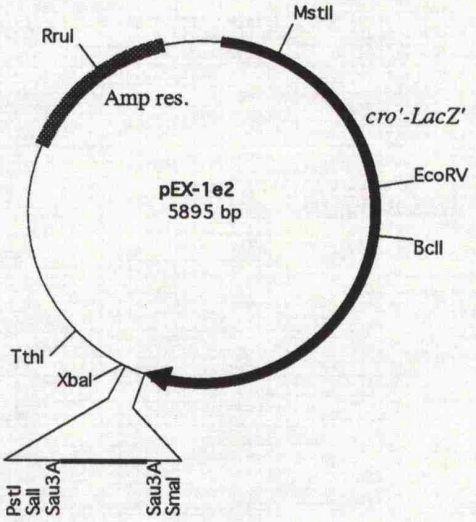
Sequencing of the pNov35 insert was carried out using the double-stranded dideoxy chain termination sequencing method, the protocol of which has been described in chapter 2. Because the insert was so small, both strands of the insert DNA could be sequenced in their entirety in two sequencing reactions using the "forward" and "reverse" universal M13 primers. The insert was found to be 240bp in length and the sequence of the insert is shown in Fig 4.4. Using the Genetics Computer Group (GCG) computer programs, the insert sequence was screened against the EMBL data base, but no obvious match was detected. When the sequence was analysed for open reading frames (ORF), one of the open reading frames was found to run through the whole insert. The predicted length of this ORF was 80 amino acid residues and the direction of the ORF was consistent with the production of a β-galactosidase fusion protein (see Fig. 4.4). The predicted 80 amino acid peptide encoded by this ORF was used to screen the Swissprot

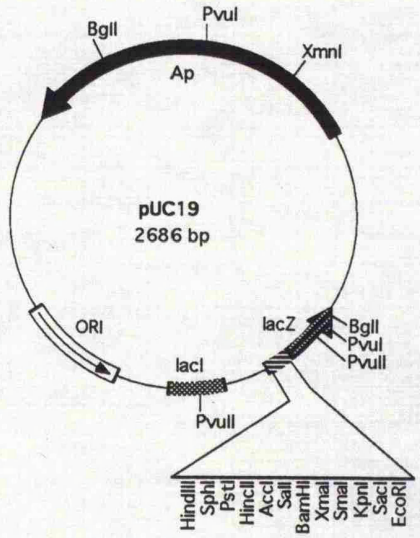
Figure 4.3 Subcloning the *Sma*I-*Pst*I fragment from pEX clone 1e2 into the pUC19 vector

The plasmid pNBP-Eco was simultaneously digested with the restriction endonucleases *Sma*I and *Pst*I. The resulting fragments were separated by electrophoresis on a 2% agarose gel run in TBE buffer. The 240bp *Sma*I-*Pst*I band was recovered from the gel by electro-elution onto dialysis membrane as described in chapter 2.

The fragment was ligated into pUC19 digested with *Sma*I and *Pst*I. Ligated plasmids were transformed into competent *E. coli* DH5α cells. The cell suspension was plated onto LB plates containing 100μg ml^{-1} ampicillin, 25μg ml^{-1} IPTG and 40μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies and incubated overnight at 37°C. Transformants were identified as white colonies against a background of blue colonies.

White colonies were randomly picked and examined for recombinant plasmids. The plasmid pUC19 containing the 240bp *Sma*I-*Pst*I insert was named pNov35.

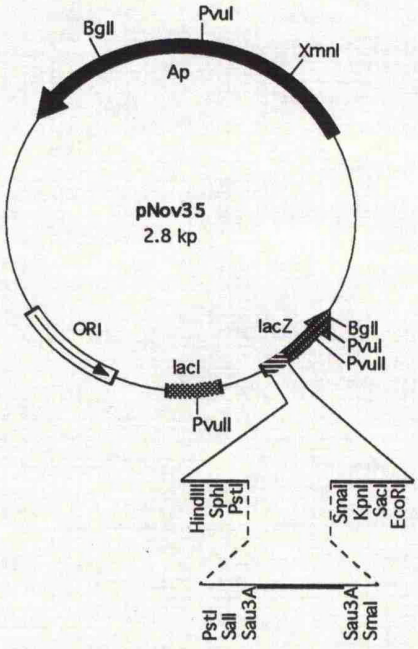




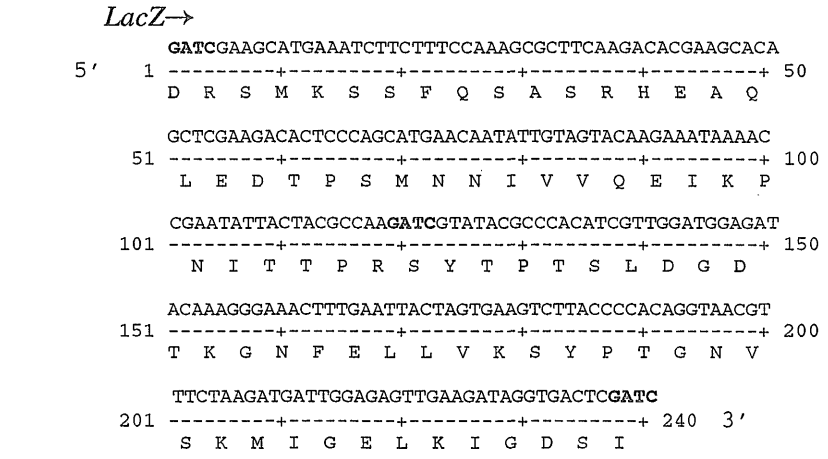
Digested with *SmaI* and *PstI*

Digested with *SmaI* and *PstI*

The pEX plasmid containing the 1e2 fragment was digested with *Sma*I and *Pst*I.
The small *Sal*I-*Hin*dIII fragment produced was ligated into thepUC19 vector.



A



B

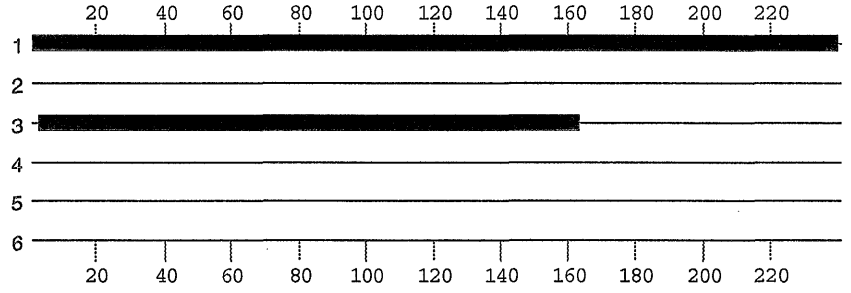


Figure 4.4 Sequence and open reading frame analysis of the pNov35 insert sequence

A Complete sequence of the pNov35 insert with *Sau*3AI sites shown in bold and the predicted amino acid sequence from frame 1. In pEX the 5' end of this fragment has been ligated to the *LacZ* gene and transcription of the lacZ fusion occurs in the 5' to 3' direction as indicated by the arrow.

B The above open reading frame analysis shows ORFs (which are larger than 25 amino acids in length) in all six potential frames. An 80 amino acid long ORF in frame 1 can be seen to run through the whole DNA sequence. The predicted amino acid sequence of this ORF shown is shown in part A above.

data base, but again no obvious match was detected. These results were consistent with the pNov35 insert being part of a novel *Saccharomyces cerevisiae* gene.

4.7 Genomic library screening

To obtain the full length gene which encodes the 35kDa protein, a yeast genomic library containing large inserts was screened, using the pNov35 fragment (Fig. 4.3) as a probe. This genomic library, already available in the laboratory, was constructed in the λ Phage NM728 (see Table 2.2) and contained *Hin*dIII partial-digested chromosomal DNA fragments from *S. cerevisiae* strain 483/1a (Table 2.1).

The NM728 library was screened by the plaque hybridisation method described in chapter 2. Briefly, the library was plated out to give approximately 3000-4000 plaques per plate. Plaques were lifted onto nitrocellulose filters, their DNA denatured and the filters baked at 80°C for 2 hours. Hybridisation was carried out in 0.5M Na_3HPO_4 at 65°C overnight, filters were washed with 40mM Na_3HPO_4 at 65°C and autoradiographed. Five positive plaques were isolated and purified through several rounds of screening. Phage DNA was isolated from the positive plaques as described in chapter 2 and digested with the restriction endonuclease *Hin*dIII. Four of the five positive plaques were found to have 4kb inserts, whereas the fifth positive was found to contain a 7kb insert. This result was somewhat puzzling because sequence data indicated that the pNov35 insert contained no *Hin*dIII site and so should only hybridise to one genomic *Hin*dIII fragment.

4.8 Genomic Southern blot analysis

It was a formal possibility that one of the two inserts isolated from the NM728 genomic library (described above) was a DNA contaminant introduced to the library during its preparation. To determine if both the 4kb and 7kb *Hin*dIII inserts isolated from the library were present in yeast genomic DNA, Southern blot analysis was carried out.

Yeast genomic DNA was digested with the restriction endonuclease *Hin*dIII, fragments were separated by agarose gel electrophoresis and the DNA blotted onto

Hybond-N™. As in the genomic library screening, the pNov35 insert was used as the probe. The probe hybridised to both 4kb and 7kb *Hin*dIII genomic fragments (Fig. 4.5). This indicated that both the 4kb and 7kb *Hin*dIII inserts, obtained in the initial genomic screening, were yeast sequences as both hybridise to the pNov35 insert. Since no *Hin*dIII site is present in the pNov35 it was unlikely that these inserts were two adjacent fragments of genomic DNA. However, it was possible that the 4kb and 7kb inserts contained genes with regions of high homology or even duplicate copies of the p35 gene.

4.9 Subcloning the *Hin*dIII genomic fragments into pUC19

The 4kb and 7kb *Hin*dIII inserts from the two positive λ phage were subcloned from the NM728 phage into the pUC19 vector. Phage DNA from the positives was digested with the restriction endonuclease *Hin*dIII and the fragments produced were separated on a 0.6% (w/v) LGT agarose gel. The bands corresponding to the 4kb and the 7kb *Hin*dIII inserts were excised and the DNA recovered from the gel by electroelution as described in chapter 2.

The fragments were ligated into *Hin*dIII-digested pUC19 plasmid and the ligated plasmids were transformed into competent *E. coli* DH5α cells. White colonies were randomly picked and restriction analysis carried out on their plasmid DNA to confirm the construction of the correct plasmids. The correct constructs in the pUC19 vector contained the 4kb and 7kb *Hin*dIII fragments as inserts and were named pNBP4 and pNBP7, respectively (Fig. 4.6).

The pNBP4 and pNBP7 inserts were mapped using a number of restriction endonucleases. Differences between the restriction maps of these plasmids (Fig. 4.7) indicated that the two fragments were not generated as the result of the loss of a *Hin*dIII site.

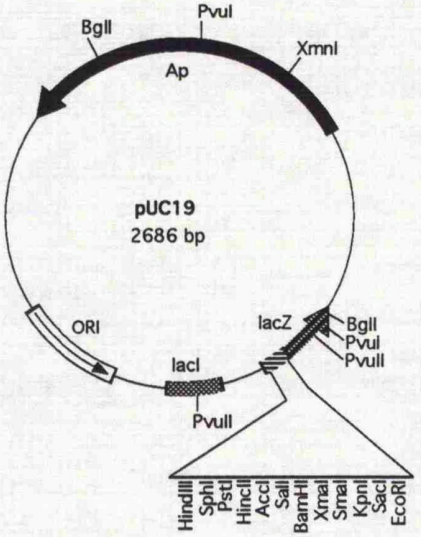
Figure 4.5 Southern blot analysis of *Hin*dIII digested yeast genomic DNA probed with the pNov35 insert

Total yeast genomic DNA was digested with the restriction endonuclease *Hin*dIII. Fragments were separated on a 1% agarose gel and blotted onto Hybond-N™. Hybridisation and washes were carried out at high stringency (see chapter 2). The probe, pNov35 insert, hybridised to two bands, a 4kb and a 7kb genomic fragment.

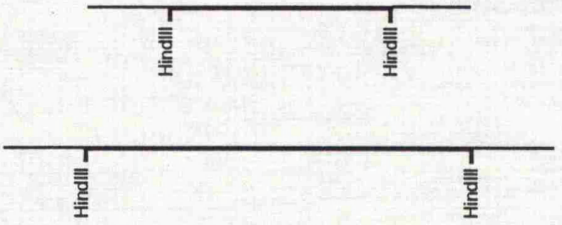
Figure 4.6 Subcloning the yeast genomic *Hin*dIII fragments from the positive λ phage into the pUC19 vector

Phage DNA was isolated from the positive plaques as described in chapter 2 and digested with the restriction endonuclease *Hin*dIII. The fragments were separated on a 0.6% LGT agarose gel. The 4kb and 7kb *Hin*dIII fragments were recovered from the gel as described in chapter 2.

The fragments were ligated separately into *Hin*dIII digested pUC19 plasmid and the ligated plasmids transformed into competent *E. coli* DH5α cells. The cell suspension was plated onto LB plates containing 100μg ml^{-1} ampicillin, 25μg ml^{-1} IPTG and 40μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies and incubated overnight at 37°C. Transformants were identified as white colonies against a background of blue colonies. White colonies were randomly picked and restriction analysis carried out to confirm the structure of recombinant plasmids. The correct constructs in the pUC19 vectors contained the 4kb or 7kb *Hin*dIII inserts and were named pNBP4 and pNBP7, respectively.

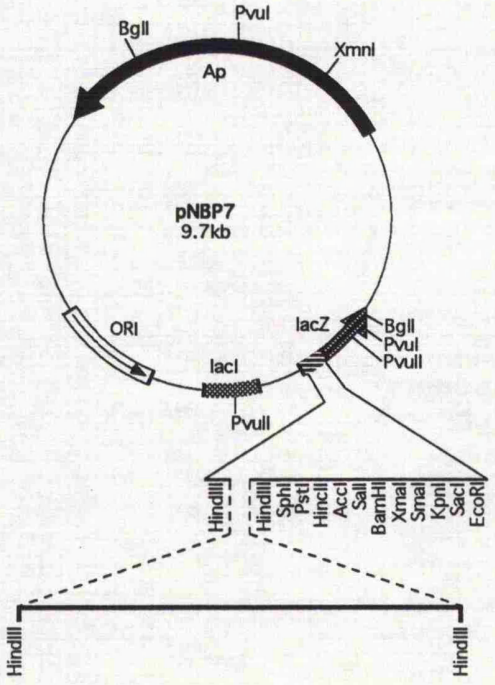


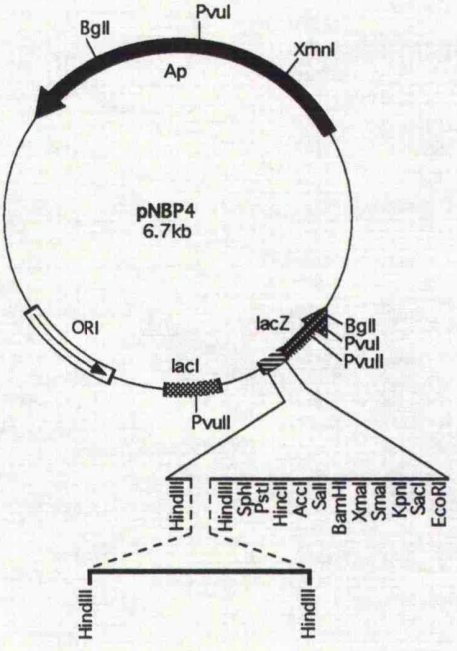
Digested with *Hin*dIII



The two λ phage NM728 positives containing the 4kb and 7kb inserts were digested with *Hin*dIII

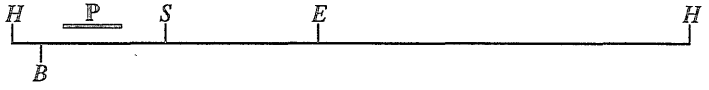
The 4kb and 7kb *Hin*dIII fragments were isolated and ligated separately to *Hin*dIII digested pUC19 vectors.





pNBP7

pNBP4



Scale: ——— = 500bp

Figure 4.7: Restriction map of the pNBP4 and pNBP7 inserts
The bar indicates 500bp and the following abbreviations were used for the restriction enzymes which cut within the fragments :
B = *Bam*HI, *C* = *Cla*I, *E* = *Eco*RI, *Ec* = *Eco*RV, *H* = *Hin*dIII, *S* = *Sal*I, *X* = *Xho*I.
The bold bar marked P shows the regions of pNBP4 and pNBP7 to which the pNov35 probe hybridises.

4.10 Southern blot analysis of the pNBP4 and pNBP7 inserts

The pNBP4 and pNBP7 plasmids were digested with a number of restriction endonucleases and the fragments produced were separated by agarose gel electrophoresis and the DNA blotted onto Hybond-N™. The blot was probed with ^{32}P-labelled pNov35 insert used in the genomic library screening which isolated the pNBP4 and pNBP7 inserts.

The results of this blot (see Fig. 4.8) indicate the regions within the pNBP4 and pNBP7 inserts to which the pNov35 probe hybridises. Analysis of these results indicate that the probe hybridises to the 1.1kb *Sal*I-*Hin*dIII fragment of pNBP4 and to the 700bp *Xho*I-*Cla*I fragment of pNBP7 (see Fig. 4.7).

4.11 Sequence analysis of the *Xho*I-*Cla*I fragment from pNBP7

The *Xho*I-*Cla*I fragment from pNBP7 was subcloned into the pIC19R vector (Fig. 4.9) and sequenced by the double-stranded dideoxy chain termination sequencing method, using both "forward" and "reverse" universal M13 primers (see chapter 2). The sequence data obtained from the *Xho*I-*Cla*I fragment was screened against sequence data bases, using the GCG programs, but no significant homology was detected.

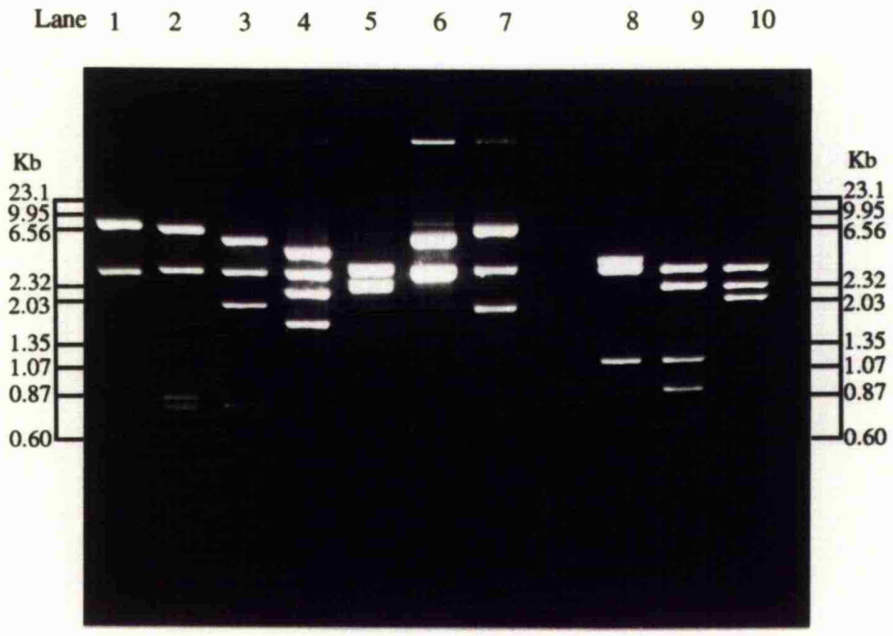
When the sequence of the *Xho*I-*Cla*I fragment was compared with that of the pNov35 insert a match was found. This match displayed 100% identity over a 120bp region which was identified as a 120bp *Sau*3AI fragment (Fig. 4.10). However, no match was found to the second *Sau*3AI fragment of the pNov35 insert (Fig. 4.10). Thus, it appears that the pNov35 insert is composed of two 120bp *Sau*3AI fragments derived from different genomic loci which have been ligated during the construction of the expression library. This conclusion is supported by Southern blot analysis which indicates that the pNBP4 and pNBP7 inserts do not hybridise to each other (results not shown) and by sequence analysis of the *Sal*I-*Hin*dIII fragment of pNBP4 described in the next chapter.

Figure 4.8 Southern blot analysis of pNBP4 and pNBP7

A The plasmids pNBP4 and pNBP7 were digested with a number of different restriction enzymes and run on a 1% agarose gel. pNBP7 was digested with: lane 1, *Bam*HI/ *Hin*dIII; lane 2, *Cla*I/ *Hin*dIII; lane 3 *Kpn*I/ *Hin*dIII; lane 4, *Eco*RI/ *Hin*dIII; lane 5, *Eco*RV/ *Hin*dIII; lane 6, *Sal*I/ *Hin*dIII; lane 7, *Xho*I/ *Hin*dIII; pNBP5 was digested with: lane 8, *Sal*I/ *Hin*dIII; lane 9, *Sal*I/ *Eco*RI/ *Hin*dIII; lane 10, *Eco*RI/ *Hin*dIII.

B The agarose gel shown was blotted onto Hybond-N™ and probed using ^{32}P labelled pNov35 insert. Comparison of the fragments which hybridised to the pNov35 insert and the restriction map allows the restriction fragment to which the probe hybridises to be determined. This analysis indicated that the probe hybridised to a 1.1kb *Sal*I-*Hin*dIII fragment of pNBP4 and to a 700bp *Xho*I-*Cla*I fragment in pNBP7.

A


Lane 1 2 3 4 5 6 7 8 9 10
Kb
23.1
9.95
6.56
2.32
2.03
1.35
1.07
0.87
0.60
Kb
23.1
9.95
6.56
2.32
2.03
1.35
1.07
0.87
0.60

B

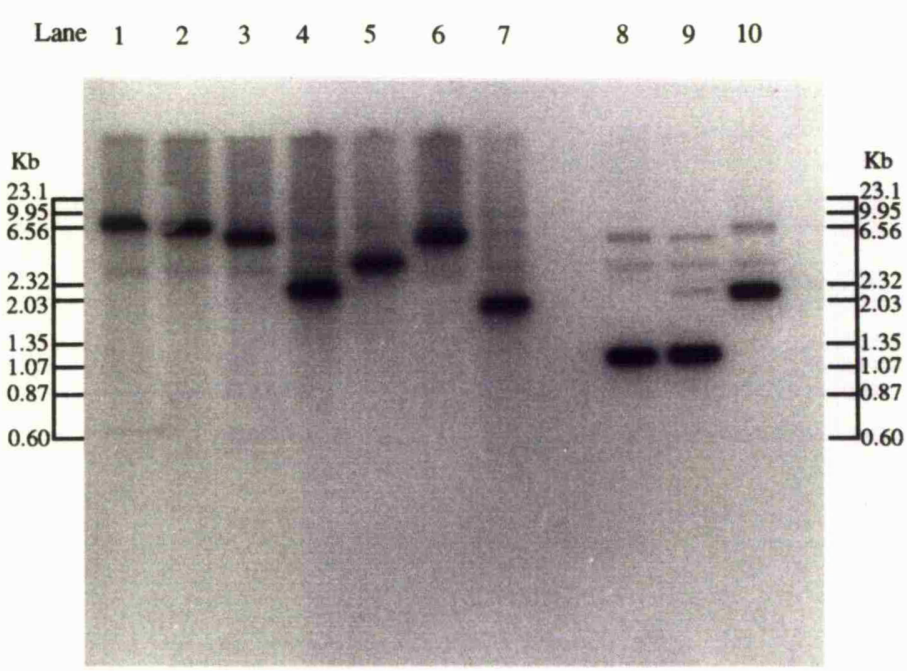
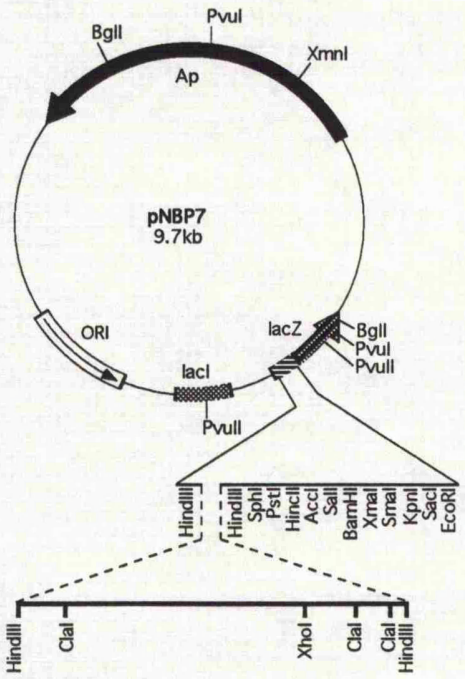

Lane 1 2 3 4 5 6 7 8 9 10
Kb
23.1
9.95
6.56
2.32
2.03
1.35
1.07
0.87
0.60
Kb
23.1
9.95
6.56
2.32
2.03
1.35
1.07
0.87
0.60

Figure 4.9 Subcloning the *Xho*I-*Cla*I fragment from pNBP7 into the pIC19R vector

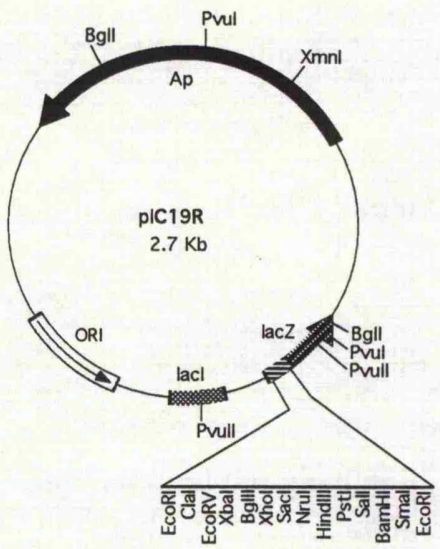
The plasmid pNBP7 was simultaneously digested with the restriction endonucleases *Xho*I and *Cla*I. The resulting fragments were separated by electrophoresis on a 1.5% agarose gel in TBE buffer. The 700bp *Xho*I-*Cla*I band was recovered from the gel by electro-elution onto dialysis membrane as described in chapter 2.

The fragment was ligated into pIC19R (see table 2.2) digested with *Xho*I and *Cla*I. Ligated plasmids were transformed into competent *E. coli* DH5α cells. The cell suspension was plated onto LB plates containing 100μg ml^{-1} ampicillin, 25μg ml^{-1} IPTG and 40μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies and incubated overnight at 37°C. Transformants were identified as white colonies against a background of blue colonies.

White colonies were randomly picked and examined for recombinant plasmids. Plasmid pIC19R containing the 700bp *Xho*I-*Cla*I insert was named pNXC.

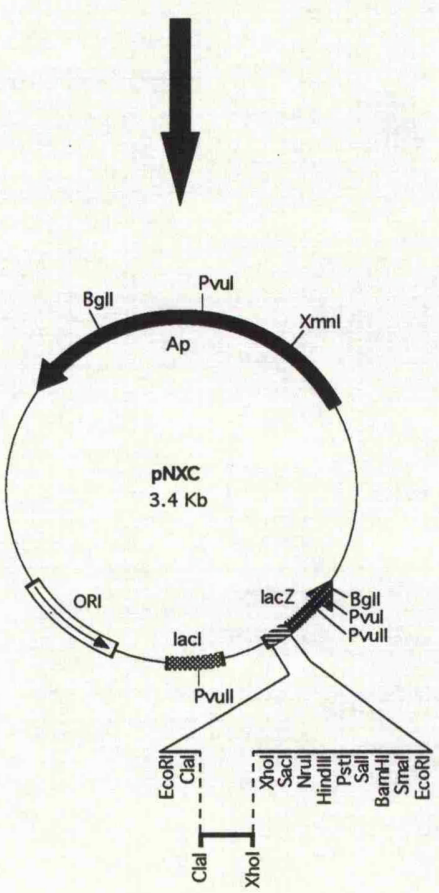


Digested with *Xho*I and *Cla*I



Digested with *Xho*I and *Cla*I

The 700bp *Xho*I-*Cla*I fragment from pNBP7 was isolated and ligated into *Xho*I-*Cla*I digested pIC19R



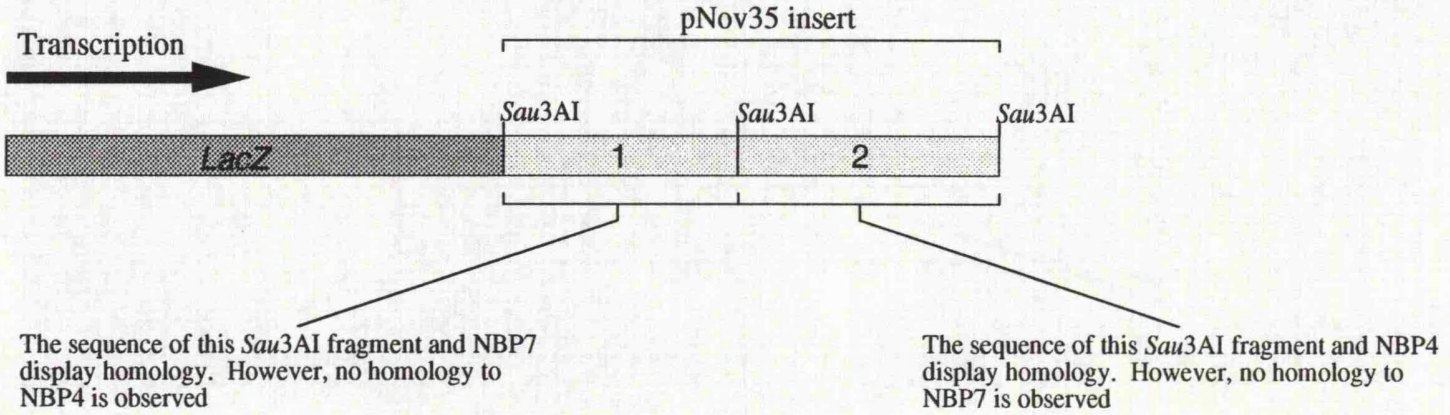


Figure 4.10 The pNov35 insert contains two independent *Sau*3AI fragments

The pNov35 insert is composed of two 120bp *Sau*3AI fragments marked 1 and 2 in the above diagram. In pEX the insert is fused to the *Lac*Z gene as shown.

When sequence data obtained from the *Xho*I-*Cla*I fragment of pNBP7 were compared with those of pNov35 only the *Sau*3AI fragment (1) was found to match. However, no homology to the second *Sau*3AI fragment (marked 2, above) was found.

When the *Sal*I-*Hin*dIII fragment of pNBP4 was sequenced (see chapter 5) homology to this second *Sau*3AI fragment was found but no homology was found to the *Sau*3AI fragment marked 1.

This indicates that the pNov35 insert is composed of two *Sau*3AI fragments which originate from different chromosomal loci.

4.12 Northern blot analysis

Northern blot analysis was carried out to determine if either the *Sal*I-*Hin*dIII fragment of pNBP4 or the *Xho*I-*Cla*I fragment of NBP7 (both hybridise to the pNov35 probe, see section 4.10) detect RNA transcripts and, if so, to determine the size of the transcript(s).

The Northern blot procedure is detailed in chapter 2. Briefly, total yeast RNA was denatured prior to electrophoresis on a 1.5% (w/v) agarose, MOPS buffer gel. Following electrophoresis, RNA was blotted onto Hybond-N™ and hybridisation was carried out in Denhardt's solution containing 50% (v/v) deionised formamide. When the *Xho*I-*Cla*I fragment from NBP7 (Fig. 4.7) was used as a probe, no hybridisation was observed (Fig. 4.11). However, when the *Sal*I-*Hin*dIII fragment from pNBP4 (Fig. 4.7) was used as a probe, hybridisation to a 1.2kb transcript was detected (Fig. 4.11). This transcript is long enough to encode a 35kDa protein and, although not conclusive, this suggested that the pNBP4 insert contains the gene encoding the 35kDa novobiocin-binding protein.

4.13 Subsequent sequence analysis of the pNBP7 *Xho*I-*Cla*I fragment

Recently, the sequence data obtained from the *Xho*I-*Cla*I fragment of pNBP7 was screened against an updated EMBL data base and a match was found. The DNA sequence of the *Xho*I-*Cla*I fragment displays 100% identity over its full length (742bp) to a region of the right arm of the *S. cerevisiae* chromosome III (see Fig. 4.12). Furthermore, when this region of chromosome III was sequenced as part of the European chromosome III sequencing project an ORF named YCR592 was located in this region (Jia *et al.*, 1991). This ORF is thought to correspond to a new gene. It shows no significant homology to any known gene and its function is unknown. The YCR592 ORF is 3678bp in length (1226 codons) and is thus capable of encoding a protein of approximately 139kDa. The possibility that the 35kDa protein is a breakdown product of a higher molecular weight protein encoded by the YCR592 ORF can be dismissed as the anti-p35 antibodies do not

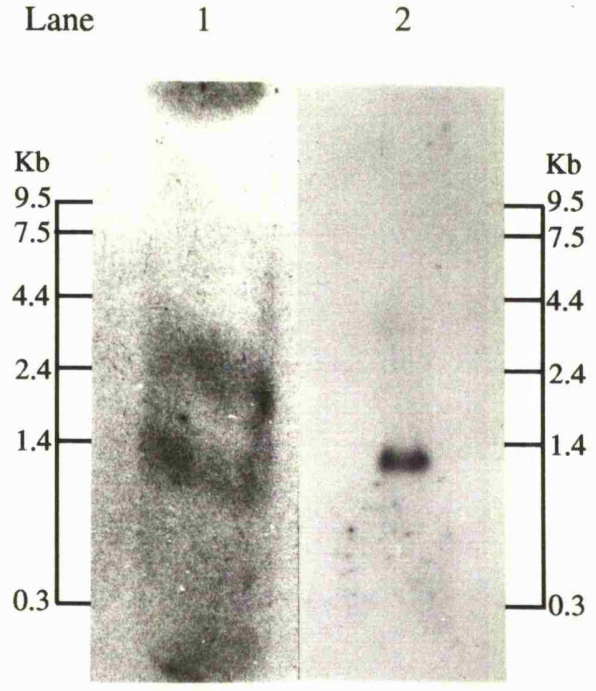


Figure 4.11 Northern blot analysis of total yeast RNA

Northern blot analysis was carried on total yeast RNA using: lane 1, *Xho*I-*Cla*I fragment from pNBP7 as a probe; lane 2, *Sal*I-*Hin*dIII fragment from pNBP4 as a probe. The *Xho*I-*Cla*I fragment from pNBP7 does not hybridise to a transcript. However, the *Sal*I-*Hin*dIII fragment from pNBP4 hybridises to a transcript of approximately 1.2kb which is compatible with the size of a gene encoding a 35kDa protein.

Figure 4.12 Lineup of the *Xho*I-*Cla*I fragment from pNBP7 with the YCR592 open reading frame

The GCG program Wordsearch was used to screen the DNA sequence of the *Xho*I-*Cla*I fragment from pNBP7 against the EMBL data base. A 100% match with the YCR592 ORF located on the right arm of the *S. cerevisiae* chromosome III was found and this match is displayed.

The *Xho*I-*Cla*I fragment sequence is labelled XC and numbered 1-742. The region which shows homology to the 120bp *Sau*3AI fragment (see section 4.11) is shown in bold.

The YCR592 sequence is labelled YCR and numbered 188229-188970 from the chromosome III position.

```
                          XC         1 ATCGATGGCTCTATAAGACCATTT 24
                                       ||||||||||||||||||||||||
                          YCR  188229 ATCGATGGCTCTATAAGACCATTT 188252

XC        25 GGGCCTGATTTTCATCGTGATACCTTTTCTAAAATTAGTGCTCCTTTAAC 74
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188253 GGGCCTGATTTTCATCGTGATACCTTTTCTAAAATTAGTGCTCCTTTAAC 188302

XC        75 CACTTTACCACCACCAAGACTACCATCTATTCAGTTTCCTCGTTCAGAAA 124
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188303 CACTTTACCACCACCAAGACTACCATCTATTCAGTTTCCTCGTTCAGAAA 188352

XC       125 TGGCAGAACCTACAGTGACAGATTTGCGTAACAGGCCCTTAGACCATATT 174
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188353 TGGCAGAACCTACAGTGACAGATTTGCGTAACAGGCCCTTAGACCATATT 188402

XC       175 GACACGTTGGCTGATGCAGCTTCGTCAGTAACAAATAATCAAAACTTCAG 224
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188403 GACACGTTGGCTGATGCAGCTTCGTCAGTAACAAATAATCAAAACTTCAG 188452

XC       225 TAATGAAAGGAATGCAATTGACATTGGCCGTAAATCGACGACAATCAGCA 274
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188453 TAATGAAAGGAATGCAATTGACATTGGCCGTAAATCGACGACAATCAGCA 188502

XC        275 ATCTATTGAATAATTCG GATCGAAGCATGAAATCTTCTTTCCAAAGCGCT 324
              ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR   188503 ATCTATTGAATAATTCGGATCGAAGCATGAAATCTTCTTTCCAAAGCGCT 188552

XC       325 TCAAGACACGAAGCACAGCTCGAAGACACTCCCAGCATGAACAATATTGT 374
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188553 TCAAGACACGAAGCACAGCTCGAAGACACTCCCAGCATGAACAATATTGT 188602

XC       375 AGTACAAGAAATAAAACCGAATATTACTACGCCAAGATCGAGTTCTATTT 424
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YCR  188603 AGTACAAGAAATAAAACCGAATATTACTACGCCAAGATCGAGTTCTATTT 188652

XC       425 CTGCATTACTAAATCCTGTAAATGGGAATGGGCAATCAAACCCAGATGGA 474
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188653 CTGCATTACTAAATCCTGTAAATGGGAATGGGCAATCAAACCCAGATGGA 188702

XC       475 AGGCCGTTGCTGCCATTTCAGCATGCTATTTCTCAAGGCACTCCTACTTT 524
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188703 AGGCCGTTGCTGCCATTTCAGCATGCTATTTCTCAAGGCACTCCTACTTT 188752

XC       525 CCCTTTACCGGCCCCTCGCACTAGTCCAATAAGTCGTGCGCCTCCAAAGT 574
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188753 CCCTTTACCGGCCCCTCGCACTAGTCCAATAAGTCGTGCGCCTCCAAAGT 188802

XC       575 TCAATTTTTCGAATGATCCGTTGGCAGCTTTGGCTGCGGTTGCCTCCGCG 624
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188803 TCAATTTTTCGAATGATCCGTTGGCAGCTTTGGCTGCGGTTGCCTCCGCG 188852

XC       625 CCAGATGCAATGAGCAGTTTTTTATCTAAAAAGGAAAATAATAATTGAAC 674
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188853 CCAGATGCAATGAGCAGTTTTTTATCTAAAAAGGAAAATAATAATTGAAC 188902

XC       675 AAACGGCTGAGACGGGCAATACATATGCTCTACTTCTTTTCCATCCAATG 724
             ||||||||||||||||||||||||||||||||||||||||||||||||||
YCR  188903 AAACGGCTGAGACGGGCAATACATATGCTCTACTTCTTTTCCATCCAATG 188952

XC       725 GTTGGTGAAACTCTCGAG 742
             ||||||||||||||||||
YCR  188953 GTTGGTGAAACTCTCGAG 188970
```

recognise any high molecular weight proteins. It is therefore unlikely that this ORF encodes the 35kDa novobiocin-binding protein.

The YCR592 is probably expressed since this region has been shown to detect a 4.2kb transcript (Yoshikawa and Isono, 1990). However, the transcript is of very low abundance which may explain the failure of the *Xho*I-*Cla*I fragment to detect a transcript in Northern blot analysis.

4.14 Gene mapping by hybridisation

In order to determine the chromosomal location of the putative p35 gene, a DNA fragment containing part of the putative p35 gene was hybridised to a chromosome blot.

This procedure was basically a Southern blot of yeast chromosomes. The yeast chromosomes were separated using pulse-field gel electrophoresis as described in chapter 2. The separated chromosomes were blotted onto a Hybond-N™ filter and probed using ^{32}P labelled pNBP4 insert (Fig. 4.6). The results of this experiment suggested that the putative p35 gene is located on chromosome IX (Fig. 4.13).

4.14 Discussion

Antibodies to both the 200kDa and the 35kDa novobiocin-binding proteins have been successfully used to clone parts of the corresponding genes from yeast expression libraries constructed in pEX.

A pEX clone which produces a peptide recognised by affinity purified anti-p35 antibodies was used to clone a genomic DNA fragment which contains at least part of the p35 gene.

Difficulties were encountered when this pEX clone was shown to contain two independent *Sau*3AI inserts. It was clear that this clone contains two 120bp *Sau*3AI fragments from different genomic loci ligated into the pEX vector. This situation was probably the result of the chance ligation of two *Sau*3AI fragments from different genomic loci into the same pEX vector molecule. This type of three-way ligation event has been

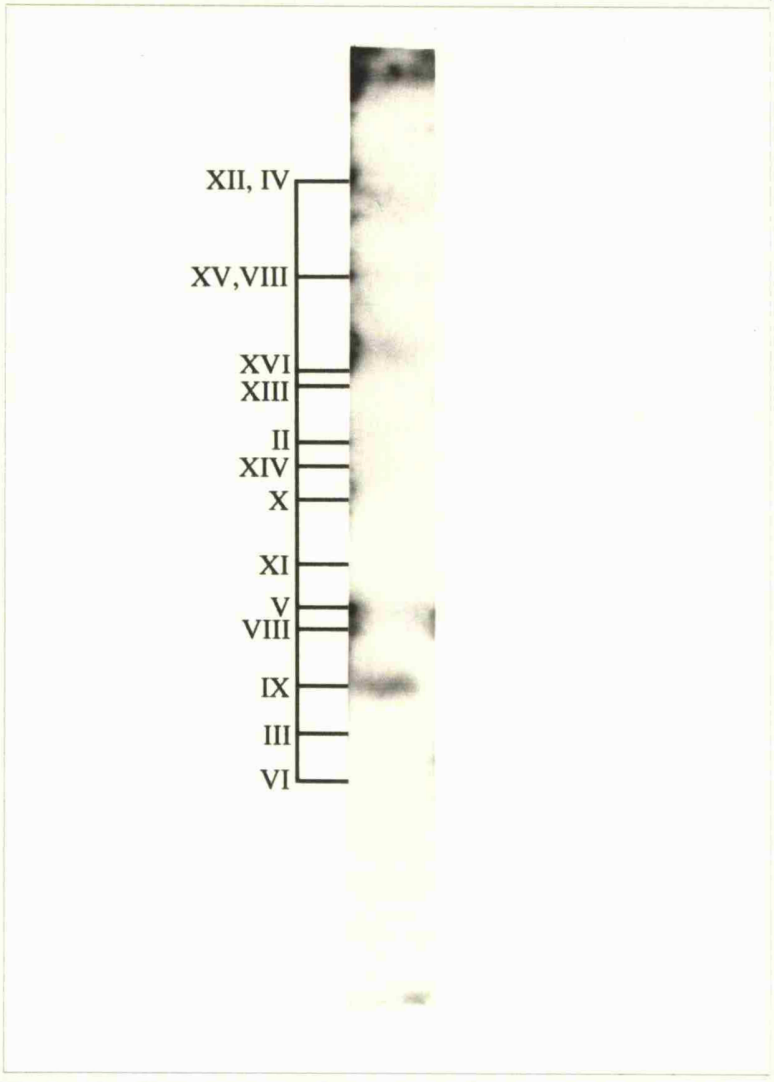


Figure 4.13 Chromosome mapping of the p35 gene

Yeast chromosomes were separated by pulse field gel electrophoresis and blotted onto Hybond-N™. The filter was hybridised with ^{32}P-labelled pNBP4 insert which contains part of the putative p35 gene. The results of this experiment suggested that the putative p35 gene is located on chromosome IX.

observed during the construction of pEX libraries by other workers (A.J. Simon, personal communication). This type of ligation may indicate that the insert to vector DNA ratio, used in the ligation reaction, was incorrectly judged. If this insert:vector ratio was biased towards insert DNA concentration, such three-way ligation events may be selected for.

Northern blot analysis was used to determine if the two *Sau*3AI pEX inserts recognised transcripts. Chromosomal DNA fragments which hybridised to each of the pEX inserts were used in this analysis. Only the *Sal*I-*Hin*dIII fragment from pNBP4 (Fig. 4.7), which hybridised to the second *Sau*3AI fragment (Fig. 4.4), identified a transcript. This 1.2kb transcript is of the correct size to encode a 35kDa protein. Although not conclusive this suggested that the p35 gene is present on the *Sal*I-*Hin*dIII fragment from pNBP4.

Recent data base searches with the *Xho*I-*Cla*I fragment of pNBP7 have shown that this fragment originates from a region on the right arm of the *S. cerevisiae* chromosome III. This region has been shown to contain a 3678bp ORF (YCR592) and is, therefore, capable of encoding a 139kDa protein (Jia *et al.*, 1991). It is unlikely that the 35kDa protein is breakdown product of a higher molecular weight protein since anti-p35 antibodies consistently failed to recognise any high molecular weight proteins. These results suggest that this ORF does not encode the 35kDa novobiocin-binding protein and that the p35 gene is present on the *Sal*I-*Hin*dIII fragment from pNBP4

The ORF appears to be expressed (Yoshikawa and Isono, 1990), but at very low levels which may explain why the *Xho*I-*Cla*I fragment did not detect a transcript in Northern blot analysis.

Additional support for the suggestion that the p35 gene is present in the *Sal*I-*Hin*dIII fragment from pNBP4 is provided in chapter 7. Briefly, using the anti-p35 antibodies no 35kDa protein is detected in Western blot analysis of crude protein extracts following the disruption of the gene found in the *Sal*I-*Hin*dIII fragment from pNBP4. Further experiments which would conclusively prove that the p35 gene is present in the *Sal*I-*Hin*dIII fragment from pNBP4 are presented in chapter 8.

Results described above suggest that the second *Sau*3AI fragment in the pEX positive 1e2 encodes the peptide recognised by the anti-p35 antibodies. Analysis of the ORFs encoded by the insert of clone 1e2 (Fig. 4.4) indicate that the second peptide was produced as a fusion with the first *Sau*3AI fragment as well as with β-galactosidase. It is quite feasible that the peptide would be recognised by antibodies in what was in effect a double fusion protein. In fact, presentation of the antigen in this manner may have enhanced the recognition of the peptide by anti-p35 antibodies.

Chapter 5

Sequencing the p35 gene

5.1 Introduction

DNA sequencing is one of the fundamental procedures in molecular biology. In many cases, determining the sequence of a cloned DNA fragment is a prerequisite for planning any subsequent manipulation of the DNA. For example, sequence information is often invaluable since it allows the identification of a gene, including its size and position within a DNA fragment. In addition, sequence information allows assessment of identity or homology with previously cloned and sequenced genes. A DNA sequence can also be used to predict the amino acid sequence of a gene product and structural motifs within the sequence often help in the identification of the function(s) of the gene product.

The pNov35 (Fig. 4.3) probe, initially isolated from pEX expression libraries, hybridised to the 1.1kb *Sal*I-*Hin*dIII fragment of clone pNBP4 (Fig. 4.8) and this fragment hybridised to a transcript of the correct size to encode a 35kDa protein (Fig. 4.10). This suggested that part of the p35 gene was present on this fragment. Unfortunately, there were no convenient restriction enzyme sites within the *Sal*I-*Hin*dIII fragment which would allow sequencing of the fragment from internal subclones. Therefore, in order to sequence the entire *Sal*I-*Hin*dIII fragment, a series of deletion subclones were produced using the double-stranded nested deletion method (Henikoff, 1984). Briefly, this method includes an exonuclease III digestion starting from a 5'-overhang or a blunt end, while a 3'-overhang end, resistant to exonuclease III digestion, protects the plasmid from digestion in the other direction.

Sequencing from nested deletions is routinely carried out in the laboratory and so it was used in preference to sequencing by gene walking as the latter method is relatively expensive and time consuming, depending on the design and synthesis of a new primer every 250bp.

5.2 Subcloning the *Sal*I-*Hin*dIII fragment into pUC19 and pIC19R vectors

In order to obtain sequence from both strands of the *Sal*I-*Hin*dIII fragment, nested deletions from both the *Sal*I and the *Hin*dIII sites were required. Thus, the *Sal*I-*Hin*dIII fragment was subcloned into pUC19 and pIC19R (Table 2.2) vectors. The pUC19 vector contains restriction endonuclease sites allowing the construction of nested deletions from the *Sal*I site, whilst subcloning into a pIC19R vector made the construction of nested deletions from the *Hin*dIII site possible.

The plasmid pNBP4 (Fig.4.6) was simultaneously cut with the restriction endonucleases *Sal*I and *Hin*dIII. The resulting fragments were separated on a 0.6% (w/v) LGT agarose gel. The lower band, corresponding to the 1.1kb *Sal*I-*Hin*dIII fragment, was recovered from the gel as described in chapter 2.

The fragment was ligated to the vectors pUC19 and pIC19R, previously digested with both *Sal*I and *Hin*dIII. The ligated plasmids were transformed into competent *E. coli* DH5α cells. White colonies were randomly picked and restriction analysis carried out on their plasmid DNA to confirm the construction of correct plasmids. The correct constructs in pUC19 and pIC19R, containing the *Sal*I-*Hin*dIII fragment, were named pNBP-UCSH and pNBP-ICSH respectively (Fig. 5.1).

5.3 Double-stranded nested deletion of pNBP-UCSH and pNBP-ICSH

The plasmid pNBP-UCSH was digested with the restriction endonuclease *Sst*I to generate a 3'-overhang end, resistant to exonuclease III digestion. The linear plasmid was then digested with *Xba*I to obtain a 5'-overhang which is a substrate for exonuclease III. Similarly, the plasmid pNBP-ICSH was digested with the restriction endonuclease *Sst*I to generate a 3'-overhang end and the linear plasmid digested with *Hin*dIII to obtain a 5'-overhang which is a substrate for exonuclease III.

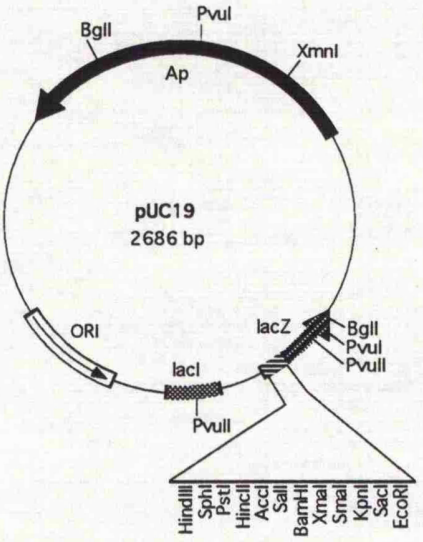
Treatment of a 5'-overhang with exonuclease III results in the removal of nucleotides from one strand of the target sequence, producing a single-stranded region

Figure 5.1 Subcloning the *Sal*I-*Hin*dIII fragment into pUC19 and pIC19R vectors

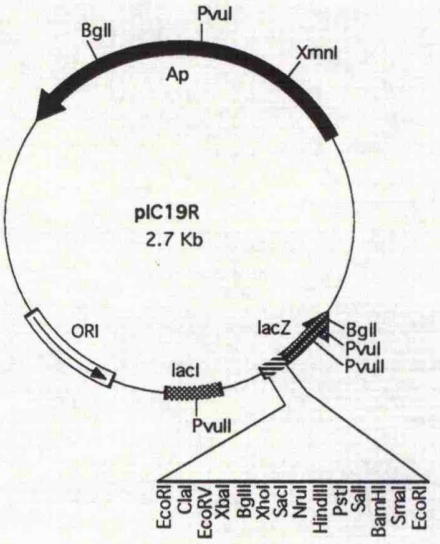
The plasmid pNBP4 was cut with the restriction endonucleases *Sal*I and *Hin*dIII in a double-digestion. The resulting fragments were separated on a 0.6% LGT agarose gel. The lower band, corresponding to the 1.1kb *Sal*I-*Hin*dIII fragment, was recovered from the gel as described in chapter 2.

The fragment was ligated to *Sal*I and *Hin*dIII double-digested pUC19 and pIC19R plasmids. The ligated plasmids were transformed into competent *E. coli* DH5α cells. The cell suspension was plated onto LB plates containing 100μg ml^{-1} ampicillin, 25μg ml^{-1} IPTG and 40μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies and incubated overnight at 37°C. Transformants were identified as white colonies against a background of blue colonies.

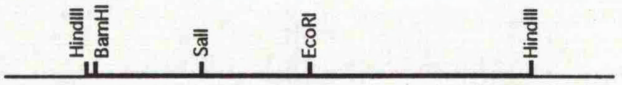
White colonies were randomly picked and examined for recombinant plasmids. The correct constructs in pUC19 and pIC19R contained the *Sal*I-*Hin*dIII fragment and were named pNBP-UCSH and pNBP-ICSH, respectively.



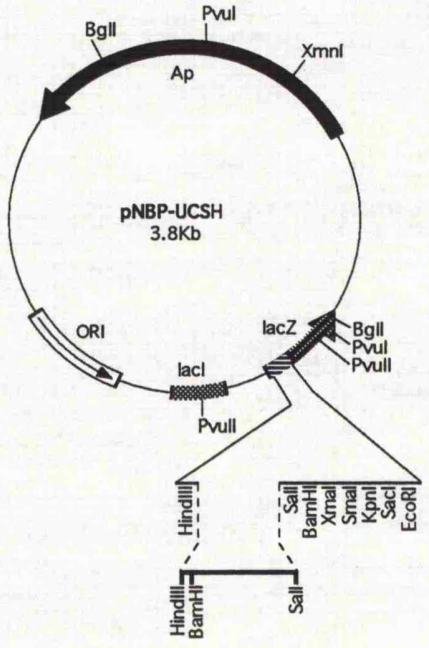
Digested with *Sal*I and *Hin*dIII

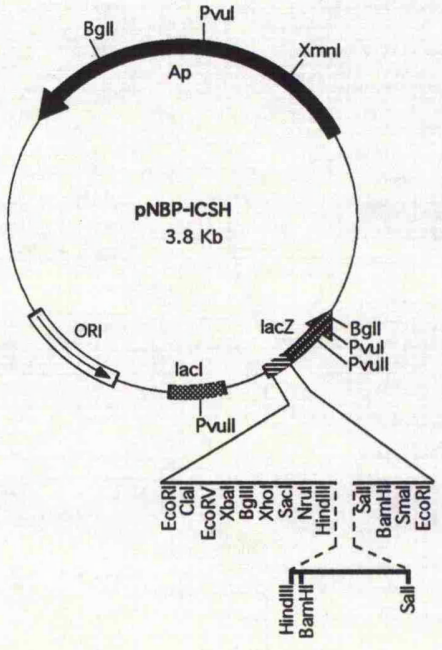


Digested with *Sal*I and *Hin*dIII



NBP4 fragment in pUC19 was digested with *Sal*I and *Hin*dIII to give a 1.1Kb *Sal*I-*Hin*dIII fragment which was ligated into pUC19 and pIC19R.





which can be removed by S1 nuclease. Following the restriction endonuclease digestions, exonuclease III digestion was carried out on each plasmid at 30°C. Samples were removed at timed intervals and treated with S1 nuclease to remove single-stranded regions generated by the exonuclease III. Analysis of each sample was carried out by agarose gel electrophoresis, allowing the length of DNA deleted over time to be estimated (Fig. 5.2). The results of this analysis were used in the selection of samples to be recircularised by blunt-end ligation. Recircularised plasmids were transformed into competent *E. coli* DH5α cells.

There is a degree of variation between individual clones obtained in the experiment, even within individual samples. Consequently, the plasmid DNA isolated from a number of clones obtained from each sample was analysed by agarose gel electrophoresis. Plasmid DNA with appropriate deletions were prepared for sequencing as described in chapter 2.

5.4 DNA sequencing of the *Sal*I-*Hin*dIII fragment

Sequencing of plasmid DNA with appropriate deletions was carried out using the double-stranded sequencing method described in chapter 2. To reduce the amount of false termination, the Klenow fragment of DNA polymerase was used in a "Klenow chase" reaction (see chapter 2).

The clones obtained following the nested deletion of pNBP-UCSH were sequenced using the "forward" universal M13 primer and clones obtained following the nested deletion of pNBP-ICSH were sequenced using the "reverse" universal M13 primer. Both strands of the *Sal*I-*Hin*dIII fragment from pNBP4 were sequenced in their entirety from these clones. The use of the double-stranded nested deletion method ensured that overlaps in sequence information from successive clones was obtained and the strategy of sequencing is illustrated in Fig. 5.3.

Assembled sequence data were analysed with the help of the Genetics Computer Group (GCG) computer programs. Analysis of the *Sal*I-*Hin*dIII fragment sequence identified a region which exactly matched the sequence of the second *Sau*3AI fragment

Figure 5.2 Analysis of double-stranded nested deletion of the plasmid pNBP-UCSH

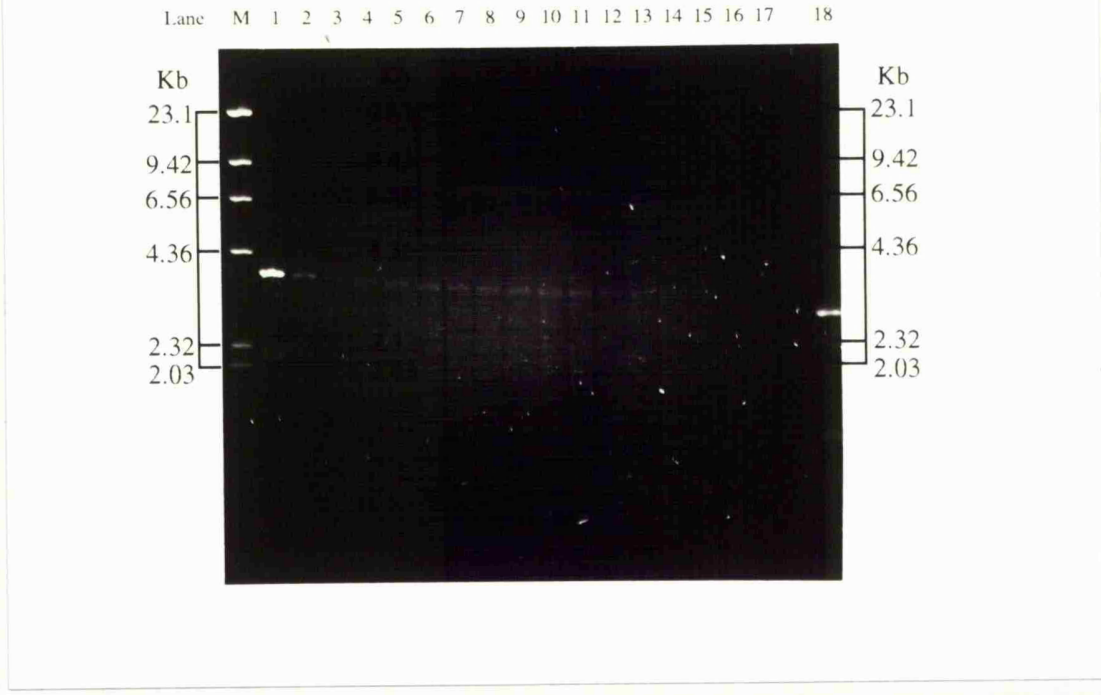
The plasmid pNBP-UCSH was digested with the restriction endonuclease *Sst*I to generate a 3'-overhang end resistant to exonuclease III digestion. The linear plasmids were digested with *Xba*I to obtain a 5'-overhang which is sensitive to exonuclease III digestion.

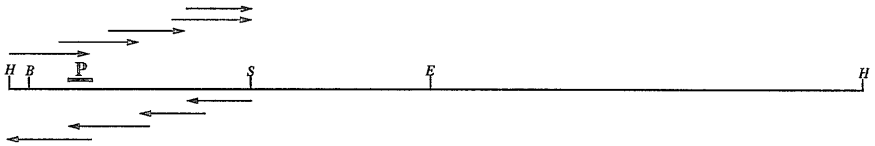
Exonuclease III digestion was carried out at 30°C. Samples were removed at 1 minute intervals and treated with S1 nuclease to remove single-stranded regions generated by the exonuclease III digestion. Analysis of the samples taken at each time point was carried out by electrophoresis using a 1% agarose gel:

Lane M: λ *Hin*dIII marker

Lane 1: Control, pNBP-UCSH digested with *Sst*I and *Xba*I

Lanes 2-17: Time point samples, samples taken at one minute intervals


Lane M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Kb
23.1
9.42
6.56
4.36
2.32
2.03
Kb
23.1
9.42
6.56
4.36
2.32
2.03



Scale:——= 200bp

Figure 5.3: Restriction map of NBP4 and sequencing strategy
The bar indicates 200bp and the following abbreviations were used for the restriction enzymes which cut within the fragment: *B* = *Bam*HI, *E* = *Eco*RI, *H* = *Hin*dIII, *S* = *Sal*I.
The arrows indicate the region and the direction of sequencing.
The bold bar marked P shows the region of NBP4 to which the original 120bp probe hybridised.

present in the pNov35 insert (Fig. 4.8). This result further supported the conclusion that the pNov35 insert is composed of two 120bp *Sau*3AI fragments derived from different genomic loci (see section 4.11). The sequence analysis also indicates the presence of a large open reading frame (ORF) in the fragment. This open reading frame, which begins upstream of the *Hin*dIII site, is 833bp in length and ends with a TAA stop codon 233bp upstream of the *Sal*I site. The DNA sequence displays no homology to any sequence in the EMBL data base. In contrast, the predicted amino acid sequence of the ORF exhibits significant homology to mammalian cytochrome b5 reductases and plant nitrate reductases. This homology will be discussed in detail in chapter 6.

5.5 Isolation of the full length p35 gene

Sequence analysis indicates that the start of the ORF is upstream of the *Hin*dIII site of the genomic clone pNBP4. Thus, a genomic fragment, overlapping the pNBP4 insert, was required to obtain the full-length p35 gene.

5.5.1 Identification of an overlapping clone

The use of an *Eco*RI genomic library to isolate an overlapping clone was considered for two reasons:

a) An *Eco*RI restriction endonulease site is present in the pNBP4 insert (Fig. 5.3). Since an *Eco*RI genomic fragment would overlap the pNBP4 insert, the *Sal*I-*Hin*dIII fragment from pNBP4 could be used as a probe for the screening of an *Eco*RI genomic library.

b) A previously constructed λ*gt*11 *Eco*RI library was available in the laboratory.

Library screening is a time-consuming process. Therefore, before a library was screened, Southern blot analysis of yeast genomic DNA was carried out to determine how much additional DNA would be obtained from an overlapping *Eco*RI fragment.

Yeast genomic DNA was digested with the restriction endonuclease *Eco*RI. Fragments were separated by agarose gel electrophoresis and the DNA blotted onto Hybond-N. Southern blot analysis was carried out using the *Sal*I-*Hin*dIII fragment from

pNBP4 as a probe. The probe hybridised to a 4kb *Eco*RI genomic fragment (Fig. 5.4). The restriction map of the pNBP4 insert (Fig. 5.3) indicates that 2kb of this *Eco*RI fragment must overlap clone pNBP4. This indicates that an additional 2kb of DNA could be obtained by cloning this *Eco*RI genomic fragment.

The ORF on the *Sal*I-*Hin*dIII fragment is 833bp in length. Given that a gene encoding a 35KDa protein would be approximately 900bp long, the 4kb *Eco*RI genomic fragment was assumed to contain the complete gene.

5.5.2 The screening of a λ*gt*11 genomic library

Based on the information obtained from the genomic Southern blot analysis (see above), it was decided to clone the genomic *Eco*RI fragment from a λ*gt*11 library constructed in our laboratory. The λ*gt*11 genomic library was screened with the *Sal*I-*Hin*dIII fragment from pNBP4 as a probe, using the plaque hybridisation method described in chapter 2. Positive phage were purified through several rounds of screening (Fig. 5.5).

5.6 Subcloning the *Eco*RI genomic fragment into the pUC19 vector

Phage DNA was isolated from a positive phage as described in chapter 2 and digested with the restriction endonuclease *Eco*RI. The fragments were separated on a 0.6% (w/v) LGT agarose gel. The lower band, corresponding to the 4kb *Eco*RI fragment, was excised and the DNA was recovered from the gel as described in chapter 2.

The DNA fragment was ligated into the pUC19 vector digested with *Eco*RI and the ligated plasmids were transformed into competent *E. coli* DH5α cells. White colonies were randomly picked and their plasmid DNA was analysed. The correct construct in the pUC19 vector containing the *Eco*RI fragment was named pNBP-Eco (Fig. 5.6).

Restriction endonuclease analysis of pNBP-Eco indicated that the two inserts NBP-Eco and NBP4 overlap (Fig. 5.7). The analysis also showed a number of restriction endonuclease sites which made subcloning and sequencing of the region upstream of the *Hin*dIII site possible.

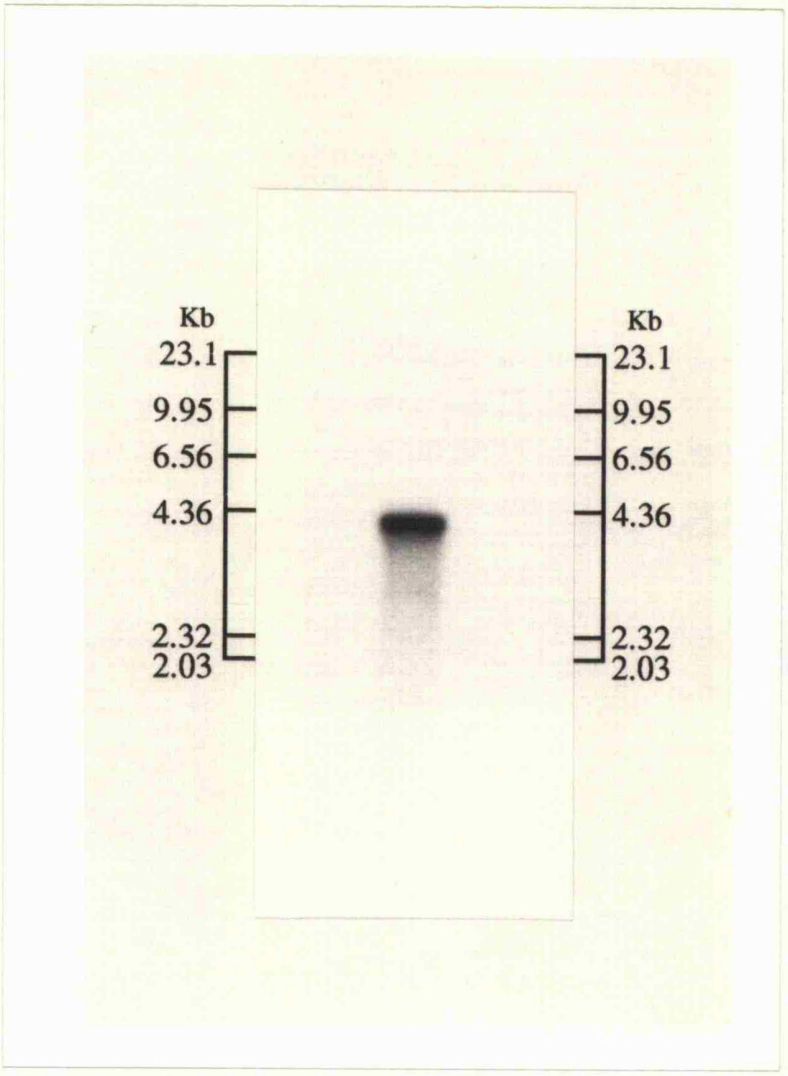


Figure 5.4 Southern blot analysis of yeast genomic DNA using *Sal*I-*Hin*dIII fragment from pNBP4 as a probe

Yeast genomic DNA was digested with the restriction endonuclease *Eco*RI. Fragments were separated by agarose gel electrophoresis and the DNA blotted onto Hybond-N. Southern blot analysis was carried out as described in chapter 2 using the *Sal*I-*Hin*dIII fragment from clone NBP4 (fig.4.6) as a probe. The probe hybridised to a 4kb *Eco*RI genomic fragment.

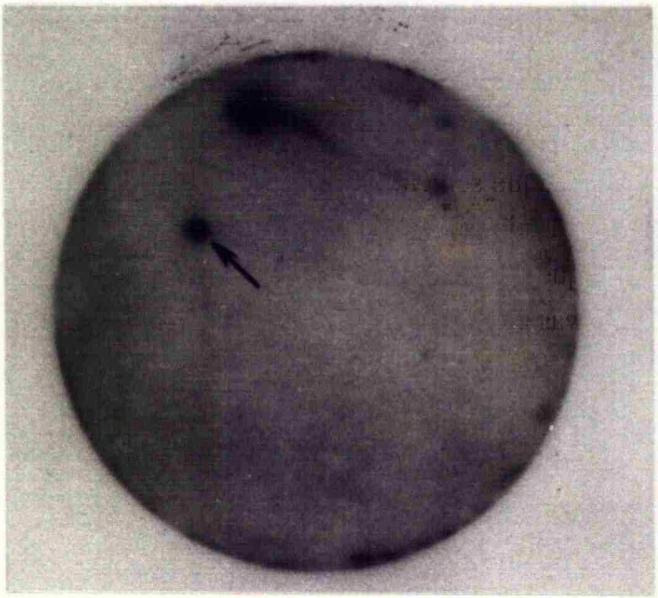
Figure 5.5 The Screening of a genomic *Eco*RI library constructed in λ*gt*11

a) Primary plaque screen; the probe hybridised to two plaques. Plaques in the area surrounding the putative positive indicated by an arrow were picked and rescreened.

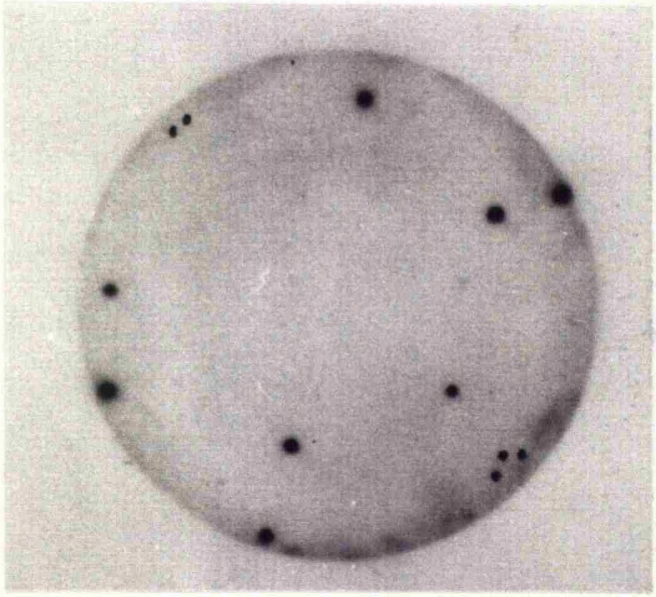
b) Secondary plaque screen; the probe hybridised to a number of plaques. An isolated single plaque was picked and rescreened.

c) Tertiary plaque screen; the probe hybridised to all the plaques indicating that the positive phage picked was pure.

A



B



C

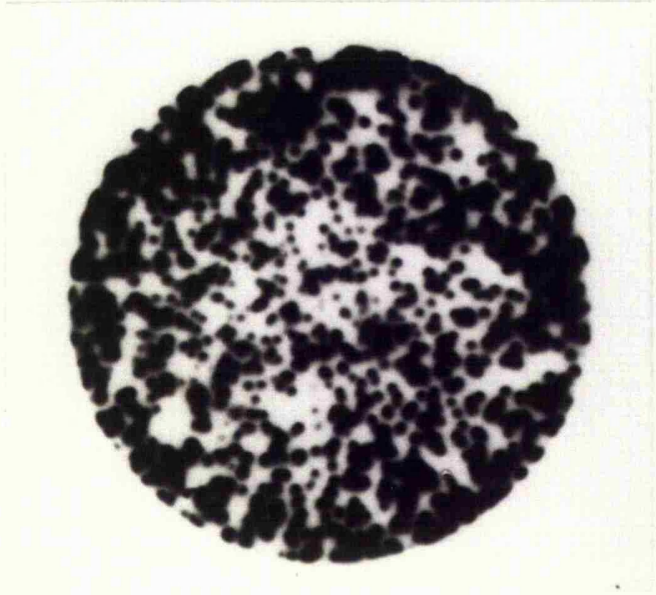
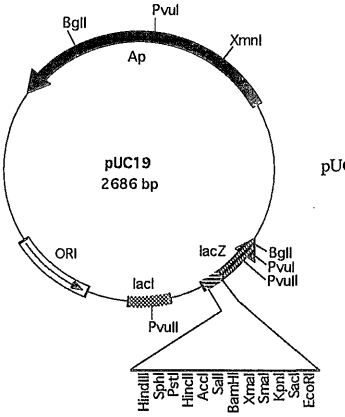


Figure 5.6 Subcloning the yeast genomic *Eco*RI fragment from λ*gt*11 into the pUC19 vector

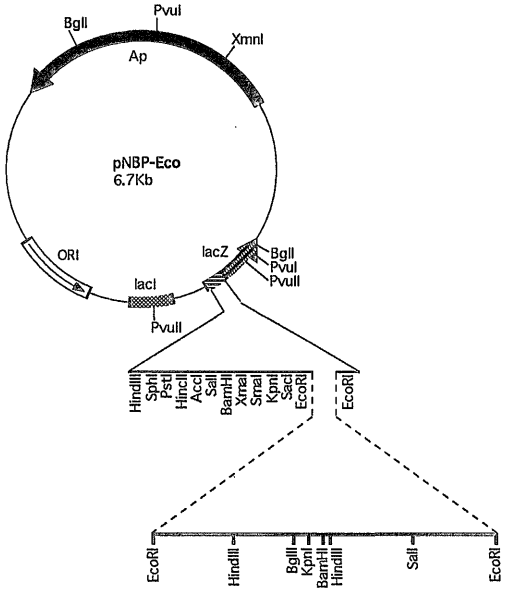
Phage DNA was isolated from a positive plaque as described in chapter 2 and digested with the restriction endonuclease *Eco*RI. The fragments were separated by electrophoresis on a 0.6% LGT agarose gel. The lower band, corresponding to the 4kb *Eco*RI fragment, was recovered from the agarose as described in chapter 2.

The fragment was ligated into *Eco*RI digested pUC19 plasmid and the ligated plasmids transformed into competent *E. coli* DH5α cells. The cell suspension was plated onto LB plates containing 100μg ml^{-1} ampicillin, 25μg ml^{-1} IPTG and 40μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies and incubated overnight at 37°C. Transformants were identified as white colonies against a background of blue colonies. White colonies were randomly picked and restriction analysis carried out to confirm the structure of recombinant plasmids. The correct construct in pUC19 containing the *Eco*RI fragment was named pNBP-Eco.



pUC19 was digested with *Eco*RI

NBP-*Eco*RI fragment in λ*gt*11 was digested with *Eco*RI to give a 4Kb *Eco*RI-*Eco*RI fragment which was ligated into pUC19



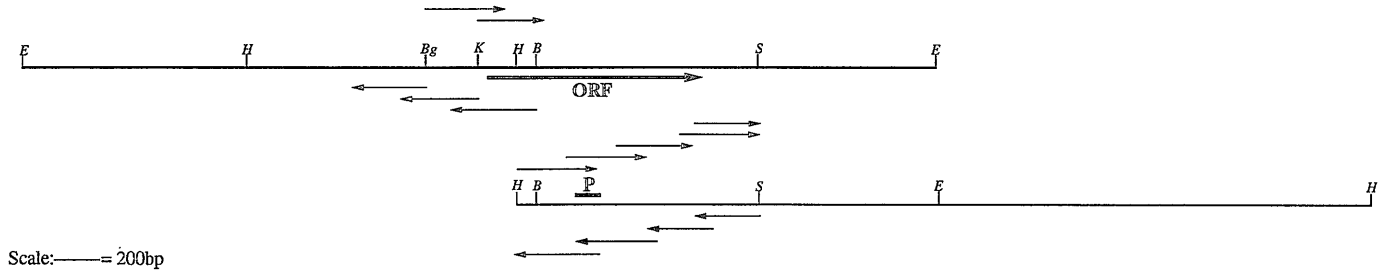


Figure 5.7: Restriction map of NBP-Eco and NBP4 and sequencing strategy
The figure shows the NBP-Eco fragment above the NBP4 fragment, indicating the region of overlap.
The bar indicates 200bp and the following abbreviations were used for the restriction enzymes which cut within the fragments: *B* = *Bam*HI, *Bg* = *Bgl*I, *E* = *Eco*RI, *K* = *Kpn*I, *H* = *Hin*dIII, *S* = *Sal*I.
The small arrows indicate the region and the direction of sequencing.
The large arrow marked ORF indicates the location and direction of the p35 open reading frame.
The bold bar marked P shows the region of NBP4 to which the original 120bp probe hybridised.

5.7 Completion of the p35 gene sequence using subclones of pNBP-Eco

The 2.1kb *Eco*RI-*Bam*HI fragment from pNBP-Eco was subcloned so that the sequence between the *Bam*HI and the *Hind*III sites could be determined (see Fig. 5.7). The *Eco*RI-*Bam*HI fragment was subcloned into the pUC19 vector *via* standard procedures (see Fig. 5.8) and designated pNBP-EB21. Sequencing was carried out as described previously using the "reverse" universal M13 primer. The sequence between the *Bam*HI and the *Hind*III sites of pNBP-EB21 and pNBP4 was found to be identical, confirming that the inserts of pNBP4 and pNBP-Eco overlap.

Four additional subclones from pNBP-Eco were constructed; two of these clones, pNBP-KK and pNBP-KE, are shown in Fig. 5.9. These clones made the sequencing upstream and downstream of the *Kpn*I site possible (Fig. 5.7). The last two subclones, pNBP-BE16 and pNBP-BE24 (see Fig. 5.10 for their construction), allowed the sequencing upstream and downstream of the *Bgl*II site (Fig. 5.7).

These sequencing data were assembled together with the previous data obtained from the *Sal*I-*Hin*dIII fragment and analysed using the GCG computer programs. Reading frame analysis of the assembled sequence showed one large open reading frame of 966bp which could encode a protein with a predicted molecular mass of 36,196 Da (Fig. 5.11).

5.8 Analysis of the p35 gene sequence

5.8.1 General features of yeast genes

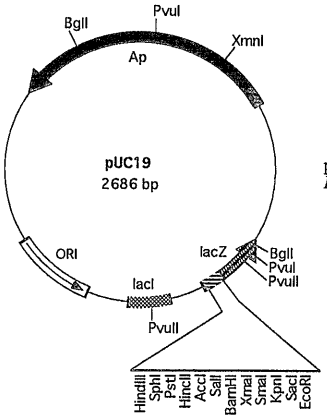
In eukaryotes, a number of features are thought to be important for transcription initiation. The first of these is an AT-rich region, similar to the Pribnow box in prokaryotic systems, named the TATA box (Gannon *et al.*, 1989). In higher eukaryotes, the TATA box has a relatively fixed location approximately 25-30bp upstream of the transcription start site. However, in *S. cerevisiae* the location of the TATA box varies from 40 to 90bp upstream of the transcription start site (Lewin, 1990).

Figure 5.8 Subcloning the *Eco*RI-*Bam*HI fragment from pNBP-Eco into the pUC19 vector

The plasmid pNBP-Eco was simultaneously digested with the restriction endonucleases *Eco*RI and *Bam*HI. The resulting fragments were separated by electrophoresis on a 0.6% LGT agarose gel. The 2.1kb *Eco*RI-*Bam*HI band was recovered from the gel as described in chapter 2.

The fragment was ligated into a pUC19 vector digested with *Eco*RI and *Bam*HI. Ligated plasmids were transformed into competent *E. coli* DH5α cells. The cell suspension was plated onto LB plates containing 100μg ml^{-1} ampicillin, 25μg ml^{-1} IPTG and 40μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies and incubated overnight at 37°C. Transformants were identified as white colonies against a background of blue colonies.

White colonies were randomly picked and examined for recombinant plasmids. Plasmid pUC19 containing the 2.1kb *Eco*RI-*Bam*HI fragment was named pNBP-EB21.



pUC19 was digested with *Eco*RI and *Bam*HI

pNBP-*Eco*RI digested with *Eco*RI and *Bam*HI, the 2.1Kb *Eco*RI-*Bam*HI fragment was isolated and ligated in pUC19

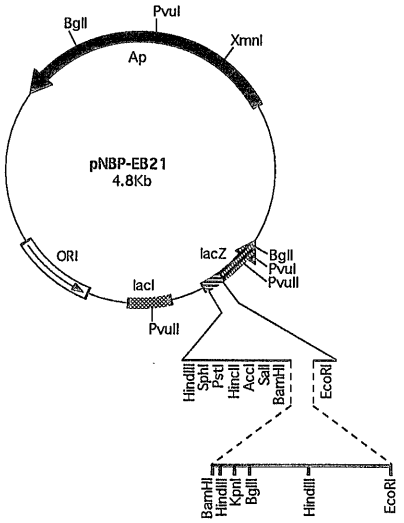
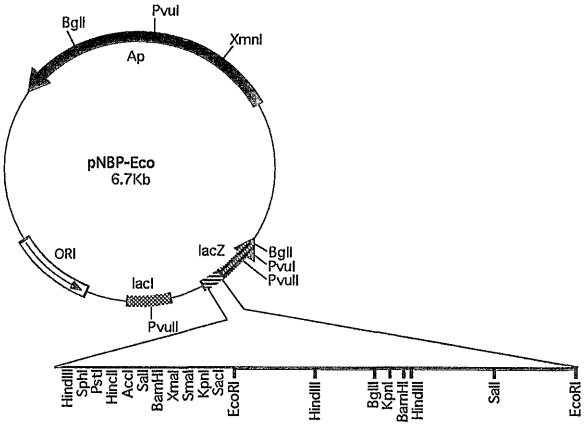


Figure 5.9 Subcloning the *Kpn*I-*Eco*RI fragments from pNBP-Eco into the pUC19 vector

The plasmid pNBP-Eco was digested with the restriction endonuclease *Kpn*I. The resulting fragments were separated by electrophoresis on a 0.6% LGT agarose gel. Both the upper band, corresponding to the linear pUC19 containing a 2.1kb insert and the lower band, corresponding to a 2kb *Kpn*I-*Kpn*I fragment, were purified from the gel as described in chapter 2.

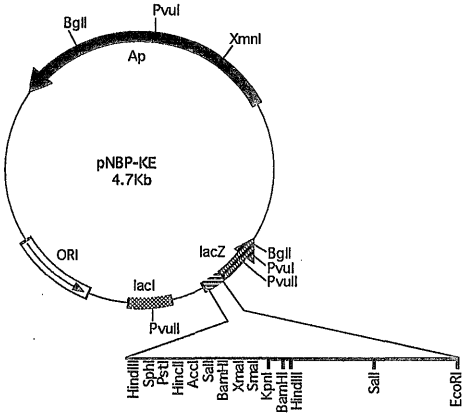
The plasmid in the upper band was recircularised by self-ligation and the *Kpn*I-*Kpn*I fragment was ligated into pUC19 vector digested with *Kpn*I. The ligated plasmids were transformed into competent *E. coli* DH5α cells. The cell suspension was plated onto LB plates containing 100μg ml^{-1} ampicillin, 25μg ml^{-1} IPTG and 40μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies and incubated overnight at 37°C.

White colonies were randomly picked and examined for recombinant plasmids. Plasmid pUC19 containing the *Kpn*I-*Kpn*I fragment was named pNBP-KK and the religated plasmid was named pNBP-KE.



pNBPE4 was digested with *Kpn*I and both the *Kpn*I-*Kpn*I fragment and plasmid were isolated

Plasmid religated



*Kpn*I-*Kpn*I fragment ligated in pUC19 cut with *Kpn*I

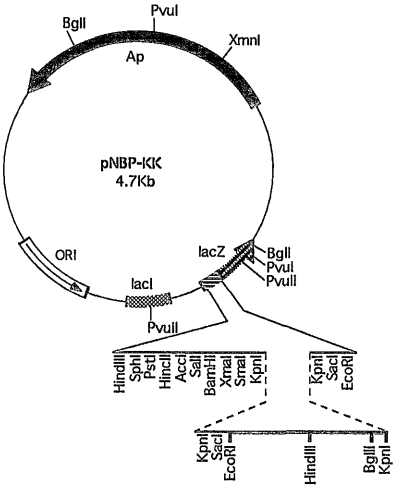
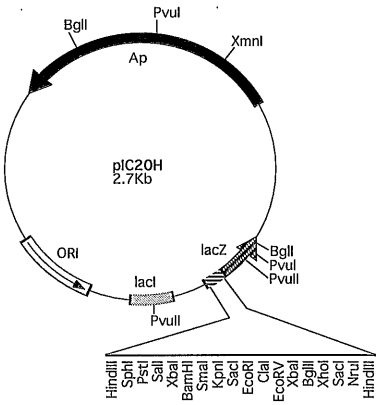


Figure 5.10 Subcloning the *Bgl*II-*Eco*RI fragments from pNBP-Eco into the pIC20H vector

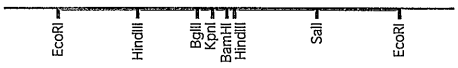
The plasmid pNBP-Eco was simultaneously digested with the restriction endonucleases *Bgl*II and *Eco*RI. The resulting fragments were separated by electrophoresis on a 0.6% LGT agarose gel. The two lower bands, corresponding to the 1.6kb and 2.4kb *Bgl*II-*Eco*RI fragments, were recovered from the gel as described in chapter 2.

The fragments were ligated into pIC20H vector digested with *Bgl*II and *Eco*RI. Ligated plasmids were transformed into competent *E. coli* DH5α cells. The cell suspension was plated onto LB plates containing 100μg ml^{-1} 25μg ml^{-1} IPTG and 40 μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies and incubated overnight at 37°C.

The white colonies were randomly picked and restriction analysis carried out on their plasmid DNA to confirm the construction of the correct plasmids. The correct constructs in pIC20H containing the 1.6kb and 2.4kb *Bgl*II-*Eco*RI fragments, were named pNBP-BE16 and pNBP-BE24, respectively.

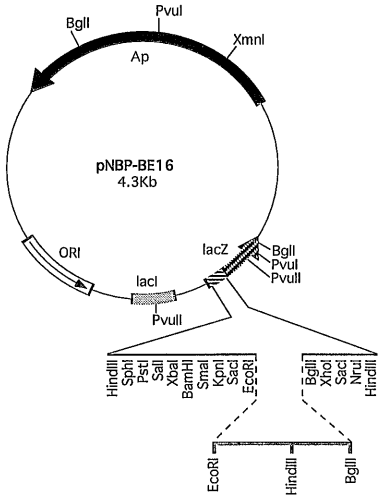


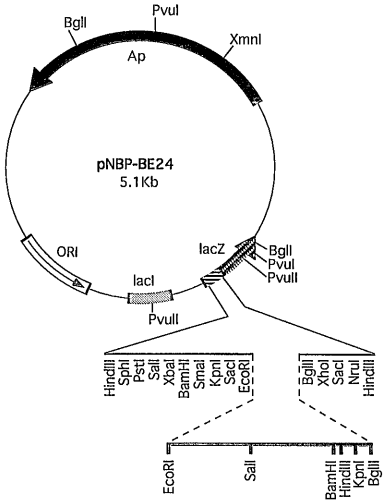
pIC20H was digested with *Bgl*II and *Eco*RI



pNBP-Eco was digested with BglII and EcoRI
the two BglII-EcoRI insert fragments were recovered

Ligation





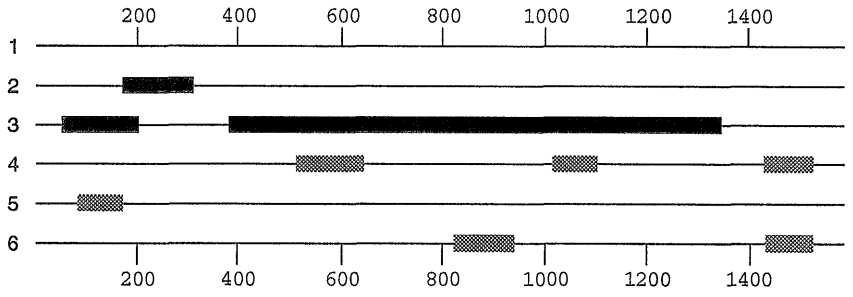


Figure 5.11 Open reading frame analysis of the assembled sequence from NBP4 and NBP-Eco

The open reading frame analysis shows ORFs in all six potential frames which are larger than 25 amino acids in length. All but the large ORF in frame three are very small. The large ORF shown in this analysis is 322 amino acids long.

A second feature important for transcription initiation is the CAAT box with the consensus sequence G G Y C A A T C T (McKnight and Kingsbury, 1982). This sequence has been found in several (but not all) promoters and in higher eukaryotes is often located 80bp upstream of the transcription initiation site. Although the function of this element is not well defined, it is thought to be a recognition sequence for transcription factors (La Thangue and Rigby, 1988). Sequences related to the CAAT box have also been described in the yeast *S. cerevisiae* (Ballance, 1986) and the region homologous to the CAAT box is often referred to as upstream activation sequence 2 (UAS2) (La Thangue and Rigby, 1988).

Dobson *et al.* (1982) described a high efficiency promoter sequence in highly expressed yeast genes. This sequence contains a block of residues rich in CT followed by CAAG, approximately 10bp downstream. In addition, mutation analysis of the *CYC1* locus has indicated that the hexanucleotide CACACA may be involved in translation initiation events (Stiles *et al.*, 1981) and, when present, the sequence is usually found 10bp to 25bp upstream of the translation start (Cigan and Donahue, 1987; Dobson *et al.*, 1982)

The 3' untranslated region of eukaryotic genes frequently contains a polyadenylation signal and additional stop codons. Mammalian polyadenylation signals are now well defined; they contain two elements, the AAUAAA sequence 20-30 nucleotides upstream and a diffuse GU-rich sequence immediately downstream of the 3' end of the mRNA (Proudfoot, 1991).

Although analysis of *S. cerevisiae* mRNA indicates that polyadenylation occurs, the AAUAAA signal is not generally found. Moreover, different sequences have been shown to be involved in the formation of correct mRNA 3' ends. For example, Henikoff and Cohen (1984), identified the octanucleotide UUUUUAUA as being essential for 3' end formation of the *Drosophila melanogaster ADE-8* gene expressed in *S. cerevisiae*. Whereas, Zaret and Sherman (1982) found that a *cyc1* mutant defective in normal *CYC1* 3' end formation lacked a 38bp region containing the tripartite sequence UAG...UA(U)GU...UUU. One or other of these sequence motifs has been found near the polyadenylation site of many yeast genes (Irniger *et al.*, 1991).

Analysis of revertants of the original *CYC1* deletion mutant described above has shown that other related sequences, such as UAUAUA and UACAUA, may also be involved in RNA polyadenylation (Russo *et al.*, 1991). In addition, the polyadenylation signal of another *S. cerevisiae* gene, *GAL7*, has been characterised as being AUAUAUAUAUAUAAUAAUGACAUCAUU, again suggesting that AU-rich sequences are important in the polyadenylation signal (Abe *et al.*, 1990). It has been suggested (Irniger *et al.*, 1991) that yeast may have a number of mechanisms for the polyadenylation of mRNA, with different sequence elements directing these processes.

In higher eukaryotes the formation of mRNA 3' termini can be divided into three distinct reactions: transcription termination, cleavage of the primary transcript at its 3' end and the addition of the poly(A) tail (Heidmann *et al.*, 1992). However, despite extensive work on yeast mRNA processing, the mechanism leading to the 3' end formation of yeast mRNA cannot be divided into distinct processing reactions. This, along with the observation that all mRNA in *S. cerevisiae* including histone mRNA (Farhner *et al.*, 1980) and aberrant transcripts (Zaret and Sherman, 1982) are polyadenylated, has led to the suggestion that transcription termination and polyadenylation may be directly coupled events (Osborne and Guarente, 1988; Rosso and Sherman, 1989).

The only efficient initiation codon in *S. cerevisiae* is AUG. A compilation of 131 yeast genes suggests that all 131 use an AUG triplet as the initiation codon (Cigan and Donahue, 1987). Quantitative measurements of the expression of various alleles containing mutations in the initiation codon show that non-AUG codons support only very low levels of protein synthesis, usually 2% or less of the wild-type allele.

The nucleotides surrounding the initiation codon are thought to be non-random. There is a strong preference (≈45%) for A nucleotides and a scarcity (≈12%) of G nucleotides in the mRNA leader regions of yeast genes (Cigan and Donahue, 1987; Hamilton *et al.*, 1987). The preference for A is particularly striking at the -3 position relative to the AUG codon (75%) and occurs with the virtual exclusion of pyrimidines which account for only 3% of the residues found at -3. In highly expressed genes, this non-random sequence distribution is even more striking, with the occurrence of A

nucleotides at the -3 position approaching 100% and well over 50% at the -1, -2, -5, -6 and -7 positions. At the latter five positions, and also at -4, a pyrimidine always occurs when an A nucleotide is not present (Cigan and Donahue, 1987).

Distribution of nucleotides downstream of the AUG codon is also non-random: 28% of all *S. cerevisiae* genes examined contain the UCU serine codon immediately following the initiation codon. Thr (10%), Leu (9.5%), Ala and Val (7.8% each), Lys and Phe (6% each) and Asn, Gly and Pro (4% each) are the next most abundant amino acid residues (Cigan and Donahue, 1987). However, the non-random distribution of amino acids seen at the +4 to +6 positions may reflect constraints on amino-terminal protein sequences, rather than sequence requirements for efficient translation initiation (Cigan and Donahue, 1987; Hamilton *et al.*, 1987).

The comparative analysis of *S. cerevisiae* initiator regions suggests a consensus sequence surrounding start codons (5'-(A/Y) A (A/Y) A (A/Y) A **A U G** U C U-3') which differs from that compiled for vertebrate mRNA (5'-G C C G C C (A/G) C C **A U G** G-3') (Kozak, 1987), except for the strong preference for A at -3 (61% in vertebrate mRNAs).

In yeast, the importance of the consensus sequence around the start codon has been illustrated by mutation analysis. For example, replacing the A at the -3 position of the *CYC1* gene (Baim and Sherman, 1988) or the *HIS4* gene (Cigan *et al.*, 1988) with a less-preferred nucleotide causes an approximately two-fold decrease in gene expression. The A at -3 appears to have a dominant role, as nucleotide replacements elsewhere in the consensus sequence affect initiation only in the absence of an A at -3 (Kozak, 1986).

5.8.2 The ATG context and 5' non-coding region of the p35 gene

The complete p35 gene, including the 5'- and 3'- non-coding regions, is shown in Fig. 5.12. As described earlier, reading frame analysis of the sequence showed one large open reading frame of 966bp. The length of this ORF was based upon the distance between the first ATG which follows an upstream stop codon, to the first in-frame stop codon. Sequence analysis of the region surrounding the first ATG (at position -114, Fig. 5.12) reveals the absence of the usually highly conserved A at the -3 (see Fig. 5.13). It was also noticed that the first codon downstream of this ATG encodes tyrosine which is

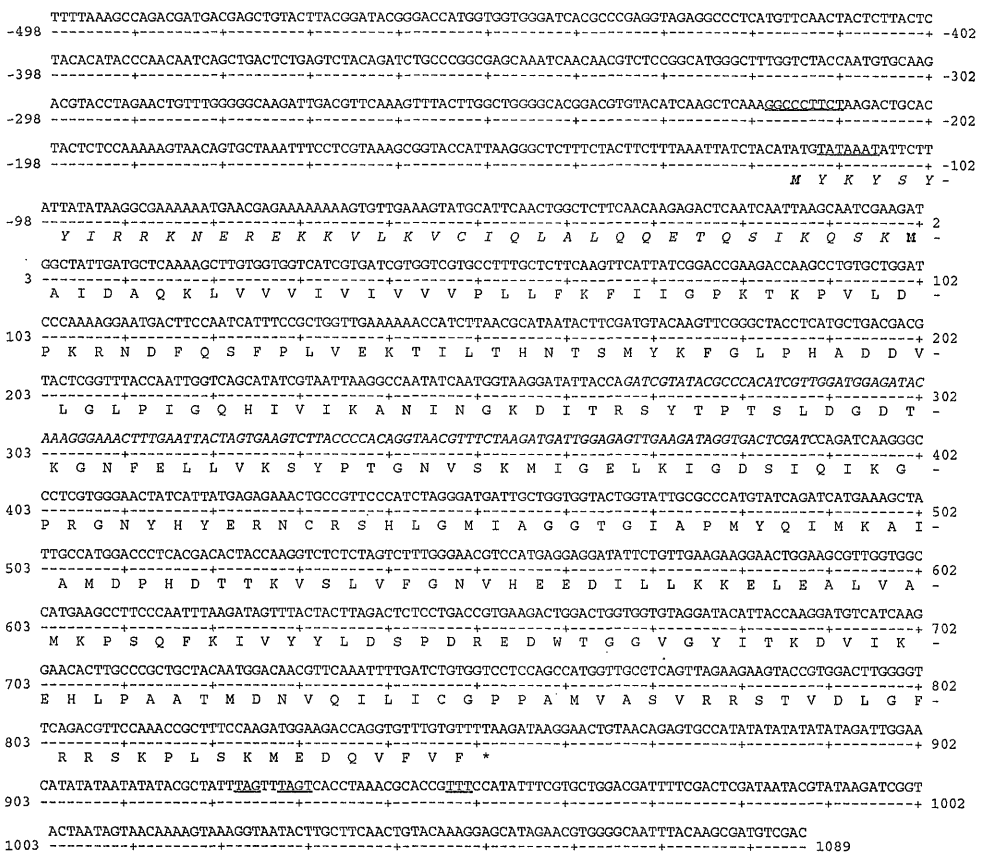


Figure 5.12: The complete nucleotide sequence of the p35 gene

The predicted amino acid sequence of the p35 protein is displayed with the two possible protein initiation methionines shown in bold.

Possible CAAT (-220), TATA (-114) and polyadenylation sequences(926, 931 & 950) are underlined.

The nucleotide sequence which matches the *Sau*3AI fragment from the pNov35 insert is shown in italics (270-390).

consensus	A/Y)	A	(A/Y)	A	(A/Y)	A	**A**	**U**	**G**	U	C	U
ATG^{-114}	C	U	A	C	A	U	**A**	**U**	**G**	U	A	U
ATG^{1}	U	C	G	A	A	G	**A**	**U**	**G**	G	C	U

Figure 5.13 Comparison of the consensus sequence surrounding the *S. cerevisiae* start codon with the sequences surrounding the putative start codons ATG^{-114} and ATG^{1} from the p35 gene

Analysis of *S. cerevisiae* initiator regions has suggested a consensus sequence surrounding start codons and this is displayed above, as are the sequences surrounding the putative start cocoons at positions -114 and 1 in the p35 gene. Comparison of these sequences suggests that the ATG at position 1 is the more likely translation start site as the sequence surrounding this ATG is closer the consensus sequence than the sequence surrounding the ATG at -114.

not a well conserved amino acid at this position. The sequence around the ATG at -114 shows a poor match to the consensus sequence indicating that it is an unlikely translation initiation site. Because of these findings, the sequence surrounding the ATG site downstream of the ATG^{-114} was analysed. The following ATG was found to be 114bp downstream, at position 1 (Fig. 5.12). Analysis of the sequence surrounding the ATG at this position identified an A at the -3 position. In addition, the first codon downstream of the ATG encodes alanine which is present at this position in 7.8% of yeast transcripts analysed so far. This analysis indicates that the sequence around the ATG^{1} is closer to the consensus sequence required for efficient translation initiation than that found around the ATG at -114.

The Mac Vector molecular biology program was used to search for the TATA and CAAT subsequences. A number of putative TATA box sequences were found downstream of ATG^{-114}, including a perfect TATA at position -111 (TATAAATA). Identification of this TATA box indicates that the transcription initiation site is likely to be downstream of the ATG at -114. In the majority of yeast genes, the ATG nearest to the 5' end of the mRNA serves as the translation start site. The position of the TATA box indicates that transcription of the p35 mRNA may start downstream of the ATG^{-114}, again suggesting that the translation start of the p35 gene is likely to be the ATG at position 1. Analysis of the predicted amino acid sequence is presented in chapter 6 and further supports this conclusion.

A putative CAAT box was identified at position -217 (GGCCctTCT). Unfortunately, since the transcription start has not been positively identified, it is difficult to assess if this CAAT box is located in a normal position. In addition, a hexanucleotide CACACA is present at position -400. However, this is approximately 380bp upstream of its normally observed position, close to the translation start and is therefore unlikely to be significant. The high efficiency promoter sequence described by Dobson *et al.* (1982) is not present in the 5' sequence.

5.8.3 Open reading frame: the codon usage in the p35 gene

The DNA sequence analysis of the 5'-region of the p35 gene (see above) indicated that the initial open reading frame analysis may have been incorrect in assigning the ATG^{-114} as the translation start. The sequence analysis strongly suggests that the ATG^{1} is likely to be the translation start of the protein. In this case, the p35 gene would have an open reading frame of 852bp extending from position 1 to 852 and encode a protein with a predicted molecular mass of 31,491.

Yeast genes generally, and highly expressed genes in particular, exhibit a bias in the codon usage (Bennetzen and Hall, 1982). In highly expressed genes, the codon most frequently used to encode a specific amino acid is complementary to the anticodon of the most abundant tRNA for that residue. This means that for any given amino acid there is a "preferred" codon, which ensures that the most abundant tRNA is used to add the residue to the growing polypeptide. The "codonfrequency" program of the molecular biology computer package (Devereux *et al.*, 1984) was used to compile a codon usage table for the p35 gene (Table 5.1). This codon usage was compared to a codon usage table compiled by Dr. M. Stark from the sequence of 19 highly expressed *S. cerevisiae* genes (Table 5.2). Several unusual codons, rarely used in genes which are highly expressed in *S. cerevisiae*, are used in the p35 gene. The most noticeable deviations in codon usage in the p35 gene occur for histidine, glutamine, phenylalanine and valine. The codon CAT is only used to encode 18% of the histidine in highly expressed genes, whereas it is used to encode 75% of the histidine in the p35 gene. In the case of glutamine, the codon CAG is not used at all in highly expressed proteins, whereas it encodes 50% of the glutamine in the p35 gene. The situation is very similar in the case of the codon GAG which encodes only 1% of valines in highly expressed genes but 42% of the valine residues in the p35 protein. Also, only 8% of phenylalanine residues in highly expressed genes are encoded by TTT, whereas in the p35 protein 55% of the phenylalanine residues are encoded by this codon. A number of other amino acids exhibiting aberrant codon usage are shown in Table 5.1. Codon usage for the remaining amino acids encoding the p35 protein appears to broadly agree with that described in the reference table.

AmAcid	Codon	Number	/1000	Fraction	AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	5.00	17.54	0.23	Trp	TGG	1.00	3.51	1.00
Gly	GGA	5.00	17.54	0.23	End	TGA	0.00	0.00	0.00
Gly	GGT	11.00	38.60	0.50	Cys	TGT	1.00	3.51	0.50
Gly	GGC	1.00	3.51	0.05	Cys	TGC	1.00	3.51	0.50
Glu	GAG	4.00	14.04	0.36	End	TAG	0.00	0.00	0.00
Glu	GAA	7.00	24.56	0.64	End	TAA	1.00	3.51	1.00
Asp	GAT	7.00	24.56	0.37	Tyr	TAT	4.00	14.04	0.44
Asp	GAC	12.00	42.11	0.63	Tyr	TAC	5.00	17.54	0.56
Val	GTG	11.00	38.60	0.42	Leu	TTG	8.00	28.07	0.33
Val	GTA	3.00	10.53	0.12	Leu	TTA	4.00	14.04	0.17
Val	GTT	6.00	21.05	0.23	Phe	TTT	6.00	21.05	0.55
Val	GTC	6.00	21.05	0.23	Phe	TTC	5.00	17.54	0.45
Ala	GCG	2.00	7.02	0.14	Ser	TCG	4.00	14.04	0.27
Ala	GCA	0.00	0.00	0.00	Ser	TCA	2.00	7.02	0.13
Ala	GCT	7.00	24.56	0.50	Ser	TCT	4.00	14.04	0.27
Ala	GCC	5.00	17.54	0.36	Ser	TCC	4.00	14.04	0.27
Arg	AGG	1.00	3.51	0.10	Arg	CGG	0.00	0.00	0.00
Arg	AGA	5.00	17.54	0.50	Arg	CGA	0.00	0.00	0.00
Ser	AGT	1.00	3.51	0.07	Arg	CGT	4.00	14.04	0.40
Ser	AGC	0.00	0.00	0.00	Arg	CGC	0.00	0.00	0.00
Lys	AAG	20.00	70.18	0.83	Gln	CAG	4.00	14.04	0.50
Lys	AAA	4.00	14.04	0.17	Gln	CAA	4.00	14.04	0.50
Asn	AAT	4.00	14.04	0.40	His	CAT	6.00	21.05	0.75
Asn	AAC	6.00	21.05	0.60	His	CAC	2.00	7.02	0.25
Met	ATG	11.00	38.60	1.00	Leu	CTG	4.00	14.04	0.17
Ile	ATA	2.00	7.02	0.08	Leu	CTA	4.00	14.04	0.17
Ile	ATT	12.00	42.11	0.48	Leu	CTT	2.00	7.02	0.08
Ile	ATC	11.00	38.60	0.44	Leu	CTC	2.00	7.02	0.08
Thr	ACG	2.00	7.02	0.13	Pro	CCG	3.00	10.53	0.17
Thr	ACA	4.00	14.04	0.25	Pro	CCA	2.00	7.02	0.11
Thr	ACT	4.00	14.04	0.25	Pro	CCT	8.00	28.07	0.44
Thr	ACC	6.00	21.05	0.38	Pro	CCC	5.00	17.54	0.28

Table 5.1: Codon usage of the p35 gene.

Codons underlined are codons rarely used in highly expressed genes but whose use has increased by 30-56% within their synonymous family.
Codons underlined are codons rarely used in highly expressed genes but whose use has increased by 20-30% within their synonymous family.
AmAcid= Amino Acid
Number= represents the number of amino acids in the gene encoded by the corresponding codon
/1000= Codon observations normalised to frequency per thousand
Fraction= The fraction a codon is used within its synonymous family

AmAcid	Codon	Number	/1000	Fraction	AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	2.00	0.46	0.01	Trp	TGG	31.00	7.17	1.00
Gly	GGA	2.00	0.46	0.01	End	TGA	0.00	0.00	0.00
Gly	GGT	342.00	79.09	0.96	Cys	TGT	27.00	6.24	0.93
Gly	GGC	9.00	2.08	0.03	Cys	TGC	2.00	0.46	0.07
Glu	GAG	4.00	0.93	0.02	End	TAG	0.00	0.00	0.00
Glu	GAA	228.00	52.73	0.98	End	TAA	1.00	0.23	1.00
Asp	GAT	80.00	18.50	0.37	Tyr	TAT	7.00	1.62	0.06
Asp	GAC	138.00	31.91	0.63	Tyr	TAC	112.00	25.90	0.94
Val	GTG	4.00	0.93	0.01	Leu	TTG	274.00	63.37	0.82
Val	GTA	0.00	0.00	0.00	Leu	TTA	42.00	9.71	0.13
Val	GTT	198.00	45.79	0.53	Phe	TTT	11.00	2.54	0.08
Val	GTC	174.00	40.24	0.46	Phe	TTC	126.00	29.14	0.92
Ala	GCG	1.00	0.23	0.00	Ser	TCG	1.00	0.23	0.00
Ala	GCA	7.00	1.62	0.02	Ser	TCA	7.00	1.62	0.03
Ala	GCT	290.00	67.07	0.73	Ser	TCT	142.00	32.84	0.57
Ala	GCC	99.00	22.90	0.25	Ser	TCC	92.00	21.28	0.37
Arg	AGG	1.00	0.23	0.00	Arg	CGG	0.00	0.00	0.00
Arg	AGA	270.00	62.44	0.86	Arg	CGA	0.00	0.00	0.00
Ser	AGT	2.00	0.46	0.01	Arg	CGT	43.00	9.94	0.14
Ser	AGC	7.00	1.62	0.03	Arg	CGC	0.00	0.00	0.00
Lys	AAG	360.00	83.26	0.86	Gln	CAG	0.00	0.00	0.00
Lys	AAA	61.00	14.11	0.14	Gln	CAA	125.00	28.91	1.00
Asn	AAT	12.00	2.78	0.07	His	CAT	17.00	3.93	0.18
Asn	AAC	153.00	35.38	0.93	His	CAC	79.00	18.27	0.82
Met	ATG	80.00	18.50	1.00	Leu	CTG	1.00	0.23	0.00
Ile	ATA	0.00	0.00	0.00	Leu	CTA	15.00	3.47	0.04
Ile	ATT	111.00	25.67	0.45	Leu	CTT	3.00	0.69	0.01
Ile	ATC	138.00	31.91	0.55	Leu	CTC	0.00	0.00	0.00
Thr	ACG	1.00	0.23	0.00	Pro	CCG	0.00	0.00	0.00
Thr	ACA	2.00	0.46	0.01	Pro	CCA	156.00	36.08	0.91
Thr	ACT	107.00	24.75	0.48	Pro	CCT	13.00	3.01	0.08
Thr	ACC	112.00	25.90	0.50	Pro	CCC	2.00	0.46	0.01

Table 5.2: Codon usage of highly expressed genes.

Compiled by Dr. M. Stark (Dundee University) from 19 highly expressed genes
AmAcid= Amino Acid
Number= represents the number of amino acids in the gene encoded by the corresponding codon
/1000= Codon observations normalised to frequency per thousand
Fraction= The fraction a codon is used within its synonymous family

The comparison of the codon usage between highly expressed yeast genes and the p35 gene shows that a number of codons found in the p35 gene are rarely used in highly expressed genes. This would seem to indicate that the p35 gene does not encode a highly expressed protein.

5.8.4 Untranslated 3' non-coding region of the p35 gene

The polyadenylation tripartite sequence UAG...UA(U)GU...UUU described by Zaret and Sherman (1982) was found at position 925, 75bp downstream of the p35 gene stop codon. In this case, the signal is in the form UAG...UAGU...UUU and is underlined in Fig. 5.12. The involvement of PolyTA regions in enhancing the use of downstream polyadenylation sites has been suggested by Russo *et al.* (1991). PolyTA regions are present in the 3' non-coding region of the p35 gene; from position 881, TA is repeated seven times and between position 905 and 917 it is repeated six times. However, Heidmann *et al.* (1992) suggested that the sequences described above, in a general AT rich environment, enhance polyadenylation events downstream but are not sufficient to direct the choice of all the actual polyadenylation sites used. They suggest that a second signal is encoded within the cleavage sites themselves and that sequence motifs comprising a pyrimidine followed by one or more adenosines ($Py(A)_n$) are used preferentially.

Following the stop codon at position 854 (Fig. 5.12), two additional termination signals are found at positions 896 and 926.

5.9 Discussion

DNA sequence analysis of the p35 gene indicates that the translation start is likely to be the ATG at position 1. Taking this ATG as the translation start, the gene has an open reading frame of 852bp, extending from position 1 to 852. As is common for many yeast genes, the p35 gene is uninterrupted by introns (Fink, 1987). The open reading frame predicts a protein of 31,491Da which is preceded by a TATA box located at -111. In *S. cerevisiae*, the TATA box is usually located 40-90bp upstream of the transcription start site

(Lewin, 1990) and so the transcription initiation site of the p35 gene may lie between positions -71 and -21. In a study of eighteen *S. cerevisiae* promoters, Hahn *et al.* (1985) found that 50% of transcription initiation occurred at the sequences TC(G/A)A and RRYRR (R=purine; Y=pyrimidine). These sequences occur a number of times within the -71 to -21 region of the p35 gene where transcription initiation is likely to occur. If time had allowed, identification of the transcription start site by primer extension (Sambrook *et al.*, 1989) or S1 mapping (Berk and Sharp, 1977) may have resolved the question of translation start position.

A CAAT box-like sequence was also identified in the 5' non-coding region at position -217 (GGCCctTCT). However, none of the yeast-specific promoter sequences were found close to the putative initiation codon.

The biological significance of codon bias involves the rate of mRNA translation, with rapid translation ensuing from the use of the preferred codons. If highly expressed genes used rare triplet codons, the pool of tRNAs with complementary anticodons could soon be depleted, resulting in all protein synthesis being slowed down due to the lack of specific tRNAs. In contrast, proteins which are not abundant could use the rarer codons without affecting highly expressed mRNA translation. It should be noted that the overall number of yeast genes used to compile the reference codon usage table is very small in comparison to the total predicted number of genes. Nevertheless, results of the codon bias analysis suggest that the p35 gene is not a highly expressed protein.

The polyadenylation signal UAG...UAGU...UUU, found at the 3' end of the message (position 926, 931 and 950), is typical of those found in other yeast genes, as is the presence of AT repeats. However the exact mechanism by which polyadenylation occurs in *S. cerevisiae* is still elusive. Thus, the significance of these motifs remains unclear.

As described above, a TATA box is located at -111 and this indicates that the translation start is 40-90 bp downstream. Taking the shorter of these distances and calculating the distance between this and the putative polyadenylation site 10 bp down-stream of the of the polyadenylation signal (UAG...UAGU...UUU) at 950 gives a rough

estimate of the transcript size for the p35 gene as 1030bp. This is a little shorter than the 1.2kb estimated by Northern blot analysis. However, neither figure is an exact measurement and probably contain a margin of error which could explain this difference.

Chapter 6

Analysis of the p35 gene product

6.1 Introduction

The GCG molecular biology computer programs (Devereux *et al.,* 1984) provide useful tools for the manipulation of DNA sequences. The programs allow the translation of DNA sequences into polypeptide sequences and both can be compared to known sequences in various protein and DNA data bases, *i.e.* Swissprot, EMBL or Genbank data bases. Thus any homology to previously identified genes can be identified. In addition, certain physical properties of translated sequences can be predicted, such as hydrophobicity, antigenicity etc. These features can often help in the identification of the biological properties of proteins

6.2 Analysis of the predicted p35 gene protein

The DNA sequence of the p35 gene was translated into a polypeptide sequence using the GCG programs. This analysis indicates that the protein encoded by the p35 gene consists of 290 amino acid residues (see Table 6.1) with a molecular mass of approximately 31491Da and a predicted isoelectric point of 8.89. The discrepancy seen here between the predicted molecular mass and the apparent molecular mass gauged from SDS-PAGE is a phenomenon observed for many proteins (Hames, 1990).

The predicted protein sequence was used to screen the EMBL data base using the GCG program TFASTA. The results showed the p35 gene product to have high homology to the FAD domain of plant nitrate reductases (30-33% identity, 53-58% similarity)(Calza *et al.*, 1987; Crawford *et al.*, 1988; Daniel-Vedele *et al.*, 1989) and to mammalian NADH-cytochrome b_5 reductases (34-37% identity, 61-63% similarity) (Ozols *et al.*, 1985; Yubisui *et al.*, 1986 & 1987; Pietrini *et al.*, 1988). The GCG dotplot program was used to compare the p35 gene product, human NADH-cytochrome b_5 reductase and tomato nitrate

Amino Acid Composition:

Non-polar:

	No.	Percent
A	14	4.93
V	26	9.15
L	24	8.45
I	25	8.80
P	18	6.34
M	11	3.87
F	11	3.87
W	1	0.35

Polar:

	No.	Percent
G	22	7.75
S	15	5.28
T	16	5.63
C	2	0.70
Y	9	3.17
N	10	3.52
Q	8	2.82

Acidic:

	No.	Percent
D	19	6.69
E	11	3.87

Basic:

	No.	Percent
K	24	8.45
R	10	3.52
H	8	2.82

Calculated Molecular Weight = 31491

Estimated pI = 8.89

Table 6.1 Amino Acid Composition of the 35kDa protein

reductase amino acid sequences; homology is indicated by diagonal lines in Fig. 6.1. The dotplot comparison of the p35 gene product and tomato nitrate reductase indicates that only the C-terminal region of the plant protein shows homology to the p35 gene product. This C-terminal region is thought to be the FAD-binding domain of plant nitrate reductases (Crawford *et al.*, 1988) and this region has previously been shown to have significant homology to mammalian NADH-cytochrome b_5 reductases (Crawford *et al.*, 1988; Daniel-Vedele *et al.*, 1989).

6.2.1 NADH-cytochrome b_5 reductase

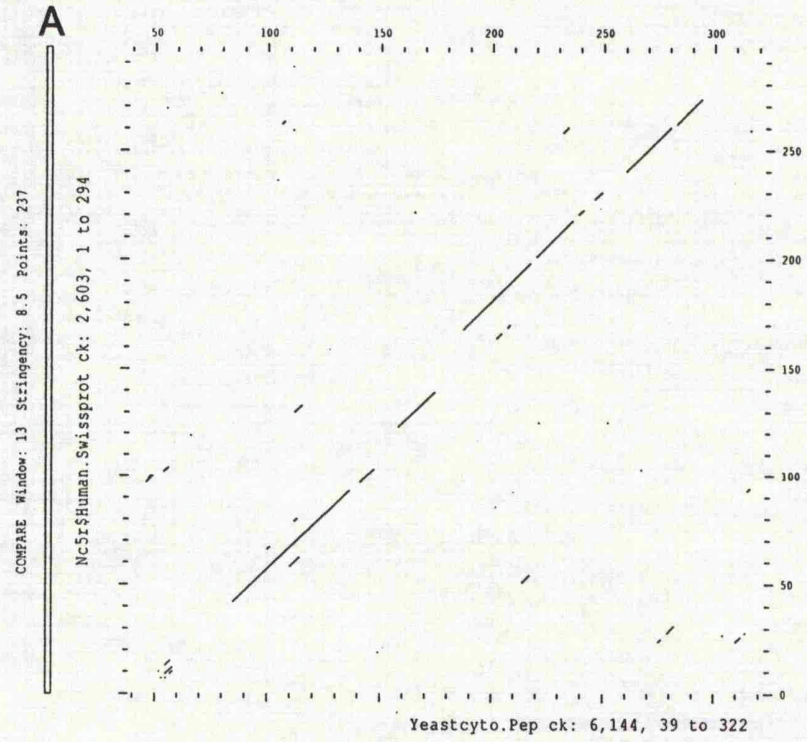
Flavoenzymes catalyse a variety of reactions by transferring one or two electrons between chemically diverse donor and acceptor molecules. NADH-cytochrome b_5 reductase (NADH: ferricytochrome-b_5 oxidoreductase) is a representative flavoprotein, having flavin adenine dinucleotide (FAD) as its prosthetic group. Cytochrome b_5 reductase is an important component of the microsomal electron transport system catalysing the reduction of cytochrome b_5 which contains the prosthetic group protoheme. The enzyme contains a large hydrophilic catalytic domain and a small N-terminal hydrophobic membrane-binding domain (Spatz and Strittmatter, 1973). The enzyme exists in two forms, a membrane-bound form composed of both the catalytic and the membrane-binding domains and a soluble form comprising only the catalytic domain. The soluble form is found in circulating erythrocytes and participates in the methaemoglobin reaction which proceeds as follows: cytochrome b_5 reductase reduces cytochrome b_5 which in turn reduces methaemoglobin to form haemoglobin (Hultquist and Passon, 1971). The membrane-bound form of the enzyme is localised mainly on the cytoplasmic side of the endoplasmic reticulum, mitochondria, nuclear and plasma membranes of somatic cells. The deficiency of cytochrome b_5 reductase in human erythrocytes and somatic cells is known to occur in hereditary methaemoglobinemia (Leroux *et al.*, 1975). Mainly, two types of enzyme deficiency are known; erythrocyte type (type I), in which the enzyme is deficient only in erythrocytes resulting in mild cyanosis, and generalised type (type II), in which the enzyme is deficient not only in erythrocytes but also in other somatic cells (Takeshita *et al.*,

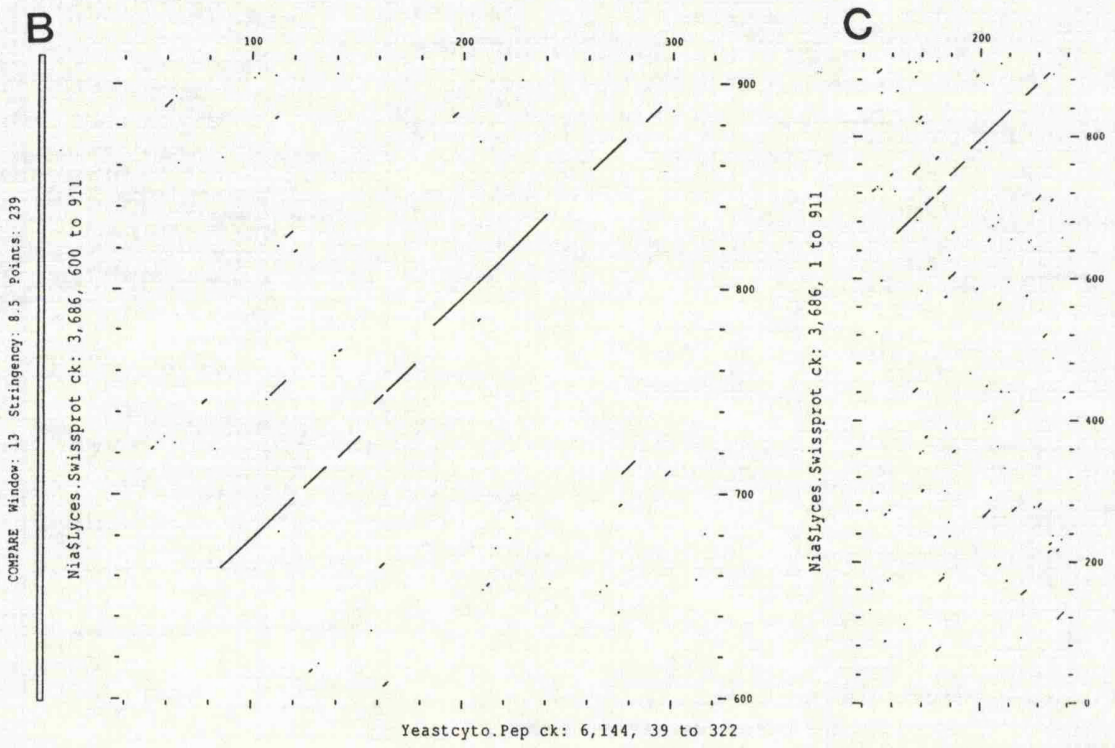
Figure 6.1 Dotplot comparisons of the predicted amino acid sequence from the p35 gene with the amino acid sequences of human NADH-cytochrome b_5 reductase and tomato NADH-nitrate reductase

A. Comparison of the predicted amino acid sequence from the p35 gene and human NADH-cytochrome b_5 reductase.

B. Comparison of the predicted amino acid sequence from the p35 gene and amino acid residues 600 to 911 of tomato NADH-nitrate reductase.

C. Comparison of the predicted amino acid sequence from the p35 gene and the full amino acid sequence of tomato NADH-nitrate reductase. Homology between the two sequences is only observed in the C-terminal region of the nitrate reductase sequence.


A
COMPARE Window: 13 Stringency: 8.5 Points: 237
Nc5r$Human.Swissprot ck: 2,603, 1 to 294
Yeastcyto.Pep ck: 6,144, 39 to 322
50
100
150
200
250
300
0
50
100
150
200
250


B
COMPARE Window: 13 Stringency: 8.5 Points: 239
Nia$Lyces.Swissprot ck: 3,686, 600 to 911
Yeastcyto.Pep ck: 6,144, 39 to 322
100
200
300
600
700
800
900
C
Nia$Lyces.Swissprot ck: 3,686, 1 to 911
200
0
200
400
600
800

1982). Type II is a severe form accompanied by mental retardation and neurological impairment (Takeshita *et al.*, 1982).

Cytochrome b_5 reductase has been shown to participate in the desaturation of fatty acids (Keyes and Cinti, 1980; Oshino *et al.*, 1971), sterol (cholesterol) biosynthesis (Reddy *et al.*, 1977) and drug metabolism (Hildebrandt and Estabrook, 1971). Desaturation of fatty acids and sterol (cholesterol) biosynthesis are all cytochrome b_5-dependent reactions. The role of cytochrome b_5 reductase in these reactions is to transfer electrons from NADH to cytochrome b_5 which, in turn, transfers the electrons to the enzyme components of the reactions (Fig. 6.2). Drug metabolism is mediated by the cytochrome P-450 dependent monooxygenase reaction (Black and Coon, 1987). In the majority of cases cytochrome P-450 is reduced by cytochrome P-450 reductase. However, under certain conditions cytochrome b_5 is also thought to donate the electrons required for cytochrome P-450 function (Black and Coon, 1987; Aoyama *et al.*, 1990). Again, the role of cytochrome b_5 reductase in this reaction is to transfer electrons from NADH to cytochrome b_5.

6.2.2 Nitrate reductase

Nitrate reductase is a key enzyme involved in the first step of nitrate assimilation in plants (for review see Solomonson and Barber, 1990). It is a multicentre electron-transfer protein catalysing the reduction of nitrate into nitrite:

$$NO_3^- + NADH + H^+ \rightarrow NO_2 + NAD^+ + H_2O$$

In higher plants, the native enzyme is a homodimer of 110-115kDa. Each subunit is associated with three prosthetic groups (flavin, haem and molybdenum) each bound by distinct domains. The flavin group is FAD, the haem is typical of those found in b-type cytochromes and the molybdenum is complexed with a small organic pterin cofactor. The deduced amino acid sequences for nitrate reductase have been derived from DNA clones of a number of higher plant nitrate reductases (Solomonson and Barber, 1990). Analysis of these amino acid sequences suggests the presence of three domains corresponding to the regions forming the molybdenum-pterin, haem and flavin-binding domains (Crawford *et al.*, 1988; Daniel-Vedele *et al.*, 1989). These domains were identified because of the

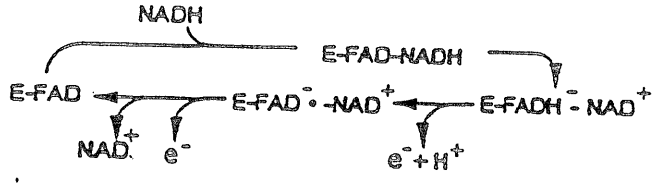


Figure 6.2 Reaction mechanism of NADH-cytochrome b_5 reductase

NADH-cytochrome b_5 reductase function is to catalyse the transfer of two electrons from NADH to two molecules of cytochrome b_5 as shown above. E indicates NADH-cytochrome b_5 reductase and FAD^- denotes the red type semiquinone (Shirabe *et al.*, 1991).

extensive conservation of their residues with similar cofactor-binding domains in other proteins. For example, the N-terminal region of nitrate reductase binds molybdenum-pterin and exhibits significant sequence similarity to the molybdenum-pterin domain of sulphite oxidase, whereas the central haem-binding domain exhibits significant sequence similarity to the cytochrome b_5 superfamily. The flavin-binding C-terminal region of nitrate reductase is very similar to the FAD-binding domain of cytochrome b_5 reductase. Thus nitrate reductase appears to be a three-redox-centre enzyme and is thought to have evolved by gene fusion between sequences coding for the one-redox-centre proteins described above (Daniel-Vedele *et al.*, 1989).

6.2.3 Line-up comparisons

A line-up of the p35 gene product against the human, rat and bovine cytochrome b_5 reductases and the FAD-binding domain of spinach, pumpkin, tobacco, *Arabidopsis*, pea and tomato nitrate reductases is shown in Fig. 6.3. This line-up only indicates homology to the p35 gene product and not homology between mammalian cytochrome b_5 reductases and the FAD domain of plant nitrate reductases. In this line-up, it is easy to identify amino acids which have been conserved between the p35 gene product and mammalian cytochrome b_5 reductases or plant nitrate reductases but not conserved between all three proteins. For example, the glycine at position 150 is conserved in both the p35 gene product and mammalian cytochrome b_5 reductases but not in plant nitrate reductases, whereas the opposite case is found for tyrosine-162. A line-up showing homology between the p35 gene product and consensus sequences derived from known mammalian cytochrome b_5 reductases and plant nitrate reductases is displayed in Fig. 6.4.

The molecular mass of the yeast gene product is similar to that of mammalian cytochrome b_5 reductases. In addition, the yeast protein contains a small highly hydrophobic N-terminal region (Fig. 6.5) which is similar to the N-terminal, membrane-binding domain of cytochrome b_5 reductases. This structural similarity with cytochrome b_5 reductases, taken along with the sequence homology and the protein size, suggests that the yeast gene product may be a functional homologue of the mammalian NADH-cytochrome b_5 reductase.

Figure 6.3 Line-up comparison of the predicted amino acid sequence from the p35 gene with mammalian NADH-cytochrome b_5 reductases and the FAD binding-domain of plant NADH-nitrate reductases.

The predicted amino acid sequence from the p35 gene (Yeastc5b) is aligned with the following cytochrome b_5 and nitrate reductases: human cytochrome b_5 reductase (CB5R:Hum) (Yubisui *et al.*, 1986), rat cytochrome b_5 reductase (CB5R:Rat) (Zenno *et al.*, 1990), bovine cytochrome b_5 reductase (CB5R:Bov) (Ozols *et al.*, 1985), the spinach nitrate reductase FAD binding-domain (Nia1:Sonr) (Prosser and Lazarus, 1990), the pumpkin nitrate reductase FAD binding-domain (Nia:Cucma) (Crawford *et al.*, 1986), the tobacco nitrate reductase FAD binding-domain (Nia2:Tobac) (Calza *et al.*, 1987), the *Arabidopsis* nitrate reductase FAD binding-domain (Nia1:Arat) (Cheng *et al.*, 1988) and the tomato nitrate reductase FAD binding-domain (Nia:Lyces) (Daniel-Vedele *et al.*, 1989).

In this alignment, only amino acid residues which show homology to the yeast p35 gene are shown in colour; red for identity, blue for conservative change.

```
                      1
                      +
Nia1:Sonr  LISTGYTSDS SSPGNSVHGG SVYSGLAGLA PITEAVPLRN VALN...PRV KI.PCKLIEK
Nia:Cucma  LITTGYASDS SSNSPNNSTH GAS..NFSHL APIREA.PVS RRVALA.PNE KI.PCKLISK
Nia2:Toba  LITTGYTSDS PGNSVHGSSS FSS....FLA PIKELV.PAQ RSVALI.PRE KI.PCKLIDK
Nia1:Arat  LITTGY..DS SPNVSVHGAS NFG...PLLA PIKEL..TPQ KNIALVNPRE KI.PVRLIEK
Nia:Lyces  LITTGYTSDS SPNSSVHGSS SIS...SFLA PIKELVQTPT RSVALI.PRE KI.PCKLVDK
Yeastc5b               MAIDAQKL VVVIVIVVVP LLFKFIIGPK TKPVLDPKRN DFQSFPLVEK
CB5R:Hum                   LGHM VLFPVWFLYS LLMKLFQRST PAITLESPDI KY.PLRLIDR
CB5R:Rat              GAQLSTLSRV VLSPVWFVYS LFMKLFQRSS PAITLENPDI KY.PLRLIDK
CB5R:Bov            M GAQLSTLGHV VLSPLWFLYS LIMKLFQRST PAITLENPDI KY.PLRLIDK
Consensus  Littgytsds s.nss.lg.s vls...flla .i..l.qr.t .aiale.pre ki.pc.Lidk

           50                                                       100
           +                                                        +
Nia1:Sonr  VSLSHDVRRF RFGLPSEDQV .LGLPVGKHI FLCANVDDKL CMRAYTPSS. TIDVVGYFDL
Nia:Cucma  TSISHDVRVF RFALPGGQDQ ALGLPVGKHI FICATVDGKL CMRAYTPTS. SIDEMGFFEL
Nia2:Toba  QSISHDVRKF RFALPSEDQV .LGLPVGKHI FLCAVIDDKL CMRAYTPTS. TIDEVGYFEL
Nia1:Arat  TSISHDVRKF RFALPSEDQQ .LGLPVGKHV FVCANINDKL CLRAYTPTS. AIDAVGHIDL
Nia:Lyces  QSISHDVRKF KFALPSEDQV .LGLPVGKHI FLCATVDDKL CMRAYTPTS. TVDEVGFFEL
Yeastc5b   TILTHNTSMY KFGLPHADDV .LGLPIGQHI VIKANINGKD ITRSYTPTSL DGDTKGNFEL
CB5R:Hum   EIISHDTRRF RFALPSPQHI .LGLPVGQHI YLSARIDGNL VVRPYTPIS. SDDDKGFVDL
CB5R:Rat   EIISHDTRRF RFALPSPQHI .LGLPIGQHI YLSTRIDGNL VIRPYTPVS. SDDDKGLVDL
CB5R:Bov   EVISHDTRRF RFALPSPEHI .LGLPVGQHI YLSARIDGNL VIRPYTPVS. SDDDKGFVDL
Consensus  .sisHdvrrf rFaLPsedqv .LGLPvGkHi flca.idgkl cmRaYTPtS. siD..GffdL


Nia1:Sonr  VVKVYFKDVH PRFPNGGVMS QHLDSLSLGS IVDVKGPLGH IEYLGKGNFT VHGKPKFA..
Nia:Cucma  VVKVYFKGVH PKFPNGGIMS QYLDSMEVGS TLDVKGPLGH IEYTGRGNFM VHGKPRFA..
Nia2:Toba  VVKIYFKGIH PKFPNGGQMS QYLDSMPLGS FLDVKGPLGH IEYQGKGNFL VHGKQKFA..
Nia1:Arat  VVKVYFKDVH PRFPNGGLMS QHLDSLPIGS MIDIKGPLGH IEYKGKGNFL VSGKPKFA..
Nia:Lyces  VVKIYFKGVH PKFPNGGQMS QHLDSLPIGA FLDVKGPLGH IEYQGKGNFL VHGKQKFA..
Yeastc5b   LVKSY..... ....PTGNVS KMIGELKIGD SIQIKGPRGN YHYERNCR.. ..........
CB5R:Hum   VIKVYFKDTH PKFPAGGKMS QYLESMQIGD TIEFRGPSGL LVYQGKGKFA IRPDKKSNPI
CB5R:Rat   VVKVYFKDTH PKFPAGGKMS QYLENMNIGD TIEFRGPNGL LVYQGKGKFA IRADKKSNPV
CB5R:Bov   VIKVYFKDTH PKFPAGGKMS QYLESMGIGD TIEFRGPNGL LVYQGKGKFA IRPDSKSDPV
Consensus  vvKvYfkdvh pkfpngGkmS qyldsmpiG. tidvkGPlGh ieYqgkgnf. vhgkpkfa..

                150                                                      200
                +                                                        +
Nia1:Sonr  ....KKLAMI SGGTGITPIY QVMQAILKDP EDKTEMHVVY ANRTEEDILL REELDKWADE
Nia:Cucma  ....RRLAML AGGTGITPIY QVVQAILKDP EDETEMYVVY ANRTEDDILL RDELDTWA.K
Nia2:Toba  ....KKLAMI AGGTGITPVY QVMQAILKDP EDDTEMYVVY ANRTEDDILL KEELDSWAEK
Nia1:Arat  ....KKLAML AGGTGITPIY QIIQSILSDP EDETEMYVVY ANRTEDDILV REELEGWASK
Nia:Lyces  ....KKDAMI AGGTGITPVY QVMQSILKDP EDDTEMYVVY ANRTEDDILL KDELDAWAEQ
Yeastc5b   ....SHLGMI AGGTGIAPMY QIMKAIAMDP HDTTKVSLVF GNVHEEDILL KKELEALVAM
CB5R:Hum   IRTVKSVGMI AGGTGITPML QVIRAIMKDP DDHTVCHLLF ANQTEKDILL RPELEELRNK
CB5R:Rat   VRTVKSVGMI AGGTGITPML QVIRAVLKDP NDHTVCYLLF ANQSEKDILL RPELEELRNE
CB5R:Bov   IKTVKSVGMI AGGTGITPML QVIRAIMKDP DDHTVCHLLF ANQTEKDILL RPELEELRDE
Consensus  ....kklaMi aGGTGItPmy Qv.qailkDP eDhTemyvvy aNrtEdDILl r.ELeewa.k

                                                         250
                                                         +
Nia1:Sonr  FRDRVKVWYV V.EKAEEGWK YDTGFISEKI LRDHVPAVGD DV.LALTCGP PPMIQFAVQP
Nia:Cucma  KNQRLKVWYV VQESIREGWE YSVGFITENI LREHIPAAAE DT.LALACGP PAMIQFAVQP
Nia2:Toba  IPERVKVWYV VQDSIKEGWK YSIGFITEAI LREHIPEPSH TT.LALACGP PPMIQFAVNP
Nia1:Arat  HKERLKIWYV V.EIAKEGWS YSTGFITEAV LREHIPEGLE GESLALACGP PPMIQFALQP
Nia:Lyces  VPNRVKVWYV VQESITQGWK YSTGFVTESI LREHIPEPSH TT.LALACGP PPMIQFAINP
Yeastc5b   KPSQFKIVYY LDSPDREDWT GGVGYITKDV IKEHLPAATM DNVQILICGP PAMVASVRRS
CB5R:Hum   HSARFKLWYT LD.RAPEAWD YGQGFVNEEM IRDHLPPPEE EP.LVLMCGP PPMIQYACLP
CB5R:Rat   HSSRFKLWYT VD.KAPDAWD YSQGFVNEEM IRDHLPPPGE ET.LILMCGP PPMIQFACLP
CB5R:Bov   HSARFKLWYT VD.KAPEAWD YSQGFVNEEM vRDHLPPPEE EP.LVLMCGP PPMIQYACLP
Consensus  h..rfKvwYv vde.apegW. ys.Gfiteei lreH.P.p.e .t.laLaCGP PpMiqfa..p

                                284
                                +
Nia1:Sonr  NLD......K MGFDIKEQLL IF*
Nia:Cucma  NLE......K MNYDTKNSLL VF*
Nia2:Toba  NLE......K MGYDIKDSLL VF*
Nia1:Arat  NLE......K MGYNVKEDLL IF*
Nia:Lyces  NLE......K MGYDIKEELL VF*
Yeastc5b   TVDLGFRRSK PLSKMEDQVF VF*
CB5R:Hum   NLD....... HVGHPTERCF VF*
CB5R:Rat   NLE....... RVGHPKERCF TF*
CB5R:Bov   NLD....... RVGHPKERCF AF*
Consensus  nle......k mgyd.kerll vF*
```

Figure 6.4 Line-up comparison of the predicted amino acid sequence from the p35 gene with mammalian NADH-cytochrome b_5 reductase and plant NADH-nitrate reductase consensus sequences.

The predicted amino acid sequence from the p35 gene (Yeastc5b) is aligned with the consensus sequences for cytochrome b_5 reductases and nitrate reductases.

The cytochrome b_5 reductase consensus sequence (CB5R:cons) was produced from the following sequences; human cytochrome b_5 reductase (Yubisui *et al.*, 1986), rat cytochrome b_5 reductase (Zenno *et al.*, 1990) and bovine cytochrome b_5 reductase (Ozols *et al.*, 1985).

The nitrate reductase consensus sequence (Nia:cons) was produced from the following sequences; the spinach nitrate reductase FAD binding-domain (Prosser and Lazarus, 1990), the pumpkin nitrate reductase FAD binding-domain (Crawford *et al.*, 1986), the tobacco nitrate reductase FAD binding-domain (Calza *et al.*, 1987), the *Arabidopsis* nitrate reductase FAD binding-domain (Cheng *et al.*, 1988) and the tomato nitrate reductase FAD binding-domain (Daniel-Vedele *et al.*, 1989).

In this alignment amino acid residues which show homology are coloured; red for identity, blue for conservative change.

```
                1
                +
 Nia:cons  GYtsDSs.ns svh.sss.s. ...flapike lv..pqrsva li.PreKI.P
CB5R:cons        GAQL STLghvVLsP vWFlYSL.MK LFQRStPAIT LEnPDIKY.P
 Yeastc5b          MA IDAQKLVVVI VIVVVPLLFK FIIGPKTKPV LDPKRNDFQS
Consensus  ......s... s.....v.s. v.....l..k l...p..... L..pr.k..p

               50
               +
 Nia:cons  ckLi.K.SiS HDVRkFrFaL Psedqv.LGL PVGKHiFlCA .vddKLCmRA
CB5R:cons  LRLIDREiIS HDTRRFRFAL PSPqHI.LGL PvGQHIYLSa RIDGNLViRP
 Yeastc5b  FPLVEKTILT HNTSMYKFGL PHADDV.LGL PIGQHIVIKA NINGKDITRS
Consensus  ..Li.K.iis Hdtr.frFaL Ps.d.v.LGL PvGqHI.l.A .idgkl..R.

               100
               +
 Nia:cons  YTPtS.tiDe vG.feLVVKv YFKgvHPkFP NGGqMSQhLD Slp.GsflDv
CB5R:cons  YTPvS.SDDD KGfVDLViKV YFKDTHPkFP AGGKMSQYLE sM.IGDTIEF
 Yeastc5b  YTPTSLDGDT KGNFELLVKS Y......... PTGNVSKMIG EcKIGDSIQI
Consensus  YTPtS...D. kG.feLvvKv Yfk..hpkfp .gG.mSq.l. sl.iGd.i..

                                          150
                                          +
 Nia:cons  KGPLGHIEYq GkGNFlVhGK pkFA...... kklAMiaGGT GITPiYQvmQ
CB5R:cons  RGPnGLLVYQ GKGKFAIRpD kKSnPvirTV KSVGMIAGGT GITPMLQVIR
 Yeastc5b  KGPRGNYHYE RNCR...... .......... SHLGMIAGGT GIAPMYQIMK
Consensus  kGP.G...Yq gkg.f..... .k........ k.lgMIAGGT GItPmyQvm.

                                            200
                                            +
 Nia:cons   aILkDPED.T EMyVVYANRT EdDILlreEL d.WAek.peR vKvWYVVqes
CB5R:cons   AimKDPdDHT VChLLFANQt EKDILLRPEL EELRneHSaR FKLWYTvD.k
 Yeastc5b   AIAMDPHDTT KVSLVFGNVH EEDILLKKEL EALVAMKPSQ FKIVYYLDSP
Consensus   AI.kDP.D.T ...lvfaN.t E.DILLr.EL e.l....p.r fK.wY.vd..

                                            250
                                            +
 Nia:cons   ikeGWkYstG FitEaiLReH iPeps..t.L ALaCGPPpMI QFAvqPNLe.
CB5R:cons   APeAWDYsQG FVNEEMIRDH LPPPeEEp.L vLMCGPPpMI QyACLPNLd.
 Yeastc5b   DREDWTGGVG YITKDVIKEH LPAATMDNVQ ILICGPPAMV ASVRRSTVDL
Consensus   ..E.W.ys.G fite..ireH lP.p.....l .L.CGPPpMi q.a..pnld.

                          284
                          +
 Nia:cons   .....KMgyd iKesLLvF*K
CB5R:cons   ......rVGH PkERCF.F*W
 Yeastc5b   GFRRSKPLSK MEDQVFVF*D
Consensus   .....k.... .ke..fvF*.
```

Figure 6.5 Computer analysis of the 35kDa protein sequence

The graphed data shown in this figure were computed using the MacVector™ sequence analysis programs and algorithms.

The hydrophilicity plot graphs the local hydrophilicity of the protein along its amino acid sequence using the Kyte-Doolittle scale (Kyte and Doolittle, 1982).

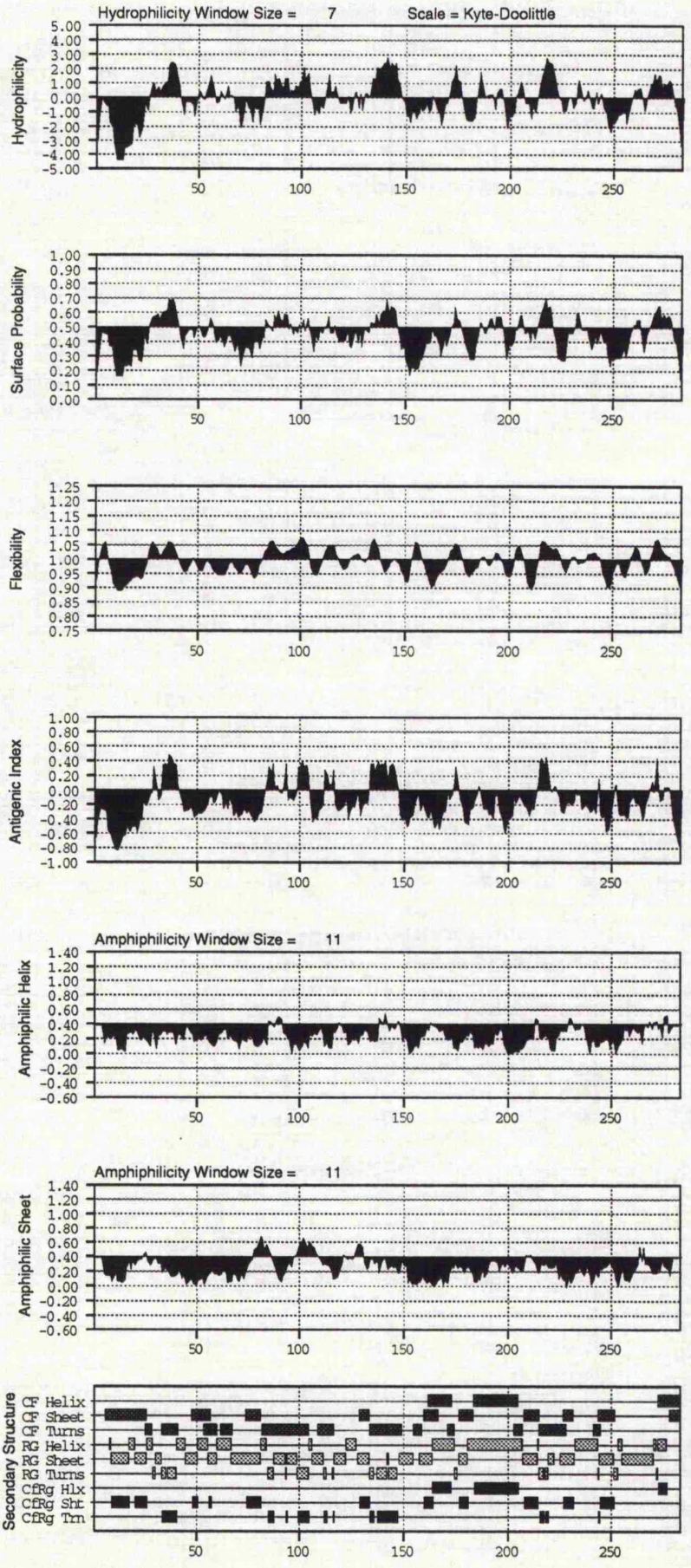
The surface probability plot shows which regions of a protein are likely to lie on the surface of the protein .

The flexibility plot reflects regional flexibility of the polypeptide chain.

The antigenicity plot indicates possible exposed surface peaks of the protein which may be antigenic sites. Analysis combines information from hydrophilicity, surface probability and backbone flexibility predictions along with the secondary structure predictions of Chou-Fasman (Chou and Fasman, 1978) and Robson-Garnier (Garnier *et al.*, 1978) in order to produce a composite prediction of the surface contour of a protein.

The amphiphilicity profiles detect regions of a protein that may form amphiphilic structures, *i.e.* structures that tend to be polar on one side and apolar on the other. Such regions are often found at protein-solvent or membrane-protein interfaces.

The secondary structure plot indicates regions of α-helix, β-sheet and reverse turn or coil. Predictions have been made using the Chou-Fasman method (CF) and the Robson-Garnier method (RG). In addition, the secondary structure predictions where both the Chou-Fasman and Robson-Garnier methods agree (CfRg).


Hydrophilicity Window Size = 7
Scale = Kyte-Doolittle
Hydrophilicity
Surface Probability
Flexibility
Antigenic Index
Amphiphilicity Window Size = 11
Amphiphilic Helix
Amphiphilicity Window Size = 11
Amphiphilic Sheet
Secondary Structure
CJ Helix
CJ Sheet
CJ Turns
RG Helix
RG Sheet
RG Turns
CfRg Hlx
CfRg Sht
CfRg Trn
50
100
150
200
250

The homology between mammalian cytochrome b_5 reductases and the FAD-binding domain of plant nitrate reductases is significantly higher (45% identity, 67% similarity) than that of the yeast protein to either the mammalian or the plant proteins. This is interesting because phylogenetic trees, constructed from a comparison of small subunit (SSU) ribosomal RNA, indicate that the kingdom Animalia diverged from the Plantae and Fungi kingdoms before they themselves separated (Johanson *et al.*, 1988). It would seem that a common ancestor contained a primeval cytochrome b_5 reductase sequence. In plants this sequence underwent gene fusion events with genes encoding molybdenum-pterin and haem binding domains to produce nitrate reductase, but retained high homology to the original sequence. The yeast cytochrome b_5 reductase homologue seems to have diverged further from the original sequence than the plant and animal proteins.

6.2.4 The flavoprotein pyridine nucleotide cytochrome reductase family

In addition to its homology with nitrate reductase, cytochrome b_5 reductase displays significant homology to two FAD-containing enzymes; ferredoxin-$NADP^+$ reductase and cytochrome P-450 reductase (Porter and Kasper, 1986). Ferredoxin-$NADP^+$ reductase is the terminal electron carrier of the photosynthetic electron transport chain and cytochrome P-450 reductase is a component of the microsomal cytochrome P-450 dependent monooxygenase system. Using X-ray crystallography, the three-dimensional structure of spinach ferredoxin-$NADP^+$ reductase has recently been described (Karplus *et al.*, 1991). Furthermore, Karplus *et al.* (1991) showed that the ferredoxin-$NADP^+$ reductase represents a new structural class of flavoenzymes. This family of flavoenzymes includes the FAD-binding domain of NADP-cytochrome P-450 reductase, the FAD-binding domain of the flavoprotein NADPH-sulphite reductase (Ostrowski *et al.*, 1989), NADH-cytochrome b_5 reductase and NADH-nitrate reductase. Hyde *et al.* (1991) have suggested that this family of flavoenzymes be called "flavoprotein pyridine nucleotide cytochrome reductases". Furthermore, Hyde *et al.* (1991) used the spinach ferredoxin-$NADP^+$ reductase structural model to produce alignments for all 30 of the known members of the flavoenzyme family. In addition to the enzymes described above, this alignment included sequences from various NADPH-nitrate reductases and the FAD-binding domain of the

unique *Bacillus megaterium* cytochrome P-450. The alignment of these 30 sequences, identified 14 invariant residues, the positions of which are indicated in an alignment of 8 representative sequences shown in Fig. 6.6. Only 7 of the 30 sequences aligned by Hyde *et al.* (1991) are shown in Fig. 6.6 in order to simplify the presentation and to emphasise the secondary structure predictions in relation to the invariant residues. The alignment also includes the sequence of the yeast cytochrome b_5 reductase homologue identified in this study and, as expected, this sequence readily aligns with the other proteins. The yeast cytochrome b_5 reductase homologue (YCB5R) contains 13 of the 14 amino acid residues indicated by Hyde *et al.* (1991) as being invariant. The fourteenth amino acid (present in the Alpha F1 region) is a conservative substitution of leucine to isoleucine.

The predicted FAD-binding domain of the cytochrome P-450 reductase and sulphite reductase is split into two segments with an intervening amino acid sequence of approximately 110 apparently unrelated residues (Porter and Kasper, 1986). Consequently, the alignment in Fig. 6.6 is divided into two parts; the first part (Fig. 6.6A) displays the alignment of the first 60 residues of the FAD-binding sequence, which Porter (1991) referred to as the FAD-PP_i region of the cytochrome P-450 and sulphite reductases, and the second part (Fig. 6.6B) shows the alignment of the remaining sequences. If the intervening residues in the cytochrome P-450 and sulphite reductases are ignored, these FAD domains have the binding sites for FAD and NAD(P)H encoded sequentially, as illustrated in the structural model for ferredoxin-$NADP^+$ reductase (Karplus *et al.*, 1991). Thus, the secondary structure features identified in Fig. 6.6 have been designated F for the FAD-binding sequence and N for the pyridine nucleotide-binding sequence. However, the structural model and some chemical modification/cofactor protection studies indicate that amino acid residues in the FAD-binding domain may contribute to the binding of NAD and conversely residues in the NAD-binding domain may contribute to the binding of FAD (Karplus *et al.*, 1991). This suggests that the cofactors bind at the interface between the NAD and FAD-binding domains.

Of the 14 amino acid residues identified by Hyde *et al.* (1991) as being invariant, only one, a Gly residue, is found in the N-terminal portion of the FAD-binding domain

Figure 6.6 An alignment of the yeast cytochrome b_5 reductase homologue with other related flavoenzymes based on the secondary structure prediction for ferredoxin: $NADP^+$ reductase

This secondary structure prediction is based on the three-dimensional conformational model of ferredoxin-$NADP^+$ (Karplus *et al.*, 1991). The amino acid sequences of the FAD domains of the following representatives of the flavoprotein pyridine nucleotide cytochrome reductase family of flavoenzymes are shown: yeast cytochrome b_5 reductase homologue (NADH:YCB5R) (this study, fig 6.3), the tomato nitrate reductase FAD domain (NADH:NR-FD) (Daniel-Vedele *et al.*, 1989), human cytochrome b_5 reductase (NADH:HCB5R) (Yubisui *et al.*, 1986), *Neurospora crassa* NADPH:nitrate reductase (NADPH-NR) (Okamato *et al.*, 1991), *S. typhimurium* NADPH:sulphite reductase flavoprotein (NADPH:SR-FP) (Ostrowski *et al.*, 1989), rat NADPH:cytochrome P-450 reductase (NADPH:CPR) (Porter and Kasper, 1986; Porter, 1991) and spinach ferredoxin:$NADP^+$ reductase (NADP:FdR) (Karplus *et al.*, 1991).

A. Alignment of the first 60 residues of the yeast cytochrome b_5 reductase homologue with the similar region of sulphite reductase and cytochrome P-450 reductase, which have an intervening sequence shown by the three periods at their ends.

B. Alignment of the remainder of the sequences extending to the C termini.

The secondary structure elements of spinach ferredoxin:$NADP^+$ reductase (Karplus *et al.*, 1991) are shown by labels beneath boxes which enclose the residues predicted to have a similar structural role in the various members of this flavoenzyme family. The secondary structure elements are numbered sequentially and designated F for the subdomain binding FAD and N for the proposed $NADP^+$-binding subdomain. The invariant residues identified in the multiple alignment of Hyde *et al.* (1991) are shown above the sequences with the leucine not conserved in the yeast cytochrome b_5 reductase homologue shown in brackets. Black blocks followed by numbers are discussed in the text. The numbering of the amino acid residues for each protein is shown to the left and right of each sequence section. (Based on alignment of Hyde *et al.*, 1991).

A.

```
INVARIANT                                                                 G
NADH:YCB5R     1 IIGPKTKPVLDPK RNDFQSFPLVE KTILTHNTS MYKFGLP HADDVLGLPIGQ HIVIK A...NINGK.DIT   89
NADH:NR-FD   641 VQTPTRSVALI.P REKI.PCKLVD KQSISHDVR KFKFALP SEDQVLGLPVGK HIFLC A...TVDDK.LCM  705
NADH:HCB5R    26 FQRSTPAITLESP DIKY.PLRLID REIISHDTR RFRFALP SPQHILGLPVGQ HIYLS A...RIDGN.LVV   90
NADPH:NR     713 ALSDPSRPIFLQS KTWN.SAILTF KESVSPDTK IFHFALS HPAQSIGLPVGQ HLMMR LPDPAKPTE.SII  776
NADPH:SR-FP  224 NDIRTSPYTKDAP LIAT.LSVNQK ITGRNSEKD VRHIEID LGDSGLRYQPGD ALGVW YQNDPALVKE...  289
NADPH:CPR    269 YENQKPPFDAKNP FLAA.VTANRK LNQ.GTERH LMHLELD ISDSKIRYESGD HVAVY PANDSALVNQ...  333
NADP:FdR      25 EGITVNKFKPKTP YVGR.CLLNTK ITGDDAPGE TWHMVFS HEG.EIPYREGQ SVGVI PDGEDKNGKPHKL   92
                                Beta F1             Beta F2             Beta F3
```

B.

```
INVARIANT       R Y   S                 ▓1            G   ▓2(L)
NADH:YCB5R     90 RSYTP TSLDGD....TKG NFELLVK SY.........PT.G NVSKMIG ELKIGD.......... SIQIK GPR  136
NADH:NR-FD    706 RAYTP TS.TVD....EVG FFELVVK IYFKGVHPKFPNG.G QMSQHLD SLPIGA.......... FLDVK GPL  760
NADH:HCB5R     91 RPYTP IS.SDD....DKG FVDLVIK VYFKDTHPKFPAG.G KMSQYLE SMQIGD.......... TIEFR GPS  145
NADPH:NR      777 RAYTP IS.DGT...LERG TLRVLVK IYY.ASPTEDIKG.G QMTQALD ALALGK.......... AVEFK GPV  831
NADPH:SR-FP   386 RLYSI AS.AQA...EVES EVHITVG VVRY.D.IEGRARAG GASSFLA DRV...EEEGEVRV.. FIEHN DN.  444
NADPH:CPR     454 RYYSI AS.SSK...VHPN SVHICAV AVEYEA.KSGRVNKG VATSWLR AKEPAGENGGRALVPM FVRKS Q..  517
NADP:FdR       93 RLYSI AS.SALGDFGDAK SVSLCVK RLIYTH.DAGETIKG VCSNFLC DLKPGA.......... EVKLT GPV  151
                  Beta F4              BetaF5                Alpha F1              BetaF6

INVARIANT                                        M    G G   P
NADH:YCB5R    137 GNYHYERNCR..............S HLGMIAG GTGI APMYQIMKAI.. AMD.P.HDTT KVSLVFG.N VHE  187
NADH:NR-FD    761 GHIEYQGKGNFLVHGKQKFA......K KDAMIAG GTGI TPVYQVMQSI.. LKD.P.EDDT EMYVVYA.N RTE  821
NADH:HCB5R    146 GLLVYQGKGKFAIRPDKKSNPIIRTVK SVGMIAG GTGI TPMLQVIRAI.. MKD.P.DDHT VCHLLFA.N QTE  212
NADPH:NR      832 GKFVYQGRGVCSVNGRERKV......K RFVMVCG GSGV TPIYQVAEAV.. AVDD..QDGT ECLVLDG.N RVE  892
NADPH:SR-FP   445 ............FRLPANPE......T PVIMIGP GTGI APFRSFMQQRA. ..AEG..VEG KNWLFFGNP HFT  493
NADPH:CPR     518 ............FRLPFKST......T PVIMIGP GTGI APFMGFIQERAW LREQG.KEVG ETLLYYGCR RSD  570
NADP:FdR      152 GKE.........MLMPKDPN......A TIIMLGT GTGI APFRSFLWKMFF EKHDDYKFNG LAWLFLG.V PTS  207
                                               Beta N1        Alpha N1              Beta N2

INVARIANT          L                                                                      CG
NADH:YCB5R    188 EDILL KKELEALVAM KPSQ.. FKIVYYL DSPDREDWTGGVGY ITKDVI KE HLPAA..T MDN. VQILICG  251
NADH:NR-FD    822 DDILL KDELDAWAEQ VPNR.. VKVWYVV QESITQGWKYSTGF VTESIL RE HIPEP..S HT.. TLALACG  884
NADH:HCB5R    213 KDILL RPELEELRNK HSAR.. FKLWYTL D.RAPEAWDYGQGF VNEEMI RD HLPPP..E EE.. PLVLMCG  274
NADPH:NR      893 GDILM KSELDELVER AKPMGR CRVKYTL .SRPGAEWEGLRGR LDKTML ER EVGEGDLR GE.. TMVLLCG  957
NADPH:SR-FP   494 EDFLY QVEWQRYVKE GV.L.. SRIDLAW .SRDQK....EKIY VQDKRL EQ GAELWRWI .NDG AHIYVCG  553
NADPH:CPR     571 EDYLY REELARFHKD GA.L.. TQLNVAF .SREQA....HKVY VQHLLK RD REHLWKLI HEGG AHIYVCG  640
NADP:FdR      208 SSLLY KEEFEKMKEK APDN.. FRLDFAV .SREQTNEKGEKMY IQTRMA QY AVELWEML KKDN TYVYMCG  273
                     Alpha N2          Beta N3                AlphaN3a   Alpha N3b     Beta N4

INVARIANT            M   ▓3
NADH:YCB5R    252 .PPA MVASVRRSTVDLGF RRSKPLSKM E.........  ...  DQVFVF    284
NADH:NR-FD    885 .PPP MIQFAINPNLEKMG YDIK.....  .........  ...  EELLVF    911
NADH:HCB5R    275 .PPP MIQYACLPNLDHVG HPT.....  .........  ...  ERCFVF    300
NADPH:NR      958 .PEG MGNMVREVLKGMGW DD.....  .........  ...  EDVLVF    982
NADPH:SR-FP   554 DARR MAADVEKALLEVIA EFGGMDLES ADEYLSELRV ERR YQRDVY    599
NADPH:CPR     641 DARN MAKDVQNTFYDIVA EFGPMEHYQ AVDYVKKLMT KGR YSLDVW S  678
NADP:FdR      274 .LKG MEKGIDDINVSLAA AEGID....  WIEYKRQLKK AEQ WNVEVY    314
                      Alpha N4a              AlphaN4b      Beta N5
```

shown in Fig. 6.6A. The alignment of NADH-nitrate reductases and cytochrome b_5 reductases shown in Fig. 6.4 has 11 invariant residues in this region of the FAD-binding domain. However, the number of invariant residues in an alignment is reduced by the addition of further sequences *i.e.* cytochrome P-450, sulphite reductases and ferredoxin-$NADP^+$ reductase sequences (Fig. 6.6). It is likely that this region of the various proteins interfaces with electron-accepting proteins or regions of their own polypeptide that bind the next internal electron-carrying cofactor (Hyde *et al.*, 1991). It is, therefore, not surprising that this portion of the sequence alignment displays low similarity. In the case of cytochrome b_5 reductase, this region of the FAD-binding domain is likely to serve as an interface with cytochrome b_5, as Glu residues in this region have been identified as potential participants in charge transfer linkages with cytochrome b_5 (Strittmatter *et al.*, 1990). Overall, part A of the alignment appears to contain three of the six major β strands of the β-barrel FAD-binding region of ferredoxin reductase (Karplus *et al.*, 1991).

The remaining three β strands and the single α helix of the FAD-binding region are present in the next 60-70 residues of the alignment (Fig. 6.6B). Five of the invariant amino acid residues identified by Hyde *et al.* (1991) are found in this region of Fig. 6.6B. The invariant Arg and Tyr residues in β F4 directly contact the FAD in ferredoxin reductase (Karplus *et al.*, 1991). The Arg (residue 93 in ferredoxin reductase, residue 90 in YCB5R) probably forms hydrogen bonds with the pyrophosphate bridge of FAD and the Tyr (residue 95 in ferredoxin reductase; residue 92 in YCB5R) probably interacts with the isoalloxazine ring system of FAD and hydrogen bonding to its ribose. The Ser-96 in this β strand of ferredoxin reductase (Karplus *et al.*, 1991), which is replaced by a Thr-93 in YCB5R, is probably hydrogen-bonded to water molecules in the vicinity of the isoalloxazine ring system of FAD. The invariant Ser residue immediately following β F4 has not been assigned a role in cofactor binding.

The black blocks 1 and 2 (Fig. 6.6B), mark Lys and Ser residues at positions 116 and 133 of ferredoxin reductase (positions 110 and 118 of YCB5R) which, although variant, appear to have important functions. The Lys is probably hydrogen-bonded to the 5'-phosphate of the 2'-phospho-AMP in the apparent pyridine nucleotide-binding site of

ferredoxin reductase; NADH binding protects this Lys (Lys-110 of bovine cytochrome b_5 reductase) against acylation in cytochrome b_5 reductase (Hackett *et al.*, 1988). The Ser probably hydrogen bonds to the pyrophosphate bridge of FAD *via* main chain interactions and its hydroxyl group (Karplus *et al.*, 1991). Site-directed mutation of this Ser (Ser-127 in Human cytochrome b_5 reductase) to Pro or Ala resulted in proteins with altered spectral properties and a 10-fold higher K_m for NADH as compared with the wild-type. This result corresponds well with the earlier finding of a Ser to Pro mutation in the cytochrome b_5 reductase of patients with hereditary deficiency of this enzyme (Yubisui *et al.*, 1991). The role of the invariant Leu present in the α F1 region which is replaced by Ile in the yeast cytochrome b_5 reductase homologue has not been determined.

The major role of the first half of the FAD domain appears to be in binding FAD and assisting in electron transfer to electron acceptors. However, the results described above indicate that some of the amino acid residues in the C-terminal sequence of the FAD domain are involved in forming portions of the pyridine nucleotide-binding site and stabilising the FAD-NAD(P)H complex during electron transfer.

The most conserved region in the NAD domain alignment shown in Fig. 6.6B is found at the beginning of the proposed pyridine nucleotide-binding site where the first β strand leads to a turn containing two invariant Gly residues. This domain may correspond to a region within the main chain which comes into close contact to the pyrophosphate bridge of NAD(P)H, as found for many dinucleotide folds (Karplus *et al.*, 1991; Sutter *et al.*, 1990).

The only invariant Cys (residue 272 in ferredoxin reductase, residue 250 in YCB5R) in this alignment of flavoenzymes is found in the C-terminus at the end of β N4, next to an invariant Gly (residue 2732 in ferredoxin reductase, residue 251 in YCB5R) (Fig. 6.6B). Although it is not possible to assign this Cys a role in $NADP^+$ binding in ferredoxin reductase, most of the enzymes in this flavoprotein family have been shown to depend on a Cys thiol group for activity (Porter, 1991; Karplus *et al.*, 1991). For human cytochrome b_5 reductase, the Cys residue marked by black block 3 was identified as essential for enzyme activity and is protected by NADH (Hackett *et al.*, 1986). The role of

this Cys residue was further investigated by Shirabe *et al.* (1991) who carried out site-directed mutagenesis of all 4 Cys residues in human cytochrome b_5 reductase. They found that mutants with any 1 of the 4 Cys residues replaced by Ser were fully active and sensitive to sulphydryl inhibitors. However, in a double mutant where both invariant Cys residues (Cys-273, identified in Fig. 6.6B) and the Cys (Cys-283) identified as essential by Hackett *et al.* (1986) were replaced by Ser, the enzyme retained only 20% of its activity and was sensitive to inhibition by sulphydryl reagents (Shirabe *et al.*, 1991). It was concluded that, although none of the Cys residues of cytochrome b_5 reductase were essential for activity, the invariant Cys-273 has a role in facilitating the reaction catalysed by the enzyme. In the light of these results, it is interesting to note that the yeast cytochrome b_5 reductase homologue contains an invariant Cys (Cys-251) but the Cys (Cys-283) present in the human enzyme has been replaced by Arg (Arg-261).

6.3 The predicted N-terminal amino acid sequence

The N-terminal amino acid sequence obtained from the p35 protein in chapter 3 was screened against the predicted amino acid sequence of the p35 gene translated from the ATG at position 1 and the ATG at position -114 (see Fig. 5.12). The N-terminal sequence obtained in chapter 3 showed no homology to either of the predicted amino acid sequences. The most likely explanation for this failure to detect homology was thought to be inaccurate N-terminal sequencing particularly as the oligonucleotide produced from the sequence did not hybridise to yeast genomic DNA (see chapter 3).

6.4 Discussion

The p35 gene has been identified as a yeast homologue of mammalian NADH-cytochrome b_5 reductase on the basis of protein sequence homology. Comparison of the protein sequence to the EMBL data base using the GCG TFASTA program reveals good homology to plant NADH-nitrate reductases and to mammalian NADH-cytochrome b_5 reductases.

Initially, comparison of the p35 predicted protein sequence indicated homology to plant NADH-nitrate reductases as well as to mammalian NADH-cytochrome b_5 reductases. However, analysis of the yeast protein indicates that it is structurally similar to the mammalian cytochrome b_5 reductase as both have small highly hydrophobic N-terminal domains. This structural similarity, taken along with sequence homology and the size of the protein, indicates that the yeast p35 gene product is a homologue of NADH-cytochrome b_5 reductase. The yeast p35 gene was therefore named *YCB5R*.

The yeast protein contains 13 of the 14 amino acid residues described by Hyde *et al.* (1991) as being indicative of a family of flavoenzymes which are termed "flavoprotein pyridine nucleotide cytochrome reductases". As well as NADH-nitrate reductase and NADH-cytochrome b_5 reductase, this family of flavoenzymes includes ferredoxin-$NADP^+$ reductases, NADPH-sulphite reductases, NADPH-cytochrome P-450 reductases and NADPH-nitrate reductase.

The yeast protein is likely to be a membrane associated NADH-cytochrome reductase, possibly having the same role as mammalian cytochrome b_5 reductase *i.e.* reducing a yeast cytochrome b_5 homologue. If the yeast cytochrome b_5 reductase homologue does perform this function then it is likely to be involved in desaturation of fatty acids and sterol (ergosterol) biosynthesis. In addition, the yeast homologue may be involved in the transfer of electrons from NADH *via* a cytochrome b_5 homologue to cytochrome P-450. These electrons are normally supplied from NADPH via cytochrome P-450 reductase. Recently, a hemoglobin has been characterised in yeast (Zhu and Riggs, 1992). Therefore, it is possible that the yeast protein is involved in a methemoglobin reaction. However, the yeast hemoglobin is a two-domain protein, the first domain being the globin-like portion and the second domain being a flavin domain which shows homology to the flavoprotein pyridine nucleotide cytochrome reductase family of flavoenzymes. The function of this flavohemoglobin remains to be determined.

A cytochrome b_5 homologue has not yet been characterised in yeast. However, the presence in yeast of a gene with homology to mammalian cytochrome b_5 reductase adds weight to the suggestion of Murakami *et al.* (1990) that a cytochrome b_5 may be present in

yeast and that it is responsible for the differences observed in the effect of overexpressed yeast P-450 reductase on the P-450c- and P-$450_{17\alpha}$-dependent monooxygenase reactions.

The analysis of the p35 gene DNA sequence suggests that translation starts at the ATG position 1 (see Fig. 5.12). The analysis described above is of the protein translated from this start site. There is the possibility that a larger translation product consisting of 322 amino acid residues with a molecular mass of 36.2kDa is produced. However, when this additional 32 amino acid are added to the sequence, no additional homology to plant NADH-nitrate reductase or to mammalian NADH-cytochrome b_5 reductase is identified in this region. Also, the structural similarity between the yeast protein and cytochrome b5 is reduced because the highly hydrophobic region of the yeast gene is no longer located at the N-terminus of the protein.

The question of which translation initiation codon is used in the p35 gene could be resolved if good N-terminal sequence was obtained for the *YCB5R* protein. Unfortunately, the N-terminal amino acid sequence obtained from the p35 protein in chapter 3 does not display any homology to the predicted N-terminal amino acid sequence of p35. However, it was thought at the time that this sequence was inaccurate as the oligonucleotide produced from the sequence did not hybridise to yeast genomic DNA.

Chapter 7

Disruption of the gene encoding the putative yeast cytochrome b_5 reductase

7.1 Introduction

The ability to introduce exogenous DNA into microorganisms has been used extensively to manipulate the genomes of these organisms (Lacks, 1989). In the late 1970s the availability of purified DNA fragments enabled researchers to demonstrate reproducible transformation of yeast for the first time (Beggs, 1978; Hinnen *et al.*, 1978). Hinnen *et al.* (1978) reported the transformation of a *LEU2* DNA fragment into a non-reverting double mutant (*leu2-3, leu112*) and showed that yeast efficiently integrates circular DNA into the genome by single homologous reciprocal exchange, resulting in a direct repeat of the target sequence.

In the absence of sequences that support autonomous replication (*ARS* sequences), DNA transformed into yeast cells integrates into the genome exclusively by homologous recombination. Therefore, sequences modified *in vitro* can be used to precisely replace the resident chromosomal copy of any cloned gene (Scherer and Davis, 1979). Furthermore, homologous recombination of transforming DNA can also be used to create null alleles of a cloned yeast gene by introducing an *in vitro*-generated deletion into the genome at the precise chromosomal position of the gene (Shortle *et al.*, 1982; Rothstein, 1983).

This ability to carry out targeted integration of DNA into yeast chromosomes is one of the most powerful techniques available to a yeast biologist. One of the most widely used disruption methods is the one-step gene disruption procedure described by Rothstein (1983). This method takes advantage of the observation that sequence integration events can be targeted by the introduction of a double-strand break within the yeast sequence carried on a plasmid (Orr-Weaver *et al.*, 1981). The introduction of a double-strand break within the yeast sequence generates free DNA ends which are recombinogenic in yeast.

These free DNA ends direct the plasmid to the homologous chromosomal region where the plasmid intergrates by a double cross-over event (Rothstein, 1983). In addition, the double-strand break appears to increase the transformation frequency compared to uncut plasmid without an *ARS* sequence.

7.2 Disrupting the gene encoding the yeast cytochrome b_5 reductase homologue

The one step gene disruption method requires the identification of a suitable restriction site(s) within the ORF of a gene (preferably close to the start of the ORF) for the insertion of a DNA fragment containing a selectable marker. In this case, a suitable *Bam*HI site was identified 98bp downstream of the putative *YCB5R* ATG start site. This *Bam*HI site was convenient for the insertion of a *LEU2* gene cassette constructed by Berben *et al.* (1991). This is one of a series of "disruption" cassettes containing various yeast phenotypic markers flanked by translational termination codons. The addition of such translational termination codons prevents the possible formation of a fortuitously functional fusion by the disrupting sequence.

Before the *LEU2* disruption cassette could be subcloned into the *Bam*HI site of *YCB5R*, any additional *Bam*HI sites within the plasmid carrying the gene had to be removed. All the plasmids containing *YCB5R* produced in this study were found to contain a *Bam*HI site within their polylinker. Therefore, a plasmid without a *Bam*HI site within its polylinker was constructed. In order to destroy the *Bam*HI site in the polylinker of pIC20H (Table 2.2), a *Bgl*II-*Eco*RI fragment from plasmid pNBP-Eco (Fig. 5.6) which contained the ORF of *YCB5R* was subcloned into the *Bam*HI-*Eco*RI sites of pIC20H (see Fig. 7.1). *Bam*HI and *Bgl*II have compatible overhanging ends but when ligated, the resulting recombinant cannot be re-cleaved by either *Bgl*II or *Bam*HI. Thus, the only *Bam*HI site in the resulting recombinant plasmid pNBP-BE (Fig. 7.1) is the one present within the *YCB5R* ORF.

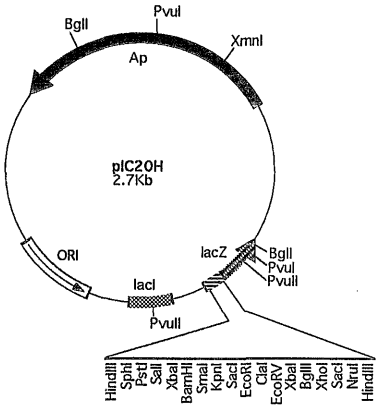
The disruption cassette, containing the *LEU2* marker flanked by translational termination codons, was subcloned from the YDp-L plasmid (Table 2.2) into the *Bam*HI

Figure 7.1 Subcloning the *Bgl*II-*Eco*RI fragment from pNBP-Eco into pIC20H

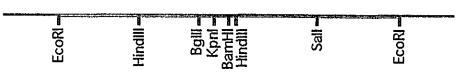
The plasmid pNBP-Eco (fig 5.6) was simultaneously digested with the restriction endonucleases *Bgl*II and *Eco*RI. The resulting fragments were separated by electrophoresis on a 0.6% LGT agarose gel and the 2.4kb *Bgl*II-*Eco*RI fragment was purified from the agarose as described in chapter 2.

The fragment was ligated into pIC20H vector digested with *Bam*HI and *Eco*RI. Ligated plasmids were transformed into competent *E. coli* DH5α cells. The cell suspension was plated onto LB plates containing 100μg ml^{-1} ampicillin, 25μg ml^{-1} IPTG and 40μg ml^{-1} X-Gal to visualise *lac*$^-$ colonies and incubated overnight at 37°C.

White colonies were randomly picked and examined for recombinant plasmids. The plasmid pIC20H containing the *Bgl*II-*Eco*RI fragment was named pNBP-BE.

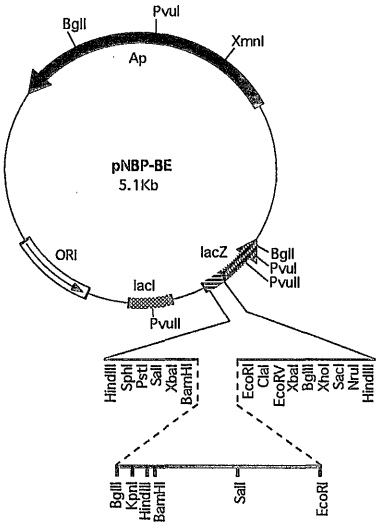


pIC20H was digested with *Bam*HI and *Eco*RI



pNBP-Eco was digested with BglII and EcoRI the larger of the two BglII-EcoRI insert fragments (2.4kb fragment) was recovered

Ligation



site of pNBP-BE, see Fig. 7.2. The resulting plasmid pNBP-BE::LEU was digested simultaneously with *Sal*I and *Xba*I. The fragments produced by the *Sal*I-*Xba*I double digest were separated by electrophoresis on a 0.8% (w/v) agarose gel and the *Sal*I-*Xba*I fragment containing the disruption cassette, flanked by recombinogenic ends, was isolated by electroelution. Approximately 1μg of the isolated *Sal*I-*Xba*I fragment was used to transform the leucine-requiring *S. cerevisiae* strain 842 (Table 2.1) by electroporation. Gene disruption was carried out in both haploid and diploid strains (8HA, 842; Table 2.1) and transformants were selected on synthetic minimal medium plates lacking leucine.

Leu^+ clones were obtained from the transformations of both diploid and haploid strains. The Leu^+ diploid transformants were induced to sporulate and tetrad analysis demonstrated that all spores were viable and that the LEU2 marker segregated with a ratio of 2:2.

7.3 Verification of gene disruption by Southern blot analysis

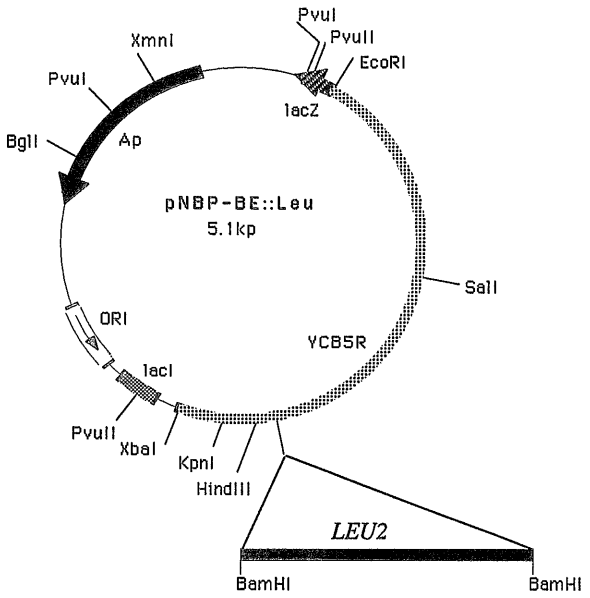
Genomic DNA was extracted from both haploid 8HA and diploid 842 Leu^+ transformants and used in Southern blot analysis. Two μg of DNA was digested with *Hin*dIII, the fragments separated by electrophoresis, DNA transferred onto Hybond-N™ and hybridisation carried out using pNBP4 (Fig. 4.6) as a probe. A single fragment of approximately 4kb was detected in *Hin*dIII digests of wild-type DNA, whereas a fragment of approximately 5.6kb was detected in four independent haploid transformants, indicating the insertion of the 1.6kb *LEU2* gene into the *YCB5R* gene. Both the 4kb and 5.6kb fragments were detected in diploid Leu^+ transformants (Fig. 7.3).

This Southern blot analysis indicated that the *YCB5R* gene had been successfully disrupted by the insertion of the *LEU2* cassette. It appears that the gene is not essential and no obvious phenotype could be determined for the *YCB5R* gene disruption when grown on YPD or minimal media supplemented with the amino acid required by the strain. Furthermore, no interference with growth is observed when the *ycb5r* strain is grown on different carbon sources. Further studies to determine the phenotypic effect of the disruption are currently being carried out.

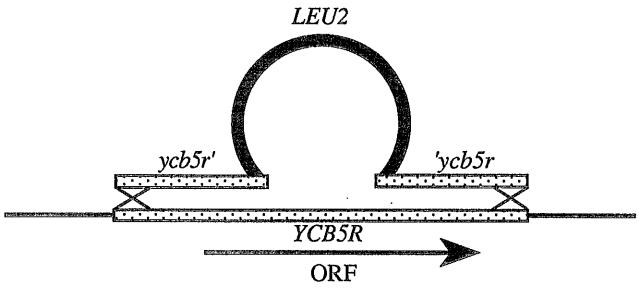
Figure 7.2 One step gene disruption

The plasmid pNBP-BE (fig 7.1) which includes a fragment containing the *YCB5R* gene was digested with *Bam*HI which cleaves within the *YCB5R* sequence. A fragment containing the yeast selectable marker *LEU2* (Berben *et al.*, 1991) was cloned into the *Bam*HI site to produce the plasmid pNBP-BE::LEU (A). The plasmid was then digested with *Sal*I and *Xba*I and the fragment containing the *LEU2* flanked by *YCB5R* sequences was isolated. The isolated fragment was transformed into yeast cells by electroporation. The homologous ends pair with the chromosome (B) and recombination results in the substitution of the wild type chromosomal sequence with the linear disrupted sequence (C).

A


PvuI
PvuII
EcoRI
lacZ
XmnI
PvuI
Ap
BglII
pNBP-BE::Leu
5.1kp
SalI
ORI
YCB5R
lacI
PvuII
XbaI
KpnI
HindIII
LEU2
BamHI
BamHI

B


LEU2
ycb5r'
'ycb5r
YCB5R
ORF

C

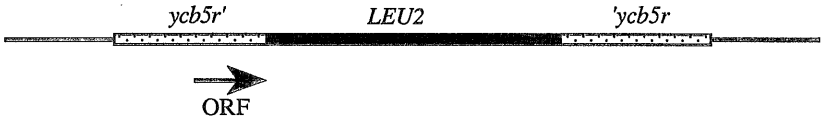

ycb5r'
LEU2
'ycb5r
ORF

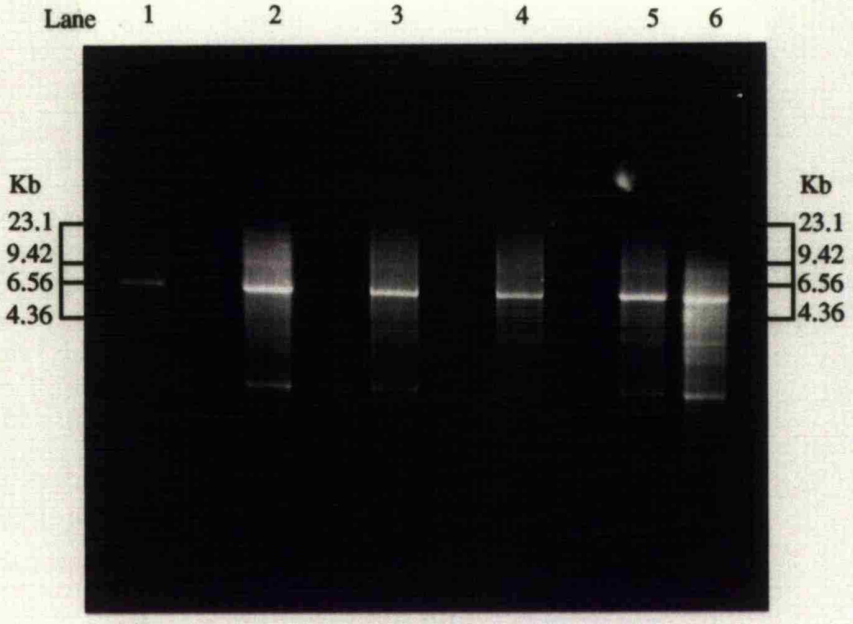
Figure 7.3 Southern blot analysis of Leu$^+$ colonies

The *YCB5R* gene in a diploid 842 strain was disrupted by the *LEU2* sequence and tetrad analysis showed the *LEU2* marker segregated 2:2. The disruption was further verified by Southern blot analysis.

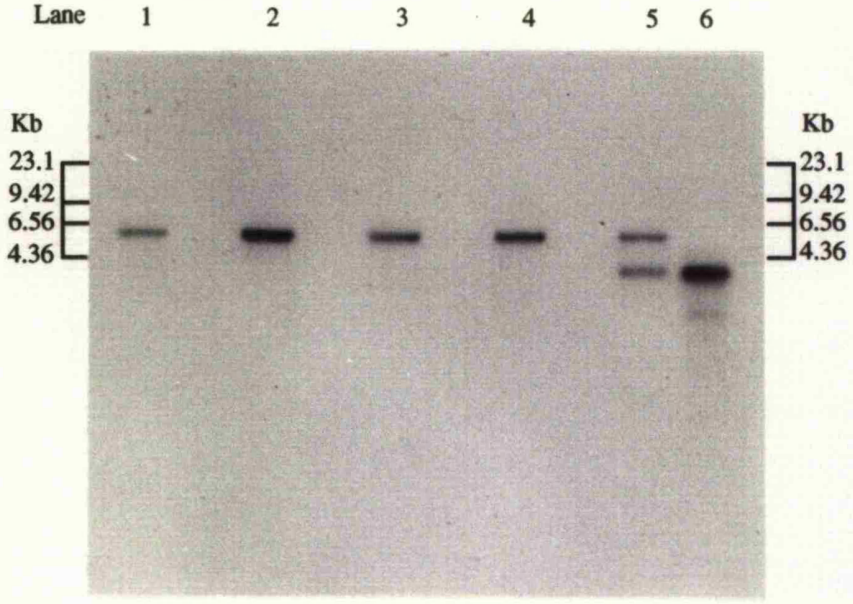
A. Genomic DNA was extracted from Leu$^+$ cells, digested with the restriction endonuclease *Hin*dIII and fragments separated by gel electrophoresis. Lane 1-4, Leu$^+$ haploid yeast; Lane 5, Leu$^+$ diploid yeast; Lane 6, Leu$^-$ haploid yeast.

B. The gel shown in A was blotted onto Hybond-N and Southern blot analysis carried out using pNBP4 as a probe (fig 4.6). Lane 1-4, Leu$^+$ haploid yeast; Lane 5, Leu$^+$ diploid yeast; Lane 6, haploid wild-type yeast .

A


Lane 1 2 3 4 5 6
Kb
23.1
9.42
6.56
4.36
Kb
23.1
9.42
6.56
4.36

B


Lane 1 2 3 4 5 6
Kb
23.1
9.42
6.56
4.36
Kb
23.1
9.42
6.56
4.36

7.4 Analysis of proteins from *YCB5R* mutants

Crude cell extracts were prepared from both wild-type and *ycb5r* strains, their proteins separated by SDS-PAGE and Western blot analysis was carried out using anti-p35 antibodies. The anti-p35 antibodies detected a 35kDa protein and the 29kDa protein (described in chapter 2) in cell extracts prepared from the wild-type strain. However, the antibodies failed to detect a 35kDa protein in extracts prepared from two independently isolated *ycb5r* colonies; whereas the 29kDa protein was still detected (Fig. 7.4). This result indicated that the *ycb5r* cells do not contain a detectable 35kDa protein and suggested that the *YCB5R* gene encodes the 35kDa protein.

7.5 Protein purification from the *YCB5R* deleted strain

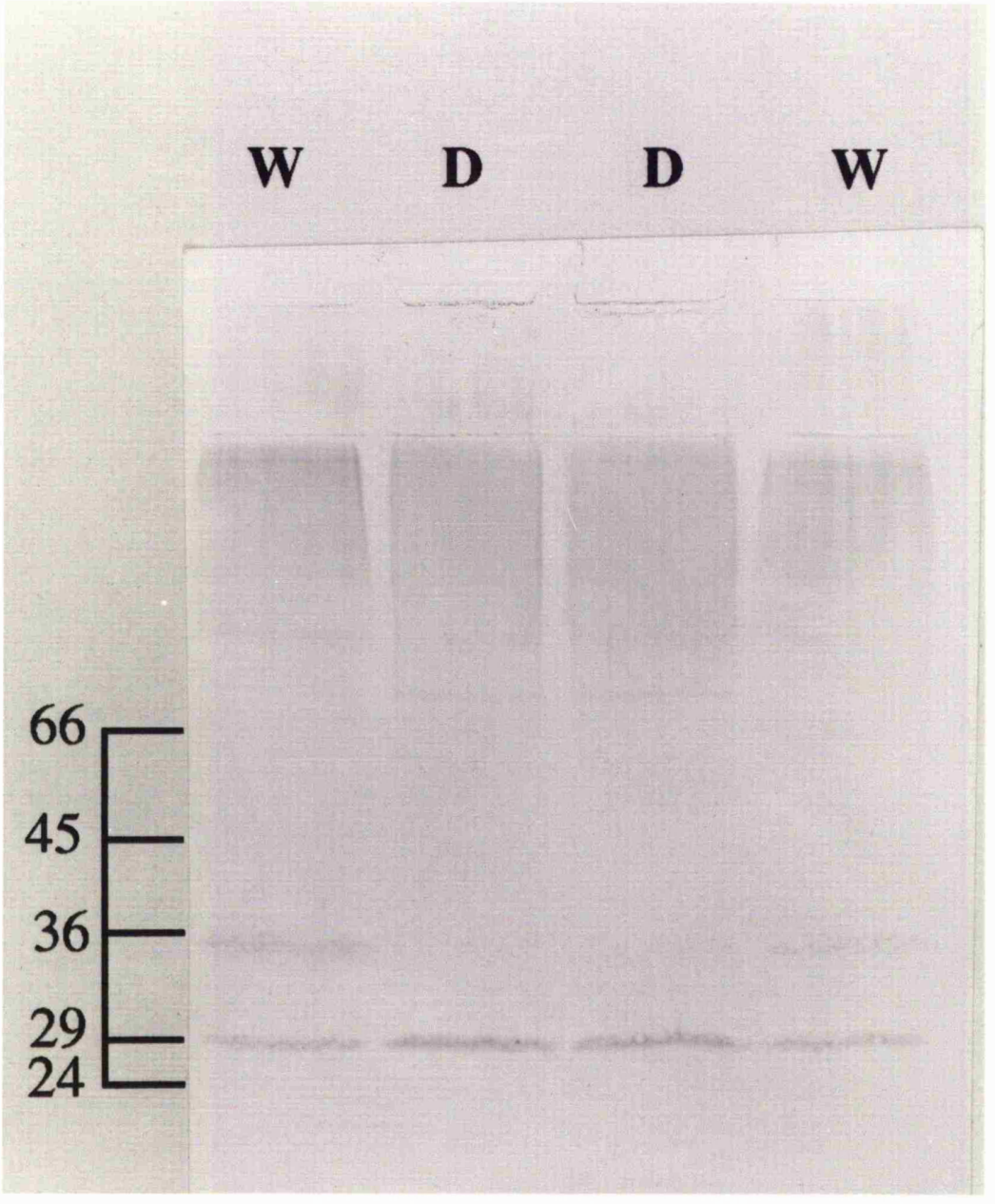
Protein extracts prepared from *ycb5r* cells were used in affinity-chromatography on novobiocin-Sepharose as described in chapter 2. Proteins, eluted from the novobiocin-Sepharose column in the 5M urea fraction, were separated by SDS-PAGE and Western blot analysis of these proteins carried out using anti-p35 antibodies (Fig. 7.5). The antibodies detected a 35kDa protein in the 5M urea fraction. This result was unexpected as no 35kDa protein could be detected in crude extracts from the *ycb5r* mutants (see above). In order to determine the nature of this 35kDa protein, additional experiments were carried out.

7.6 N-terminal sequencing of the 35kDa protein isolated from the *ycb5r* strain

The N-terminal sequence of the 35kDa protein isolated from the *ycb5r* mutant strain was carried out using the PVDF method (chapter 2) and the amino acid sequence of 5 residues was determined (Fig. 7.6). This sequence was compared to the original sequence obtained for the 35kDa protein (chapter 3). It is evident that the two sequences originated from the same protein. Using the TFASTA program, the new N-terminal amino

Figure 7.4 Western blot analysis of the *YCB5R* disruptant strain

Total yeast protein extracts were electrophoresed on a SDS polyacrylamide gel (10%) and blotted onto a nitrocellulose filter. The blotted proteins were incubated with anti-p35 antibodies, followed by goat anti-rabbit IgG and rabbit PAP, then developed. Lanes W, total yeast protein extracts from wild-type yeast; Lanes D total yeast protein extracts from disruptant yeast. As expected the anti-35kDa protein antibodies detect a 35kDa protein and a 29kDa protein in the wild type strain, whereas only the 29kDa protein was detected in the disruptant strain. A 35kDa protein no longer appears to be present in the disruptant strain.


W
D
D
W
66
45
36
29
24

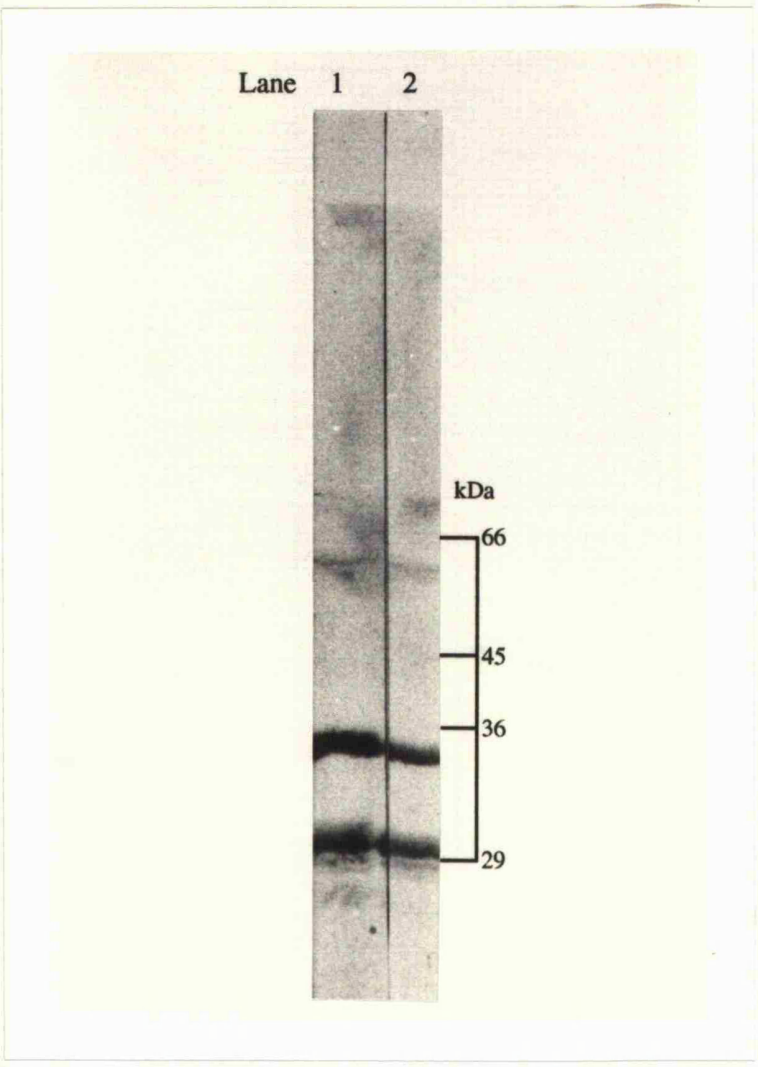


Figure 7.5 Western blot analysis of affinity purified protein from the *YCB5R* disruptant strain

The 5M urea fractions purified from wild type-yeast (lane 1) and the *YCB5R* disruptant strain (lane 2) were run on a SDS polyacrylamide gel (10%) and blotted onto a nitrocellulose. The blotted proteins were incubated with anti-p35 antibodies, followed by goat anti-rabbit IgG and rabbit PAP, then developed. Despite the evidence supplied by Western blot analysis (fig 7.4) that the anti-p35 antibodies do not detect a 35kDa protein in the crude extract of the *YCB5R* disruptant strain, the anti-p35 antibodies detected a 35kDa protein in the 5M urea fractions from both wild-type and the *YCB5R* disruptant strains.

amino acid No.	1st N-terminal amino acid sequence	2nd N-terminal amino acid sequence	coproporphrynogen oxidase
1	Ser*	Pro	Pro
2	Ala	Ala	Ala
3	Pro	Pro	Pro
4	Gln	Gln	Gln
5	Asp	Asp	Asp
6	Pro		Pro
7	Val*		Arg
8	Asn		Asn
9	Leu		Leu
10	Pro		Pro

Figure 7.6 N-terminal amino acid sequence of the 35kDa protein isolated from the *YCB5R* disruptant strain.

The N-terminal amino acid sequence for the 35kDa protein isolated from the *YCB5R* disruptant strain is shown above marked 2nd N-terminal sequence. This sequence is identical to the N-terminal amino sequence of coproporphyrinogen oxidase. The original N-terminal sequence of the 35kDa protein (see chapter 2) is also shown above. Other than the residues marked with asterisks this sequence matches the coproporphyrinogen oxidase sequence, suggesting that the 35kDa protein sequenced in the *YCB5R* disruptant and non-disruptant is coproporphyrinogen oxidase.

acid sequence was screened against the EMBL data base and a perfect match to the N-terminal sequence of coproporphyrinogen oxidase (Zagorec *et al.*, 1988) was found. This match was not found in the original screening described in chapter 2 because the coproporphyrinogen oxidase sequence was added to the data base after this first search (1989).

The coproporphyrinogen oxidase DNA and protein sequences were screened against both the *YCB5R* DNA and protein sequences but no significant homology was detected. Furthermore, no homology was found between the coproporphyrinogen oxidase and the sequence obtained from the *Xho*I-*Cla*I fragment of NBP7 (section 4.11).

The N-terminal sequence data from the *YCB5R* disruptant and non-disruptant strains indicate that the coproporphyrinogen oxidase, a 35kDa protein, binds to the novobiocin-Sepharose column. Further verification of this conclusion was obtained from Western blot analysis using anti-coproporphyrinogen oxidase antibodies (obtained from Dr. R. Labbe-Bois; Institut Jacques Monod, Paris) which detected a 35kDa protein in the 5M urea fraction following affinity purification of proteins from the *YCB5R* disruptant strain (data not shown).

Coproporphyrinogen III oxidase acts at the sixth enzymatic step in the haem biosynthetic pathway, catalysing the sequential oxidative decarboxylations of the 2- and 4-carboxyethyl side chains in coproporphyrinogen III to yield the two vinyl groups in protoporphyrinogen IX (Fig. 7.7). In *S. cerevisiae*, coproporphyrinogen oxidase is thought to be located in the cytosol and the purified enzyme is a homodimer, with a subunit M_r of 35,000 (Camadaro *et al.*, 1986). The enzyme contains two iron atoms per molecule of native protein and requires sulphydryl group(s) for its activity; however, the involvement of iron in the oxidative catalytic activity of the enzyme has not been demonstrated (Camadaro *et al.*, 1986). The mammalian enzyme requires oxygen for its activity. Poulson and Polglase (1974) claimed that the yeast coproporphyrinogen oxidase functioned at the same velocity under aerobic and anaerobic conditions provided $NADP^+$, ATP and methionine were present under the latter conditions. However, Camadaro *et al.* (1986) were unable to measure any anaerobic coproporphyrinogen oxidase activity with crude

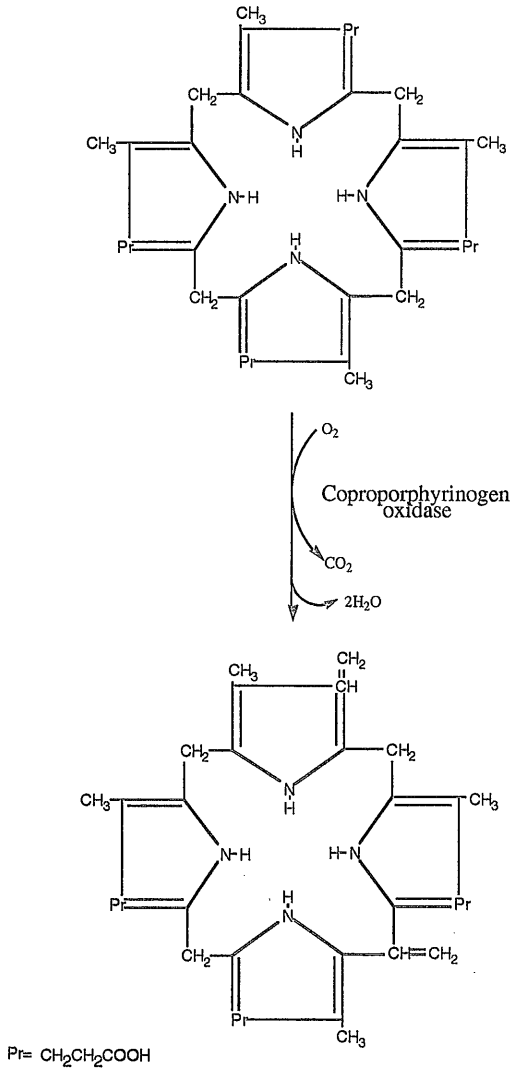


Figure 7.7 Proposed mechanism of coproporphyrinogen oxidase
After Granick and Beale (1978)

yeast extracts or purified coproporphyrinogen oxidase even in the presence of the factors described above. If the data obtained by Camadaro *et al.* (1986) are correct, the question of how haem is synthesised in anaerobically grown yeast cells remains unanswered (Zagorec and Labbe-Bois, 1986).

7.7 Analysis of proteins from the coproporphyrinogen oxidase deletion mutant

In order to determine if the coproporphyrinogen oxidase is the only 35kDa protein present in the 5M urea fraction obtained from the affinity purification procedure on novobiocin-Sepharose, a coproporphyrinogen oxidase deletion mutant (*hem13*) was obtained from Dr. R. Labbe-Bois.

The *hem13* mutant is an auxotrophic mutant which can only grow on a fermentable carbon source with the addition of ergosterol or with the addition of hemin. The inability of this mutant to synthesise haem leads to cytochrome deficiency resulting in the inability to grow aerobically and the requirement for ergosterol the principle sterol present in yeast membranes (Fieser and Fieser, 1959).

Protein extracts were prepared from the coproporphyrinogen oxidase deletion mutant for affinity-chromatography on novobiocin-Sepharose as described in chapter 2. During protein purification of the coproporphyrinogen oxidase disruptant strain the lipid pelical normally found on the surface of the soluble protein fraction following ultra-centrifugation was observed to be abnormally opaque. This feature could be due to abnormal membrane synthesis in the *hem13* mutant demonstrated by its requirement for ergosterol. The soluble protein fraction used for affinity chromatography and the 2M KCl and 5M urea fractions obtained following the affinity chromatography were separated using SDS-PAGE. Western blot analysis of these proteins was carried out using anti-p35 antibodies (Fig. 7.8).

No 35kDa protein was detected by the anti-p35 antibodies in the soluble protein fraction, 2M KCl or 5M urea fractions. However, the anti-p35 antibodies detected a 35kDa protein in a lane containing total yeast protein extract prepared from the

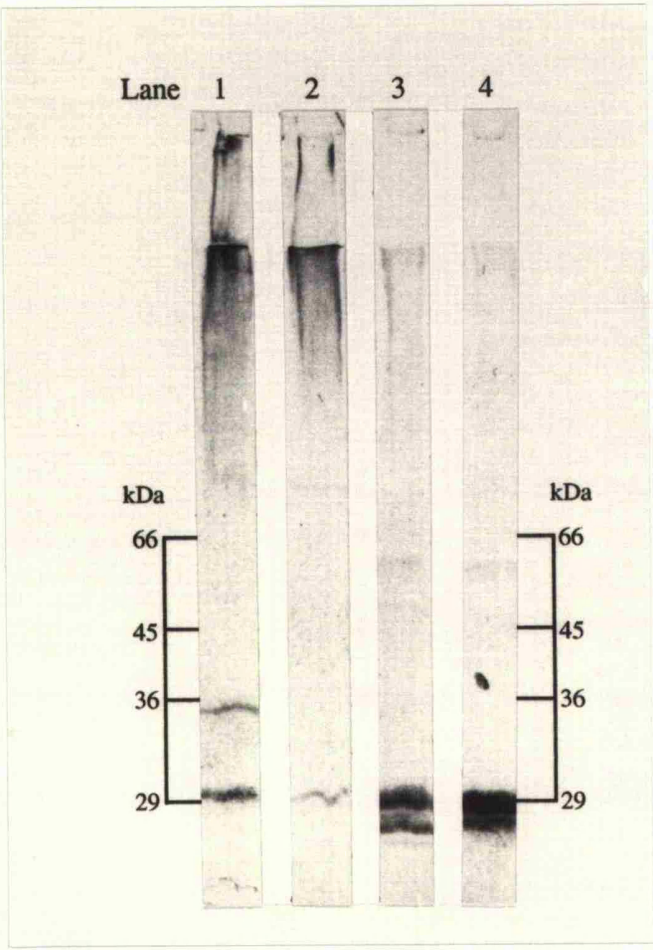


Figure 7.8 Western blot analysis of affinity purified protein from the coproporphyrinogen oxidase deletion strain

Lane 1	total yeast protein extract from the *hem13* deletion strain
Lane 2	soluble protein fraction prepared from the *hem13* deletion strain
Lane 3	2M KCl fraction eluted from the novobiocin-Sepharose column
Lane 4	5M urea fraction eluted from the novobiocin-Sepharose column

The anti-p35 antibodies do not detect a 35kDa protein in the soluble protein fraction loaded onto the novobiocin-Sepharose column nor do they detect a 35kDa protein in the 2M KCl or 5M urea fractions eluted from the novobiocin-Sepharose column. However, a 35kDa protein is detected in a total yeast protein extract prepared from the *hem13* deletion strain, indicating that the anti-35kDa antibodies recognise two 35kD proteins in yeast.

coproporphyrinogen oxidase deletion mutant. This result indicated that the anti-p35 antibodies detect two 35kDa proteins. However, it appears that the second protein is not solublised in this strain by the procedures used to prepare proteins for affinity chromatography from wild-type strains. Anti-coproporphyrinogen oxidase antibodies were used in Western blot analyses in order to verify that the 35kDa protein, detected in the crude extract prepared from the *hem13* deletion, was not coproporphyrinogen oxidase (Fig.7.9). No 35kDa protein was detected by the anti-coproporphyrinogen oxidase antibodies in the *hem13* deletion strain indicating that the protein detected in the crude extract is not coproporphyrinogen oxidase. It is obvious from these results that the anti-p35 antibodies do, in fact, recognise two different proteins, one of these being coproporphyrinogen oxidase, while the second is very likely to be the yeast cytochrome b_5 reductase homologue.

As described above, the membrane structure of the coproporphyrinogen oxidase disruption strain is different from the non-disruptant strain normally used for protein purification. This is demonstrated by the requirement of ergosterol for the growth of the *hem13* mutant and by the observation that the membrane fraction produced during protein purification from this strain was abnormally opaque. This feature could be significant as the membrane-bound form of mammalian cytochrome b_5 reductases contain a small N-terminal hydrophobic membrane-binding segment. The yeast cytochrome b_5 reductase homologue also contains a small highly hydrophobic N-terminal region, suggesting that this protein also binds membranes. It is possible that in cells with an altered membrane structure this protein will not be solublised in the processes used to prepare extracts for purification using novobiocin-Sepharose. This may explain why the yeast cytochrome b_5 reductase homologue is not present in the soluble protein fraction prepared from the *hem13* strain.

7.8 Discussion

Disruption of the yeast cytochrome b_5 reductase gene demonstrates that the gene product is not essential for growth. So far, no obvious phenotype has been observed for

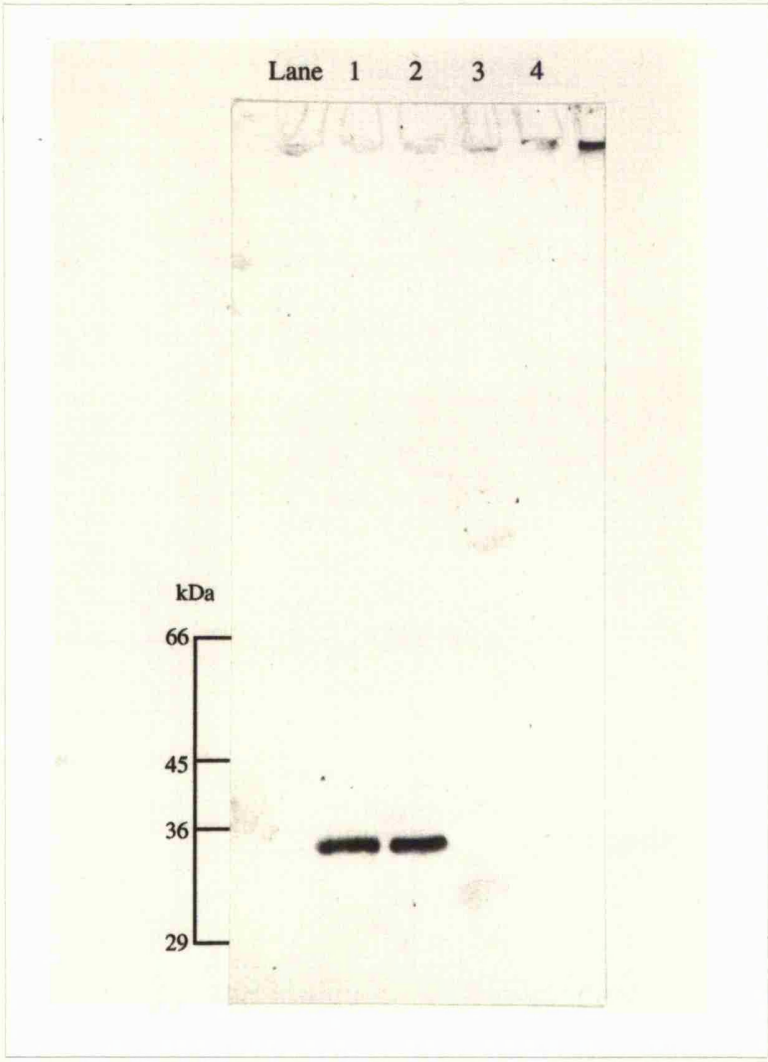


Figure 7.9 Western blot analysis of the crude protein extract prepared from the *hem13* deletion strain

Lanes 1 & 2 Total yeast protein extract prepared from wild-type yeast

Lanes 3 & 4 Total yeast protein extract prepared from the *hem13* deletion strain

The total yeast protein extracts were run on a SDS polyacrylamide gel (10%) and blotted onto a nitrocellulose filter. The blotted proteins were incubated with anti-coproporphyrinogen oxidase antibodies, followed by goat anti-rabbit IgG and rabbit PAP, then developed. The anti-coproporphyrinogen oxidase antibodies detected a 35kDa protein in wild-type protein extract, whereas no protein was detected in the *hem13* deletion protein extract.

the *ycb5r* disruptant when grown under a variety of conditions. The lack of an obvious phenotype is not too surprising, because a number of *in vitro* studies suggest that the cytochrome b_5 reductase activity can be replaced by cytochrome P-450 reductase (Oshino *et al.*, 1971; Reddy *et al.*, 1977; Keyes and Cinti, 1980; Dailey and Strittmatter, 1980). This type of functional substitution may also occur *in vivo* in a *S. cerevisiae* strain in which the cytochrome P-450 reductase gene has been disrupted (Sutter and Loper, 1989). In this strain, cytochrome P-450 is still active and so reducing equivalents must be provided by an enzyme other than cytochrome P-450 reductase. In this case, it is possible that the electron flow to cytochrome P-450 is mediated by cytochrome b_5 reductase through cytochrome b_5. This is quite feasible since cytochrome b_5 has been shown to transfer electrons to cytochrome P-450 (Hildebrandt and Mannering, 1971; Black and Coon, 1987; Aoyama *et al.*, 1990). Due to the importance of the monooxygenase system, it would be very interesting to study the effects of a cytochrome b_5 reductase/cytochrome P-450 reductase double disruption since such experiments may provide some further information as to the method of electron transfer to cytochrome P-450.

The most surprising result of this study was the detection of another 35kDa protein in the 5M urea fraction following the use of the *ycb5r* deleted strain in the affinity purification procedure. This was particularly surprising since no 35kDa protein was detected by the anti-p35 antibodies in crude protein extracts prepared from the *ycb5r* disruptant strain. The N-terminal amino acid sequence of the 35kDa protein isolated from the *ycb5r* deleted strain was used to identify the protein as coproporphyrinogen oxidase, a previously identified enzyme involved in haem biosynthesis. Thus coproporphyrinogen oxidase has been identified as a novel novobiocin-binding protein.

Why was the *YCB5R* gene cloned if the 35kDa protein is coproporphyrinogen oxidase? There are two feasible explanations for this :

a) anti-p35 antibodies recognise both proteins; this could occur if the 35kDa band obtained in the 5M urea fractions following affinity purification is not pure and actually consists of two proteins, coproporphyrinogen oxidase and the *YCB5R* gene product.

b) the p35-antibodies raised against coproporphyrinogen oxidase recognise the *YCB5R* gene product due to similarity of the two proteins.

The latter explanation can be ruled out since no sequence similarity exists between coproporphyrinogen oxidase and the *YCB5R* gene product. It was therefore concluded that antibodies were raised against both proteins. This conclusion is further supported by results which showed that a 35kDa protein is recognised by anti-p35 antibodies in total yeast extracts prepared from the *hem13* strain, whereas no proteins are recognised by anti-coproporphyrinogen oxidase antibodies.

A second protein, present in the 35kDa band, might have been expected to interfere with the N-terminal amino acid sequencing of the protein in this fraction. However, a second protein can be present without causing an aberrant effect on protein sequencing if it is blocked at its N-terminus or is present in very small molar ratio (John Kyte, personal communication). It is quite likely that the yeast cytochrome b_5 reductase homologue is blocked at the N-terminal since the mammalian cytochrome b_5 reductase contains a N-terminal myristic acid blocking group (Ozols *et al.*, 1984; Murakami *et al.*, 1989). Although the presence of an N-terminal blocking group has not been demonstrated in the yeast protein, its presence remains a strong possibility.

The antibodies used in this study were raised against proteins found in the 5M urea fraction, following affinity chromatography on novobiocin-Sepharose. Therefore, the yeast cytochrome b_5 reductase homologue must either bind to the column directly or be associated with coproporphyrinogen oxidase which is bound to the column. Further work is required in order to ascertain which of these two possibilities is correct. It is possible that the use of a non-ionic detergent during the protein extraction procedure will yield soluble protein extracts containing the 35kDa protein present in the *hem13* mutant. If such an extract can be produced it could be used in the affinity purification procedure to determine if the protein binds independently to novobiocin-Sepharose. Should the protein bind novobiocin it will be possible to identify it by obtaining N-terminal amino acid sequence and presumably this will identify the protein as the yeast cytochrome b_5

reductase homologue. Even if the protein is blocked at the N-terminal, cyanogen bromide cleavage products could be sequenced, allowing identification of the protein.

It is interesting that, although the p35 antibodies recognise coproporphyrinogen oxidase, no clones encoding part of this protein were obtained from the expression libraries. This may be due to the relatively small number of clones isolated and analysed. However, it is puzzling that, although coproporphyrinogen oxidase was present in the *YCB5R* deletion strain, Western blot analysis of crude protein extract using p35 antibodies failed to detect the protein. Coproporphyrinogen oxidase is recognised well in 5M urea fractions following affinity chromatography. Antibodies were raised against the coproporphyrinogen oxidase following treatment with urea. Thus, antibodies were raised against the denatured coproporphyrinogen oxidase rather than native protein. It is, therefore, possible that the p35 antibodies do not efficiently recognise coproporphyrinogen oxidase which has not been treated with urea as epitopes accessible in denatured protein may be inaccessible to the antibodies in the native protein.

A number of groups have suggested that novobiocin binds to the nucleotide binding sites of target proteins (Mizuuchi *et al.*, 1976b; Sugino *et al.*, 1978; Staudenbauer and Orr, 1981). Further circumstantial evidence for this theory is provided by this work since the yeast cytochrome b_5 reductase homologue is likely to bind both NADH and FAD and AMP-Sepharose has been used in the purification of cytochrome b_5 reductase (Yabisui and Takeshita, 1981). Although coproporphyrinogen oxidase has not been shown to possess an active nucleotide binding site, it binds to blue-Sepharose resin, a substrate originally designed as an affinity support for NAD-dependent enzymes (Taketani and Tockunaga, 1981).

Chapter 8

General Discussion

8.1 The potential re-emergence of novobiocin as a therapeutic agent

As described in the introduction, novobiocin was widely used as an antimicrobial agent during the nineteen sixties. However, due to the number of side effects following treatment with the drug and the development of other antibiotics, the use of novobiocin diminished to a level where it no longer featured in antimicrobial therapy.

The recent emergence of multi-drug resistant Staphylococcal strains which are also methicillin resistant has created major problems in many hospitals due to the extreme difficulty in treating such infections. The search for new antibiotic therapies for the control of drug-resistant strains has lead to renewed interest in the antimicrobiol applications of novobiocin which, in combination with sodium fusidate or rifampicin, has been suggested as a treatment for methicillin-resistant Staphylococcal infections (Jensen, 1968; Drusano *et al.*, 1986; Walsh, 1986).

In addition, interest in novobiocin as an anti-proliferation agent has recently been revived by reports that it inhibits the growth of melanoma cells (Nordenberg *et al.*, 1990) and reduces the growth of murine tumour cells, synergistically with three different alkylating agents (Eder *et al.*, 1989 & 1991). Novobiocin, in conjunction with butyrate, also promotes the differentiation of hepatoma cells, reducing their proliferation and altering their pattern of gene expression (Kaneko *et al.*, 1990). The drug has also been shown to inhibit the cytotoxicity mediated by cytotoxic T-cells in target cells (Wood and Stansfield, 1992), presumably through its effect on either transcription or chromatin structure. These striking effects on mammalian cells, and particularly cancer cell lines, call for the identification of the cellular targets of novobiocin.

8.2 Novobiocin-binding proteins

In this study, three novel novobiocin-binding proteins of 200kDa, 35kDa and 20kDa were purified from the yeast *S. cerevisiae* using affinity purification on novobiocin-Sepharose, a method described by Staudenbauer and Orr (1981). Previously, only three novoiocin-binding proteins have been identified in yeast: a 200kDa protein identified as the yeast heavy chain myosin (Watts *et al.*, 1985), the *SUP45* gene product (Pocklington *et al.*, 1990) and the β-subunit of the yeast mitochondrial F_1 ATP synthetase (Jenkins *et al.*, 1990). The former two proteins are eluted from novobiocin-Sepharose in high salt buffer, whereas, in common with the novel proteins, the latter can only be eluted from novobiocin-Sepharose by denaturing conditions.

8.3 The 200kDa novobiocin-binding protein

The results in this study indicate that the 200kDa protein is a novel yeast protein. When used in indirect immunofluorescence microscopy of yeast, anti-p200 antibodies appear to recognise novel structures which do not colocalise with known cytoskeletal elements, the nucleus or mitochondria. The anti-p200 antibodies were successfully used to screen yeast expression libraries. Further work on clones isolated in this study has been carried out by Dr. J.R. Jenkins and Dr. M. Murray. Sequence data obtained from these clone indicate that the p200 is indeed a novel yeast protein (Dr. M. Murray, personal communication).

Further indirect immunofluorescence microscopy studies have shown that the p200 antibodies cross-react with human fibronectin and detect fibronectin in primary cell lines, but not transformed cells (Dr. J.R. Jenkins, personal communication). However, it is likely that this cross-reactivity reflects the ubiquitination of both proteins as the DNA sequence of the gene encoding the p200 does not display any homology to fibronectins (Dr. M. Murray, personal communication).

Data with regard to the p200 is preliminary and the function of the protein is still unknown. Although the protein has high affinity to novobiocin, a considerable amount of

work will be required before any conclusions can be drawn as to the effects of such an interaction.

8.4 The 35kDa novobiocin-binding protein

The majority of the work in this study has been carried out on the 35kDa novobiocin-binding protein. The results suggest that the 35kDa protein fraction isolated by affinity chromatography on novobiocin-Sepharose is, in fact, composed of two proteins. These proteins have been identified as coproporphyrinogen oxidase and a yeast cytochrome b_5 reductase homologue.

The coproporphyrinogen oxidase was identified from N-terminal amino acid data obtained from the 35kDa protein, whereas the cytochrome b_5 reductase homologue was identified by cloning the corresponding gene, using anti-p35 antibodies to screen yeast expression libraries. It is interesting that these two proteins were identified by two different methods and possible reasons for this have already been addressed in chapter 7. The identification of two proteins from what was originally thought to be one protein highlights an advantage of the dual methodology used in this project. If only one of the two methods had been followed, it is quite likely that one of the proteins would not have been identified. Considering the data, the main question which should be addressed in this work is how the identification of these two proteins can help in obtaining a fuller picture of the effects of novobiocin on eukaryotic cells.

As described previously, cytochrome b_5 reductase is located in the membrane of the endoplasmic reticulum, where it is involved in the desaturation and elongation of fatty acids, cholesterol biosynthesis and is a component of the eukaryotic cytochrome P-450 monooxygenase system. A disruption of the yeast cytochrome b_5 reductase gene was carried out, but no striking growth defects were observed. This was not unexpected since a number of *in vitro* studies indicate that removal of cytochrome b_5 reductase can be compensated in the presence of cytochrome P-450 reductase (Oshino *et al.*, 1971; Reddy *et al.*, 1977; Keyes and Cinti, 1980; Dailey and Strittmatter, 1980). Similar compensatory effects have been observed when proteins associated with the actin cytoskeletal have been

deleted (De Lozanne and Spudich, 1987; Haarer and Pringle, 1987; André *et al.*, 1989; Liu and Bretscher, 1989; Schleicher *et al.*, 1988).

Thus, results from the gene disruption suggest that even if novobiocin does inhibit the yeast cytochrome b_5 reductase activity such inhibition would probably not result in any phenotypic effect.

Coproporphyrinogen oxidase acts at the sixth enzymatic step in the haem biosynthetic pathway, catalysing the sequential oxidative decarboxylations of the 2- and 4-carboxyethyl side chains in coproporphyrinogen III to yield the two vinyl groups in protoporphyrinogen IX (Fig. 7.7). In *S. cerevisiae*, coproporphyrinogen oxidase is thought to be located in the cytosol and the purified enzyme is a homodimer, with a subunit M_r of 35,000 (Camadaro *et al.*, 1986). Gene disruption of coproporphyrinogen oxidase (*hem13*) renders cells incapable of synthesising haem, leading to the secession of all cytochrome-mediated reactions since haem is an essential cytochrome prosthetic group. Lethality of the gene disruption can be prevented by the addition of hemin or ergosterol to growth media. The addition of hemin allows the production of functional cytochromes, thus relieving the effects of the mutation. Ergosterol is the major sterol component of yeast membranes (Fieser and Fieser, 1959) and functional cytochrome is required for its synthesis (Bard *et al.*,1974). The ability of an ergosterol supplement to relieve the lethality of the *hem13* mutant indicates that sterol depletion, leading to membrane disfunction, is the cause of lethality.

The implications of the binding of novobiocin to coproporphyrinogen oxidase *in vivo* are difficult to interpret with regard to the phenotypic effects of novobiocin on yeast. Novobiocin inhibits DNA replication and cell cycle progression from G_0 to S phase, as well as transcription and DNA repair. In view of the phenotype exhibited by cells treated with novobiocin, it is difficult to envisage how inhibition of coproporphyrinogen oxidase could cause any of these phenotypic effects on its own. It would be interesting to observe the terminal phenotype of the coproporphyrinogen oxidase deletion following the removal of the growth supplements (hemin or ergosterol). This may help to indicate if any of the novobiocin-induced effects are due to inhibition of coproporphyrinogen oxidase.

As mentioned previously, one of the major effects of novobiocin may be the inhibition of ATP synthesis caused by the binding of the β-subunit of the F_1 ATP synthetase. However, this inhibition does not account for all the activities of novobiocin since yeast ρ^o strains, which lack functional mitochondria, can grow by fermentation alone and yet novobiocin still inhibits their cellular growth. The inhibition of cellular growth could result from the dual inactivation of coproporphyrinogen oxidase and ATP synthesis but it is more difficult to explain the inhibitory effects of novobiocin on the cell cycle. However, the cell cycle arrest observed in yeast could be explained by the effect of novobiocin on the pheromone receptors (see chapter 1). It is quite possible that the only reason that the α-factor receptor has not been identified as a novobiocin-binding protein is that sample preparation for affinity chromatography excluded integral membrane proteins.

Obviously higher eukaryotes do not have an α-factor receptor. Nevertheless, the α-factor receptor is structurally similar to a group of receptors which include the β-adrenergic and the muscarinic acetylcholine receptors (Nakayama *et al.*, 1985; Burkholder and Hartwell, 1985; Hagen *et al.*, 1986). It is therefore possible that some of the novobiocin-induced phenotypic effects observed in higher eukaryotes may be due to the interaction of novobiocin with these or other as yet unidentified receptor proteins.

8.5 What constitutes a novobiocin-binding site?

The binding of novobiocin to proteins has long been suspected to be associated with nucleotide binding sites. With this in mind, it is interesting to note that the yeast cytochrome b_5 reductase homologue is likely to bind NADH and FAD and that AMP-Sepharose has been used in the purification of mammalian cytochrome b_5 reductases (Yabisui and Takeshita, 1981). Furthermore, although coproporphyrinogen oxidase has not been shown to contain an active nucleotide binding site, the ability of the enzyme to bind blue-Sepharose (a nucleotide analogue) has been used to isolate the protein. However, when the sequences of coproporphyrinogen oxidase and the yeast cytochrome b_5 reductase homologue were compared with that of the β-subunit of the F_1 ATP synthetase and bacterial DNA gyrase, no obvious primary sequence similarity was observed. Therefore,

despite this further piece of circumstantial evidence for the association of novobiocin-binding with nucleotide binding sites, the only convincing of evidence for this remains the competitive binding of ATP and novobiocin observed for DNA gyrase and the elution of the β-subunit of the F_1 ATP synthetase from novobiocin-Sepharose by ATP.

8.6 Future work

The yeast homologue of cytochrome b_5 reductase was cloned in this study. However, it has not yet been shown if this protein binds to novobiocin directly or if it was found in the preparations used to raise antibodies due to some type of association with the coproporphyrinogen oxidase. In order to determine if the yeast cytochrome b_5 reductase homologue does bind to novobiocin on its own, a protein extraction procedure which releases the cytochrome b_5 reductase from the membranes of the coproporphyrinogen oxidase deletion strain must be developed. Such an extract could then be used in affinity purification experiments. An extract containing membrane associated proteins may also be useful in determining if the yeast α-factor receptor binds novobiocin.

As mentioned earlier, no striking growth defects were observed following the disruption of the yeast cytochrome b_5 reductase gene. Future work is required to determine if any subtle phenotypic changes do occur.

In vitro, the removal of cytochrome b_5 reductase can be compensated by the presence of cytochrome P-450 reductase (Dailey and Strittmatter, 1980) This type of functional substitution may also occur *in vivo*. Furthermore, functional substitution may also occur in a cytochrome P-450 reductase mutant of *S. cerevisiae* (Sutter and Loper, 1989). In this strain cytochrome P-450 is still active, indicating that reducing equivalents must be obtained through the activity of an enzyme other than cytochrome P-450 reductase. The electron flow to cytochrome P-450 may in this case be mediated by cytochrome b_5 reductase through cytochrome b_5 which has been shown to transfer electrons to cytochrome P-450 (Aoyama *et al.*, 1990). Due to the importance of the monooxygenase system, I constructed a double mutant defective in both cytochrome b_5 reductase and

cytochrome P-450 reductase. Initial studies suggest that the double disruption causes lethality but further work is required before this observation can be formally presented.

In this study, a number of proteins with high affinity to novobiocin have been isolated in an attempt to identify the target of the drug. However, it is important to note the limitations of such analysis. For example, it should be remembered that in this type of biochemical analysis proteins which are not affected by the drug *in vivo* may be isolated as targets of the drug. It was for such reasons that both biochemical and genetic studies were carried out. As yet, no clear link between the genetic and biochemical data has been firmly demonstrated, although a possible explanation for this has been discussed above. My feeling is that the phenotypic effects of novobiocin are a consequence of its binding to a number of targets. Despite this, it is possible that the main target of novobiocin has not been identified and that the 200kDa and 20kDa proteins, whose functions have still to be determined, may play an important role in the effect of novobiocin on eukaryotic cells.

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