THE EFFECTS OF NALIDIXIC ACID IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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by

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To:

My Family

My Parents

ABSTRACT

Mutants which are simultaneously resistant to nalidixic acid and are temperature-sensitive, have been isolated. Genetic analysis demonstrated the mutation is nuclear. Phenotypic analysis showed that the cell cycle of the mutant [*nal(ts)*] is arrested at or around the S phase at the non-permissive temperature, although the arrest is not immediate. The mutant is hypersensitive to drugs and chemicals that affect the plasma membrane, suggesting the mutation might alter membrane permeability.

The wild-type gene has been cloned by complementation of the Ts phenotype and sequenced. Gene mapping using Southern blots indicates that the *NAL* gene is on the chromosome XII, although its precise location is still unresolved. The reading frame corresponding to the *NAL* gene was unambiguously identified by subcloning. The Nal protein, the predicted protein of the *NAL* gene, has a molecular mass of 63.5 kDa. The Nal protein has a high homology to both the β-amino-γ-ketobutyrate CoA ligase of *Escherichia coli* and δ-aminolevulinic acid synthetase of various organisms, suggesting that they may share the similar substrates glycine and acyl-CoA.

Polyclonal antibodies against the Nal protein have been raised in a chicken and rabbit. These antibodies recognise in yeast extract protein with a molecular mass of approximately 70 kDa, a protein in line with the predicted size of the protein. Indirect immunoflourescence microscopy demonstrated that the Nal protein is mainly localised in the mitochondria.

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Chapter I : Introduction

1.1. 4-Quinolones

Nalidixic acid, a 1.8-naphtyridine (Figure 1.1), is the first 4-quinolone to have been synthesized. It was originally identified as a by-product in the production of chloroquine, an antimalaria drug. Analogues later synthesized include oxolinic acid and cinoxacin (Figure 1.1). All 4-quinolone agents known to date are purely synthetic; no structurally related compounds have been identified as metabolites of living organisms. A major advantage of 4-quinolones is that the transmission of bacterial resistance against them is predominantly via chromosomes rather than via plasmids or transposons (Burman, 1977).

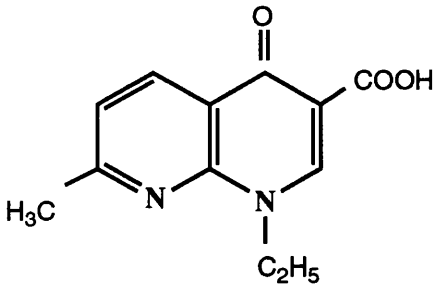
New generations of 4-quinolone antibacterial agents have been designed to increase their activities and reduce the probability of selecting for resistant microbes. Among the most potent 4-quinolone agents are the fluoroquinolones, a new 4-quinolone generation including drugs such as enoxacin, norfloxacin and ciprofloxacin (Figure 1.1). These fluoroquinolones are up to 600 times more active than their predecessor nalidixic acid. The introduction in nalidixic acid of fluorine atoms at position C6, as well as at position C1 and C7 in the other side chains, has resulted in an elevation of antimicrobial activity and longer duration (Lode *et al.*, 1990), thus reducing the concentration used. Furthermore, the toxicity and side-effects of these newly synthesized drugs are considerably reduced (Stahlmann, 1990).

4-Quinolone agents are readily absorbed gastrointestinally, and they are predominantly eliminated through the kidneys, although the

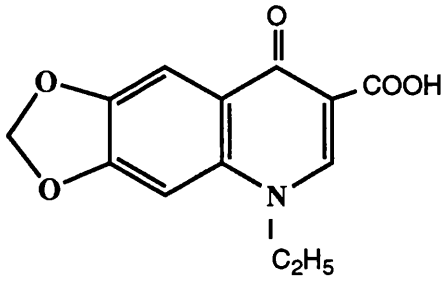
Figure 1.1 : Structure of 4-quinolones

Older agents include a) nalidixic acid b) oxolinic acid c) cinoxacin

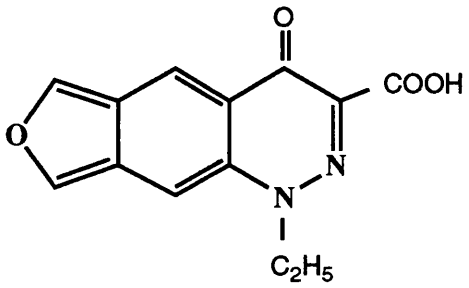
Newer agents include d) enoxacin e) norfloxacin f) ciprofloxacin



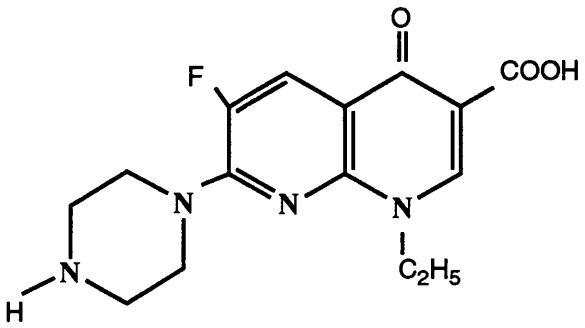
a) **Nalidixic acid**



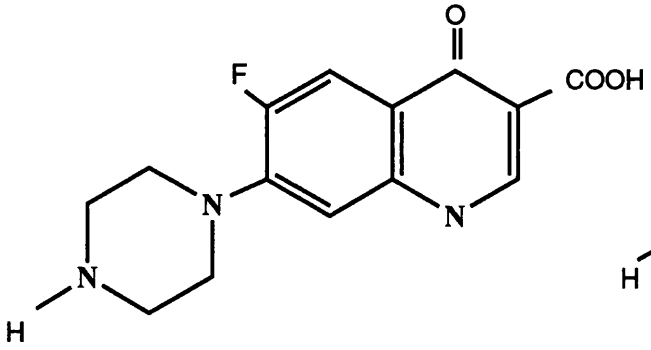
b) **Oxolinic acid**



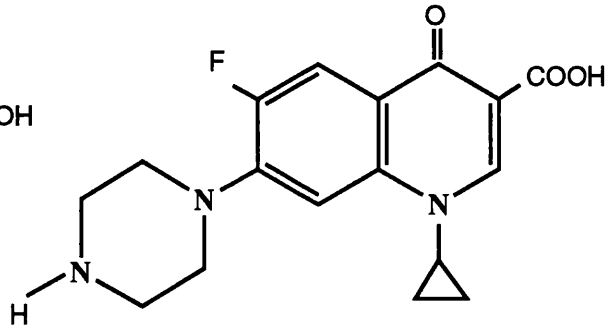
c) **Cinoxacin**



d) **Enoxacin**



e) **Norfloxacin**



f) **Ciprofloxacin**

Figure 1.1. : Structures of 4-quinolone agents

metabolism of various 4-quinolones differ considerably (Lode *et al.*, 1990).

1.2. The effects of 4-quinolones in prokaryotes

Nalidixic acid inhibits the growth of prokaryotes. It has been demonstrated that the drug inhibits DNA synthesis in bacteria by interfering with DNA gyrase, a type II topoisomerase, (Gellert *et al.*, 1976b) with a further inhibitory effect on the synthesis of RNA and proteins (Goss *et al.*, 1965, Bourguignon *et al.*, 1973). The drug differs significantly from other bacterial agents with respect to its inhibitory doses. Bacterial death is reduced, paradoxically, by increasing the concentration of nalidixic acid; above 100 $\mu g ml^{-1}$, the bacteriostatic effect is more predominant (Winshell and Rosenkranz, 1970, Crumplin and Smith, 1975). Winshell and Rosenkranz, (1970) found that nalidixic acid at a concentration of 20 $\mu g\ ml^{-1}$ exhibited the most severe effect on *E.coli*, whereas Crumplin and Smith, (1975) showed that the drug at a concentration up to 100 $\mu g\ ml^{-1}$ is still bactericidal on various *E.coli* strains. Both groups found that the addition of protein synthesis inhibitors, e.g. chloramphenicol and erythromycin decreased the bactericidal effect of nalidixic acid. They suggested that, at high concentrations, nalidixic acid inhibits RNA and protein synthesis as well as DNA synthesis, preventing bacterial growth, and so resulting in resistance to the drug.

Crumplin and Smith, (1975) observed that cells surviving an exposure to nalidixic acid at higher concentrations than 100 $\mu g\ ml^{-1}$, are still sensitive to nalidixic acid following their subculturing. This phenomenon is common to other quinolones. The inhibitory effect of nalidixic acid on DNA synthesis is reversible, i.e. after being exposed

to nalidixic acid, the cells resume DNA synthesis when transferred to a drug-free medium (Deitz *et al.*, 1966).

Dougherty and Saukkonen (1985) discovered that nalidixic acid both increased the susceptibility to detergent and changed the membrane permeability of *E.coli*. Cultures of this bacterium were resistant to sodium dodecyl-sulfate (SDS) at a concentration of 5%, but became sensitive to 0.1% detergents 50 minutes after the addition of 20 $\mu g\ ml^{-1}$ nalidixic acid. These scientists attributed the quinolone-induced changes to the loss of membrane integrity, to the ensuing leakage of cytoplasmic constituents and to eventual cell death.

Similar to EDTA and gentamicin, 4-quinolones induce lipopolysaccharide release, increase cell hydrophobicity and increase outer membrane permeability to β-lactams in *E.coli*. 4-Quinolones are in fact chelating agents and are thus capable of binding to divalent cations in the outer membrane, most notably to Mg^{2+} which has been known to antagonize the antibacterial activity of the 4-quinolones. This binding is followed by dissociation of the quinolone-magnesium complex from the outer membrane (Chapman and Georgopapadakou, 1988). The chelating effect leads to a disorganization of the bacterial surface layer, facilitating the entry of hydrophobic compounds, including quinolones, and diffusing through the newly created hydrophobic patches in a self-promoted pathway (Hancock, 1984).

Hirai *et al.* (1986a) indicated that 4-quinolones can also penetrate the outer membrane of *E.coli* through OmpF and OmpC porins. The minimum inhibitory concentration (MIC) of 4-quinolones in porin-deficient mutants increases less than four-fold relative to the wild-type. Hirai *et al.* (1986a) further noticed that the MIC of quinolones decreased in lipopolysaccharide-deficient (LPS) mutants as compared to the wild type. The decrease in the MIC was correlative to the

hydrophobicity of the quinolones: the more hydrophobic the compounds, the larger the decrease in the MIC. These observations suggested that the passage of 4-quinolones through the outer membrane was not limited to porins.

While the activity of nalidixic acid and oxolinic acid decreased in the LPS-deficient mutants, the activity of the new 4-quinolones (the fluoroquinolones) was not affected (Hirai *et al.*, 1986b). Treatment of *E.coli* with EDTA caused the disruption of the permeability barrier of the outer membrane by hydrophobic and hydrophilic antibiotics (Hardaway and Buller, 1979). However, Diver *et al.*, (1990) showed treatment with EDTA to have no effect on the accumulation of fluoroquinolones, suggesting that the outer membrane of *E.coli* does not act as a permeability barrier against these quinolones. Moreover, the experiment showed no direct correlation between the accumulation and antibacterial activity of 4-quinolones, suggesting that other factors are involved in determining the potency of quinolones.

Indeed, other factors influencing the uptake of 4-quinolones have been reported. These include:

a) temperature

Temperature reduction leads to a decrease in 4-quinolone uptake (Bedard *et al.*, 1987; Diver *et al.*, 1990). This phenomenon is probably due to an altered expression of pore-forming proteins, or to the size of the pores participating in the uptake.

b) pH

The uptake of 4-quinolones is very low in acid pH compared to that in neutral or alkali pH. At neutral pH, most 4-quinolones exist in two forms: approximately 90% in a zwitterionic form,

the remainder in an undissociated acid, a singly positively charged form. Data suggest that the outer membrane is more readily penetrated by the zwitterionic form than by the undissociated form. An increased acidity (a lower pH) reduces the amount of the zwitterionic form available, and hence the 4-quinolone uptake.

In vitro, 4-quinolones have been reported to eliminate plasmids of *E.coli* at subinhibitory concentrations (Weisser and Wiedeman, 1986). This activity is probably due to the failure of DNA gyrase to complete its function in mediating the decatenation of plasmid DNA during plasmid replication.

Vincent *et al.* (1991) found that after exposure to ofloxacin and pefloxacin at concentrations close to MIC (~0.40 $\mu g ml^{-1}$), *E.coli* developed filamentation, followed by a lytic event. It has been suggested that this lysis was correlated with changes in the peptidoglycan chain length. At higher concentrations of the drugs, no lysis occured and the growth was completely inhibited. The peptidoglycan composition exhibited an increase in the average glycan chain length, due to reduction in the activity of the lytic transglycosylase.

1.3. The mode of action of 4-quinolones in prokaryotes

4-Quinolones have been shown to inhibit or interfere with DNA gyrase, a type II topoisomerases. Despite extensive efforts on the part of scientists all over the world, the precise mode of action of 4-quinolones has yet to be fully explained.

Gellert *et al.* (1976a, 1976b) described a DNA gyrase activity that is responsible for the introduction of negative supercoils into *E.coli*. The bacterial enzyme can also relax negatively supercoiled DNA and

can decatenate intertwined DNA complexes (Wang, 1982). DNA gyrase is a prokaryote type II topoisomerase, consisting of two pairs of non-identical protomers: two A subunits with a molecular mass of 105 kDa which are encoded by the *gyrA* gene (48 minutes on the *E.coli* genetic map), and two B subunits with a molecular mass of 95 kDa, encoded by the *gyrB* gene (83 minutes). Although subunit A has been found to be more abundant in the cells than subunit B, the enzyme does require an equal number of protomers to be active (Cozzarelli, 1980). DNA gyrase and other type II topoisomerase change the linking number by two, and hence alter the topological state of nucleic acids by passing an intact DNA helix through a transient double-stranded break generated by the enzyme (Cozzarelli, 1980, Wang, 1982). The model of the process is presented in figure 1.2 (from Morrison and Cozzarelli, 1981).

The breakage-rejoining process, resulting from type II topoisomerase activity is initiated by the binding of the enzyme to its nucleic acid subtrate. Although type II topoisomerase interact with preferred sequences, the specificity is not as stringent as that of restriction endonucleases. This phenomenon, allowing the enzyme to act at numerous sites within the DNA strand might be crucial 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 equlibrium. This equilibrium highly favours the re-ligation process (Osherroff, 1989). The breakage of each strand of the double-helix DNA is thought to be mediated by a single protomer (GyrA) of the enzyme.

After hydrolysis of the DNA, tyrosine 122 of the subunit A of DNA gyrase becomes covalently bound to both newly generated 5' ends via

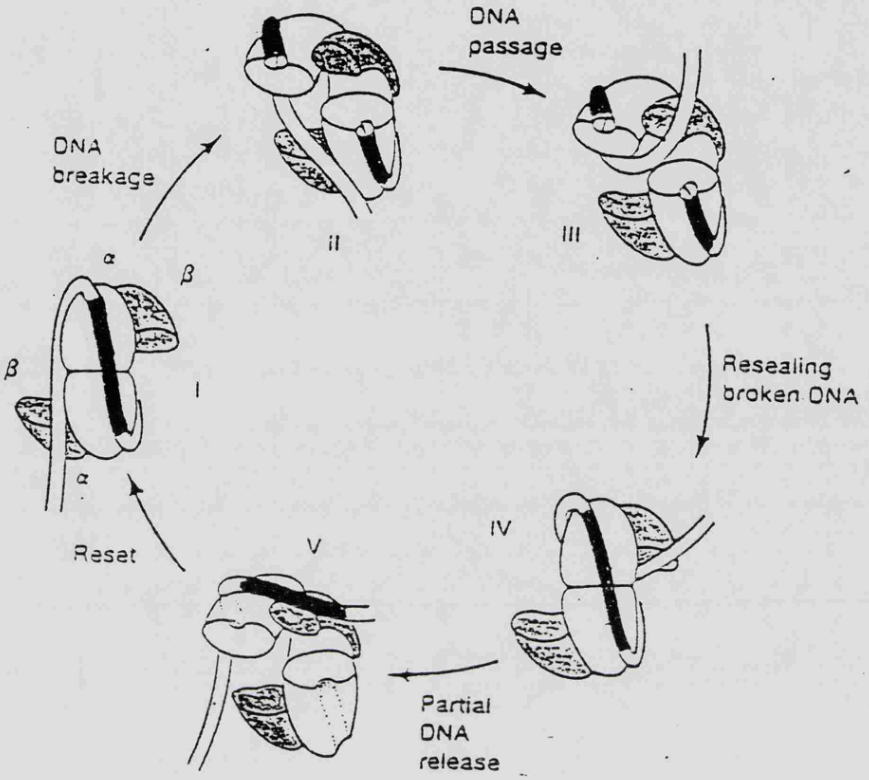


Figure 1.2: A model for negative supercoiling of DNA by gyrase

Step I represents a gyrase molecule consisting of two A and two B monomers with a circular duplex DNA wrapped in a right-handed superhelix. Step II, the DNA is cleaved by the enzyme. Step III, the unbroken segment of the DNA is passed through the breakage. Step IV, the broken DNA is resealed and finally, step V, the DNA strand is released from the enzyme (Morrison and Cozzarelli, 1981).

positive supercoils could be generated ahead of it, and it is possible that their accumulation is prevented by the activity of topoisomerase II. On the other hand, negative supercoils built up behind the replicating fork could be removed by topoisomerase I. The removal of negative supercoiled accumulation is possibly necessary as this accumulation might lead to the arrest of many physiological machineries, e.g. DNA replication, transcription and RNA translation. In yeast, either the topoisomerase I or topoisomerase II enzyme suffices for DNA replication to proceed (D'Arpa and Liu, 1989). DNA topoisomerase II also has the ability to decatenate or separate newly replicated progeny molecules at the end of DNA replication (Wang, 1985, Vosberg, 1985). Inactivation of topoisomerase II prior to mitosis results in chromatin breakage. This enzyme has been shown to be involved in chromosome condensation and decondensation (Uemura *et al.*, 1987).

Novobiocin has been demonstrated to inhibit all steps performed by subunit B e.g. its ATPase activity. Nalidixic acid and all other quinolones, on the other hand, only inhibit the activity which involves the breaking and rejoining of the DNA strand performed by subunit A. These activities do not require ATP. The mode of action of quinolones has yet to be satisfactorily explained (Brown and Cozzarelli, 1979).

It has been reported that the pre-strand passage DNA breakage/rejoining equilibrium is both a vulnerable and a notable target for several classes of antineoplastic drugs (Lock and Ross, 1987; Liu, 1989 and Osheroff, 1989). Accumulation of an intermediate reaction complex, named the "cleavable complex" occurs (or is trapped) in the presence of the drugs. The exposure of the "cleavable complex" to a protein denaturant such as SDS results in DNA breakage and the covalent linking of the topoisomerase to each end of the

Figure 1.3: Proposed reaction mechanisms for mammalian DNA topoisomerases

I. DNA topoisomerase I

Mammalian DNA topoisomerase I is proposed to form at least two different complexes with DNA that are in rapid equilibrium: the non-cleavable (B) and cleavable (C) complexes. The cleavable complex is presumed to be the transient covalent reaction intermediate. Exposure of the cleavable complex to a strong protein denaturant, such as SDS or alkali, results in single-strand DNA breaks and the covalent linking of the topoisomerase I polypeptide chain to the 3'-phosphoryl end of the broken DNA strand through a phosphotyrosyl bond (Liu, 1989).

II. DNA topoisomerase II

Mammalian DNA topoisomerase II is proposed to form at least two different complexes with DNA that are in rapid equilibrium: the non-cleavable (B) and cleavable (C) complexes. Antitumour drugs (4-quinolones, in the case of prokaryotic DNA topoisomerase II) can stabilize the cleavable complexes. Exposure of the cleavable complex to a strong protein denaturant results in double-strand DNA breaks and the covalent linking of the topoisomerase II polypeptide chain to the 5'-phosphoryl end (Liu, 1989).

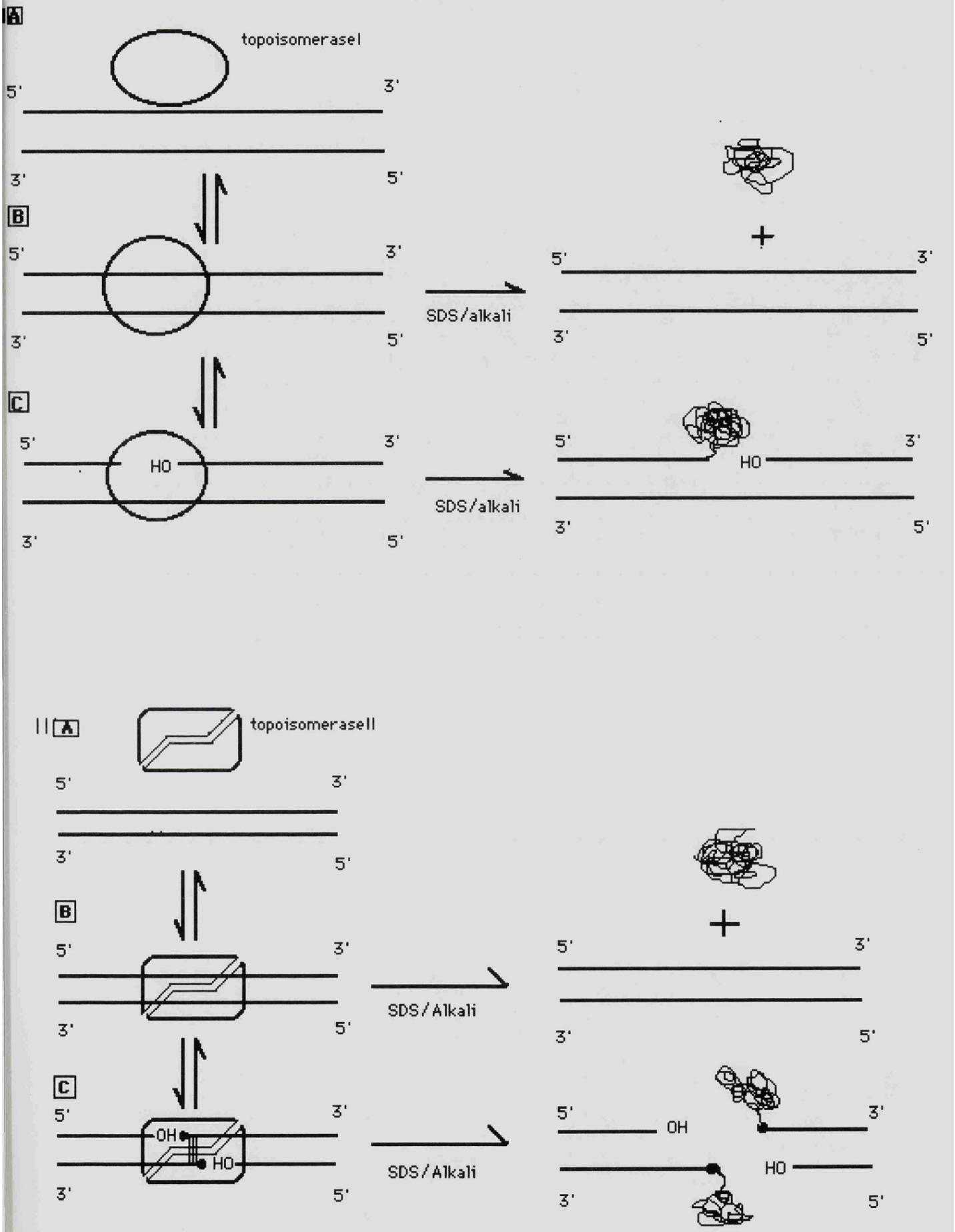

A
topoisomerasel
5'
3'
3'
5'
B
5'
3'
3'
5'
SDS/alkali
5'
3'
3'
5'
C
5'
3'
HO
3'
5'
SDS/alkali
5'
3'
HO
3'
5'
II A
topoisomerasell
5'
3'
3'
5'
B
5'
3'
3'
5'
SDS/Alkali
5'
3'
3'
5'
C
5'
3'
OH
HO
3'
5'
SDS/Alkali
5'
3'
OH
HO
3'
5'

Figure: 1.3

broken DNA strand (figure 1.3). These drug-induced cleavable complexes are reversible, and they are readily dissociated prior to the addition of the protein denaturant (D'Arpa and Liu, 1989).

When 4-quinolones, such as nalidixic acid, are applied to a reaction mixture containing *E.coli* gyrase and DNA, the covalent product is increased upon the addition of SDS. The quinolone trap seems to abort gyrase-DNA cleavable complexes. Shen and Pernet (1985) proposed that the drug, enzyme and DNA all form a ternary complex in which the drug interacts with both the enzyme and the DNA. The formation of this abortive ternary complex seems to be the cause of the rapid cessation of DNA replication (Deitz *et al.*, 1966; Drlica, 1984).

The quinolone-induced DNA damage is believed to lead to the induction of the SOS response in *E.coli* (Piddock *et al.*, 1990). Piddock further suggested that filamentation caused by the inhibition of cell division prior to cell death, may be the consequence of the SOS response. The reversibility of this DNA damage, suggests that factors other than the cleavable complexes might cause cell death. Exposure to high doses of drugs for long periods, however, does cause the damage to become irreversible.

1.4. The effect of 4-quinolones in eukaryotes.

The effects of 4-quinolones in eukaryotes have been extensively investigated *in vitro* and *in vivo*. Miller *et al.* (1981) found that nalidixic and oxolinic acid had 50% inhibitory concentrations (IC_{50}s) of 500 and 100μg ml^{-1}, respectively, for the decatenation activity of topoisomerase II isolated from Hela cell nuclei. Hussy *et al.* (1986) found that the IC_{50}s for inhibiton of this enzyme, purified from calf thymus, by ciprofloxacin, norfloxacin, nalidixic acid and ofloxacin were

150, 300, 1000 and 1300 μg ml^{-1}, respectively. The concentration of the drugs required to inhibit topoisomerase I is even higher. These concentrations are from 100 to 1000 times higher than that to inhibit prokaryotic topoisomerases. The above data show that 4-quinolones are not at present regarded as potent inhibitors of eukaryotic topoisomerases.

Additional effects have, however, been observed (Singer & Johnston, 1979; Hussy *et al.*, 1986; Forsgren *et al.*, 1987a, 1987b). Ofloxacin and ciprofloxacin at a concentration of 100 μg ml^{-1} inhibit cell growth of mammalian lymphoblast. At a concentration of 1000 μg ml^{-1} these quinolones kill the cells (Forsgren *et al.*, 1987b). Moreover, Hussy *et al.* (1986) showed that 4-quinolones at a concentration of more than 100 μg ml^{-1} reversibly inhibit DNA synthesis, catalysed by the 9S DNA polymerase α primase complex from calf thymus. Oomori *et al.* (1988) investigated the effects of 4-quinolones on Hela cell growth and topoisomerase II. They confirmed that the enzyme is inhibited only by high concentrations of the 4-quinolones tested. They further found a strong correlation between the inhibitory effects of the drugs on topoisomerase II and their cytotoxic effects.

An increase in the incorporation of [^{3}H]thymidine in human lymphocytes *in vitro* was observed in the presence of 4-quinolones (nalidixic acid, cinoxacin, pipemidic acid). Nevertheless, these drugs stimulated the uptake of [^{3}H]thymidine into trichloroacetic acid (TCA)-precipitable material only at high concentrations (Forsgren *et al.*, 1986; 1987b). Paradoxically, despite the apparent increase in DNA synthesis, lymphocyte growth was inhibited by ciprofloxacin at a concentration as low as 20 $ugml^{-1}$. In another investigation, Forsgren *et al.* (1987a) showed that ciprofloxacin failed to increase the uptake of [^{14}C]hypoxanthine, indicating a selective effect on pyrimidine and

not purine nucleotide biosynthesis. They suggested that ciprofloxacin at low concentrations might affect *de novo* pyrimidine biosynthesis, and at high concentrations (80-160 $\mu g ml^{-1}$) inhibit cell growth *in vitro*. Altered pyrimidine biosynthesis in the presence of 4-quinolones might result from the inhibition of the enzyme involved in the biosynthesis.

Upon the addition of camptothecin, a topoisomerase I antagonist, DNA synthesis and RNA synthesis are inhibited. A similar effect is observed when cells are exposed to a high concentration of cancer chemotherapeutic drugs which affect topoisomerase II. These drugs can be place into two categories:

a) DNA intercalators such as acridines, actinomycins and anthracyclines

b) Non-intercalator such as epipodophyllotoxin.

The cellular effects of topoisomerase II inhibition include rapid inhibition of DNA and RNA synthesis, as well as chromosome abberations. This enzyme is required for separating intertwined daughter chromosomes prior to mitosis in yeast, as it is in other eukaryotic cells. Inhibition of type II topoisomerase prior to this point leads to cell death, possibly due to chromatin tearing by the spindle apparatus which attempts to separate intertwined sister chromatids (Uemura, 1987).

The effects of 4-quinolones have been studied in lower eukaryotes such as *S.cerevisiae* and *Euglena gracilis*. These drugs have been shown to eliminate chloroplast from *E.gracilis* producing 100% chloroplast-less cells at concentrations which do not affect cell viability (Kraycovic *et al.*, 1989).

Michels *et al.* (1973) identified a transient inhibition of DNA, RNA and protein synthesis upon the addition of nalidixic acid to

logarithmically growing yeast cultures. Without removing the drug from the culture, this macromolecular synthesis arrest started to recover 2 hours after the addition of nalidixic acid. The drug did not inhibit mitochondrial DNA synthesis. In contrast, Luha *et al.* (1971) showed that nalidixic acid inhibits mitochondrial DNA synthesis in the yeast *Kluyveromyces lactis,* and Whittaker *et al.* (1972) found that the drug prevents the generation of petite mutants of *S.cerevisiae,* either by ethidium bromide or by 5-fluorouracil.

Carnevali *et al.* (1976) reported that yeast petite mutants selected by ethidium bromide are more resistant to sodium nalidixate than their isogenic wild-type strains. The growth rate of the wild-type strain was inhibited by sodium nalidixate at a concentration of 100 $\mu g ml^{-1}$, whereas the petite mutant was only partially inhibited. The wild-type strain, however, resumed its normal growth rate after being transferred to a nalidixate-free medium. It was consequently suggested that the growth inhibition could directly result from an inhibition of DNA synthesis or be caused by the drug-sensitivity of a mitochondrial gene product, normally involved in the control of cell division.

Nalidixic acid at low concentrations (<25 μg) exhibits a differential effect on the expression of mitochondrial genes *in vivo* e.g. the drug reduces the expression of cytochrome oxidase genes (Mahler and Johnson, 1979). Studies *in vitro* indicate that nalidixic acid inhibits yeast DNA polymerase I activity as well as the replication of the 2μm plasmid (Nakayama and Sugino, 1980). It was therefore suggested that as no effect of the drug on DNA topoisomerase II could be detected, the target of nalidixic acid in yeast was DNA polymerase I.

In a synthetic medium, the addition of nalidixic acid to yeast causes an immediate and complete inhibition of DNA and RNA

synthesis, though some residual protein synthesis can still be detected (Whittaker and Carnevali, 1977). This phenomenon also occurs in a rich medium, but without a complete inhibiton of macromolecular synthesis (Whittaker and Carnevali, 1977).

Finally, Singer and Johnston (1979) confirmed that at a concentration of 100 $\mu g\ ml^{-1}$, nalidixic acid leads to a cell cycle arrest at the G1 phase in *Saccharomyces cerevisiae*. Nevertheless, the cell cycle does resume after 2 hours of nalidixic acid exposure. They also found that 35S ribosomal preRNA was much reduced in nalidixic acid-treated cells compared to that of untreated cells. No effect on the synthesis of either tRNA or 5S RNA could be detected.

1.5. Drug resistance in general

Drug resistance can be classified into two major groups:

a) intrinsic resistance, which is also known as natural, *de novo* or primary resistance, whereby an organism, or cell, possesses a characteristic "feature" allowing all normal members of the population to tolerate a particular drug or chemical agents (Hayes and Wolf, 1990);

b) acquired or secondary resistance, whereby a resistant strain or cell line emerges from a previously drug-sensitive population (Hayes and Wolf, 1990).

1.5.1. Intrinsic resistance

The characteristic "feature" allowing all normal members of any given species to tolerate a particular drug or chemical agent has most likely been developed through the processes of evolution. The level of exposure and the nature of the agents involved are the primary factor

to have determined which resistance mechanisms have evolved. This explains the variability in cell sensitivity or in cell resistance to drugs and chemicals. In the case of intrinsic resistance, the characteristic "feature" seems to be widely spread within populations (10-100%), and response to drug or chemical induction is quite high. On the other hand, the protective "feature" in acquired resistance arises from a spontaneous mutation within a population, only a very rare occasion (10^{-6}); this feature is not therefore widely spread before exposure to the drug.

Drugs and a wide variety of toxic agents induce biochemical changes in cells, allowing them to overcome severe effects. The ability to resist toxic effects can be immediate or take a considerable length of time, after exposure to the agents. These changes in the cells include alterations in the membrane permeability, in enzymes and DNA structure, or in metabolic capability. For example, the exposure of β-lactam antibiotics to microorganisms can induce the production of β-lactamase to deactivate the toxic effect of drugs.

In some cases, the toxic chemicals are produced by certain microorganisms as a defense mechanism. Consequently, these agent-producing microorganisms require their own defense against the toxic effect of the agents they produce. In the case of antibiotic-producing microorganisms such as *Streptomyces* sp., two possible drug resistance mechanisms have been proposed by Cundliffe (1984). First, the antibiotic-producing microorganism possesses an enzyme capable of detoxicating certain antibiotics, e.g. phosphotransferase and acetyltransferase inactivate streptomycin and neomycin, respectively. Secondly, the drug resistance results from modification of the target site within the cell, e.g. in *Streptomyces erythreus*, which is an

erythromycin-producing microorganism, ribosomal RNA, which is the target of this antibiotic, is protected by methylation.

Species-specific selective toxicity of the toxic agents can raise intrinsic resistance. Albert (1985) defined this selectivity in terms of three categories:

a)Differences in drug accumulation

The differences in drug accumulation between a parasite and its host exemplifies the first category as the basis for selective toxicity. For example, phenothiazine is readily absorbed by helminths, but is poorly absorbed by the gastrointestinal system of the host; therefore, the helminth is more affected by the drug;

b)Differences in intermediary metabolism

The resistance mechanism to sulphonamide compounds is an example of the differences in intermediary metabolism between bacteria and its host. The sulphonamide agents inhibit the enzyme dihydropteroate synthetase and prevent the synthesis of dihydropteroic acid which would be converted to the nucleotide precursor dihydrofolic acid. As mammalian cells lack the dihydropteroate synthetase, they are resistant to the toxic effect of sulphonamides;

c)Differences in structure of the target

The resistance of mammalian cells to β-lactam antibiotics exemplifies resistance which results from differences in cell structure. The effect of β-lactam antibiotics is to inhibit cell wall synthesis by inhibiting the synthesis of peptidoglycan. Lacking cell walls, mammalian cells are resistant to β-lactam antibiotics, whereas bacteria are susceptible to the drug.

Physiological stress such as U.V irradiation, heat and osmotic shock can stimulate a genetic reflex to confer tolerance against subsequent exposure to the same physiological stress. In some cases, this adaptive response can lead to resistance to drugs or chemical agents. Krueger and Walker (1984) reported that in *E.coli*, the *groEL* and *dnaK* heat-shock proteins are induced by UV irradiation and 4-quinolones. Resistance to heat-shock treatment renders the cells resistant to 4-quinolones consistent with their observation.

1.5.2. Acquired drug resistance

The protective "feature" emerges from the previously drug-sensitive population. This development might be due to genetic changes following an exposure to drugs or chemical agents. Genetic change can in this case be defined in the following three ways:

a) Mutation or amplifications of specific genes directly involved in a protective pathway: for example, in *E.coli*, nalidixic acid and novobiocin inhibit cell growth by affecting DNA gyrase. Changes in the DNA gyrase structure lead to resistance either to nalidixic acid or novobiocin (Gellert *et al.*, 1976a; Gellert *et al.*, 1977). In tumour cell lines, amplification of the gene producing P-glycoprotein makes the cell resistant to anticancer agents (Riordan *et al.*, 1985; Scotto *et al.*, 1986).

b) Mutations in the genes which regulate stress-response processes leading to alterations in the expression other genes: e.g. mutations changing the LexA repressor protein affect its binding to its target genes, which are involved in the SOS response in *E.coli* (Little and Mount, 1982). Such alteration leads to the expression of genes responsible for the SOS

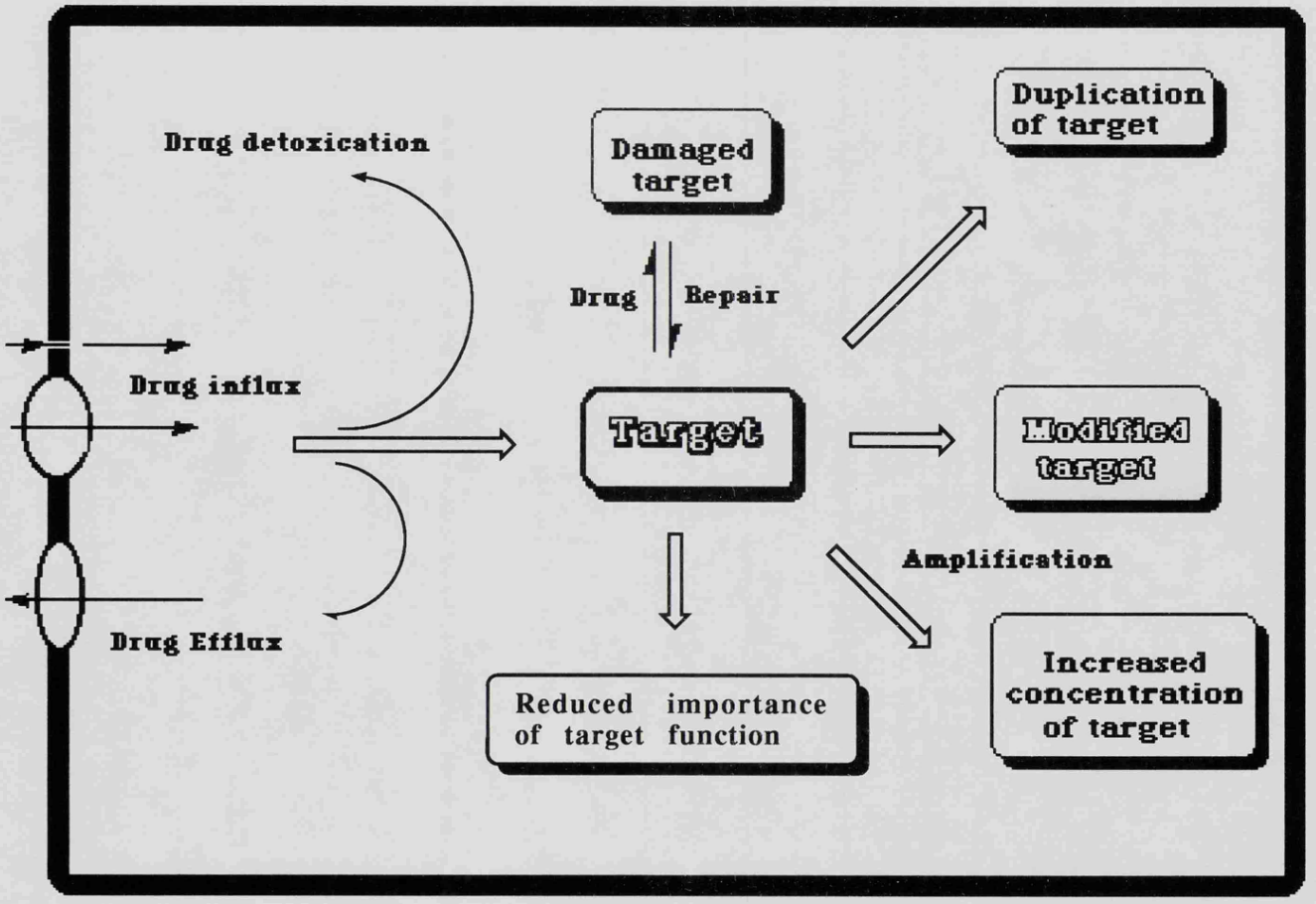


Figure 1.4 : Biochemical mechanism of drug resistance

response thus conferring of the cells resistance to nalidixic acid and UV irradiation.

c) Plasmid-mediated drug resistance, in bacteria resistance to drugs such as chloramphenicol and ampicillin, is acquired through the transfer of genes residing on mobile plasmids.

1.5.3. The biochemical mechanism of drug resistance

An outline of the biochemical mechanism of drug resistance is presented in figure 1.4. These mechanisms include:

a) Preventing an access to the target

Permeability of the membrane is generally considered as the barrier preventing a drug from reaching the target. This type of mechanism therefore depends on the hydrophobicity of the drug and the structure of the cell membranes. For example, hydrophobic antibiotics are more effective against Gram-positive than Gram-negative bacteria. Unlike Gram-positive bacteria, Gram-negatives have two layers of cell membranes, lipopolysaccharides and glycerophospholipids in the outer and inner layer, respectively. The rigidity of the outer layer might prevent hydrophobic antibiotics from penetrating the cell membrane, whereas hydrophilic antibiotics can penetrate the cell membrane through porin proteins which form channels in the membrane. Not unexpectedly, however, the loss or alteration of porins (e.g OmpF and Omp C) leads to antibiotic resistance in *E.coli* (Nikaido, 1989).

b) Structure modification of the target

Alteration or substitution of target proteins can decrease the formation of drug-target complexes which then confer resistance to the drug. Such resistance can result from point

mutation in the structural gene(s) encoding the target. Mutation in the subunit A of DNA gyrase confers resistance to 4-quinolones (Courtright *et al.*, 1988).

c) Synthesis of enzymes to deactivate/detoxicate drugs or toxic agents

A wide variety of bacteria are capable of synthesizing enzymes that specifically inactivate drugs. These enzymes can either destroy the drug by covalently binding to it, or substitute essential residues chemically, making the drug inactive. The β-lactamase inactivates β-lactam antibiotic by hydrolysing the lactam-ring of the antibiotic (Sabath *et al.*, 1965). Chloramphenicol transacetylase detoxifies chloramphenicol by acetylating it using acetyl-CoA as an acetyl donor. The resulting product is 3-acetoxy-chloramphenicol, an inactive form antibiotic (Gale *et al.*, 1981).

d) Reduction in the physiological importance of the target

In bacteria sensitive to β-lactam antibiotics, reducing the physiological importance of peptidoglycan as a structural component of the cell wall leads to insensitivity to β-lactam antibiotics (Gale, 1981).

e) Duplication of the target

Duplication of the target is a further manner in which the toxic effects of certain drugs can be avoided. Trimethoprim-resistant bacteria possesses two different dihydrofolate reductases performing the same function. One of these enzymes is unaffected by trimethoprim and hence remains active (Smith and Amyes, 1984; Amyes, 1989).

f) Increasing in the concentration of the intracellular target

It has been observed an increase in glutamine synthetase enzyme concentration in a methionine suphoximine-resistant CHO cell line (Sanders and Wilson, 1984)

g) Increased repair of the damaged target site

Cell death following exposure to drugs and toxic agents is sometimes due to DNA damage. The SOS system enhances cell survival and allows DNA repair, therefore enabling bacteria to overcome the effects of drugs such as nalidixic acid (Walker, 1984).

h) Reducing in the concentration of the intracellular toxic agent

Reducing the drug concentrations in the cell can minimize its ability to reach the target. In tumour cell lines such as the Chinese Hamster Ovary (CHO), the P-glycoprotein in the plasma membrane acts as an efflux pump extruding drugs and toxic agents from the cell, thus rendering the tumour cells more resistant to anti-cancer agents (Ling & Thompson, 1974). Another mechanism involved in the reduction of intracellular drug concentration is sequestration. Metallothionin, a low molecular mass cystein-rich protein, binds to the heavy metals of the antitumour drug, cis-diaminedichloroplatinum, thus inactiving it (Kelley *et al.,* 1988).

1.5.4. Genetic mechanism of drug resistance

Drug resistance can result from genetic changes such as gene amplification, gene transfer and gene modification. Gene modification includes a point mutation and deletion of the gene. The former

modification produces a different protein from the previous one, sufficient to change its affinity for an inhibitor.

The location of genes conferring resistance to drugs or toxic agents can be found either in bacterial chromosomes or plasmids, an extrachromosomal element. Any resistance pattern depending upon modification of existing genetic material is likely to be chromosomal, whereas resistance genes code for entirely specific enzymes which inactivate antibiotics, and are usually carried on bacterial plasmids (Gale *et al.*, 1981).

1.5.5. Multiple drug resistance

Introducing a particular drug to cells can lead to resistance not only to that drug but also to others. This phenomenon is called multiple drug resistance.

In human tumours, exposure to an anti-cancer agent such as vincristine may lead to the over-expression of a cell membrane glycoprotein, called P-glycoprotein, which in turn confers resistance to other anti-cancer agents. Juliano and Ling (1976) showed that the level of resistance to colchicine in CHO cells correlated with the level of a 170 kDa membrane protein (P-glycoprotein). The amount of this protein was greatly reduced in drug-sensitive revertants (Riordan and Ling, 1979). Examination of numerous mammalian cell lines expressing multidrug resistance have demonstrated a correlation between this phenomenon and the increased expression of P-glycoprotein. In drug sensitive parental cell lines, however, the level of P-glycoprotein is very low (Kartner *et al.*, 1983).

From the amino acid sequence of the protein, it can be deduced that P-glycoprotein contains 12 membrane-spanning helices and two ATP-binding sites (Chen *et al.*, 1986; Gros *et al.*, 1986). The membrane

(transmembrane) spanning segments might provide a channel-like structure through which the drug is extruded. The presence of two highly conserved ATP-binding sites is consistent with the role of P-glycoprotein as an energy-dependent drug efflux pump. The ATP-binding regions are involved in ATP hydrolysis required to pump out the drug against the concentration gradient. Substitution of critical amino acids in one of the ATP-binding sites removes the pump function (Azzaria *et al.,* 1989).

Mammalian P-glycoproteins are encoded by a multigene family in which the number of numbers varies between species. On the basis of sequence homologies, the P-glycoprotein genes from mammal can be divided into two groups: called *mdr1* and *mdr2*. *Mdr1* is further divided into *mdr1a* and *mdr1b* (Kane and Gottesman, 1989). Unlike *mdr1a* and *mdr1b*, *mdr2* might not function as a drug efflux pump.

P-glycoprotein-like proteins are found in a wide variety of organisms. *Plasmodium falciparum* and *Saccharomyces cerevisiae* both possess genes that are homologous to *mdr1* (Foote *et al.*, 1989; McGrath and Varshavsky, 1989). In the case of *P.falciparum,* the mdr-like genes might be responsible for resistance to anti-malarial drugs such as chloroquine. The yeast P-glycoprotein-like protein, encoded by *STE6* gene, is involved in the secretion of the mating type factor, a-factor pheromone. The HlyB protein, a 66kDa *E coli* membrane protein responsible for secreting a haemolysin also shares high homologies with P-glycoprotein at the C-terminal region (Gerlach *et al.,*1986).

In human tumours, another type of resistance has been described. Cell resistance to anthracyclines shows cross-resistance to ellipticine, acridines, actinomycinD, mitoxantrone and epipodophylotoxins. No cross-resistance to vinca alkaloid and colchicine has been observed (Borst, 1991; Liu, 1989; Rose, 1988). This

is generally considered not to be true multidrug resistance, but rather the probable result of alteration in the DNA topoisomerases which those anti-cancer agents inhibit. The alteration of DNA topoisomerase II that leads to drug-resistance might be due either to a decrease in the concentration of the normal DNA topoisomerase II or to a replacement of normal DNA topoisomerase II with mutated DNA topoisomerase II which is less sensitive to drugs (Pommier *et al.*,1986; Tan *et al.*, 1989; Deffie *et al.*, 1989).

1.5.6. Multidrug resistance in yeast

Pleiotropic drug resistance (pdr) refers to multidrug resistance in yeast. This sort of resistance results from mutations in gene(s) responsible for resistance to a great number of structurally and functionally unrelated subtances. McGrath and Varshavsky (1989) identified a yeast gene, *STE6,* which has high homologies to the mammalian multidrug resistance gene. *STE6* gene is involved in the secretion of the mating type pheromone, a-factor (Kuchler *et al.*, 1989). However, whether this gene has a function in multidrug resistance mechanism remains unclear.

Linnane *et al.* (1968) and Bunn *et al.* (1970) reported a series of mutants which confer pleiotropic drug resistance to unrelated inhibitors such as chloramphenicol, tetracyclin, erythromycin, carbomycin, oleandomycin, spiromycin, and which interfere with mitochondrial protein synthesis. Thomas and Wilkie (1968) classified the resistance of the mutants to erythromycin in two groups:

a) mitochondrial inheritance, which was thought to result from an alteration of the mitochondrial protein synthesizing machinery;

b) Mendelian inheritance, which was thought to result from changes either in the plasma membrane permeability or in mitochondrial membrane composition.

Avner and Griffiths (1973a, 1973b) isolated mutants resistant to oligomycin. They defined these mutants as belonging to two classes:

a) class I mutants, nuclear mutations, show cross-resistance to a wide variety of unrelated drugs such as cytoplasmic protein inhibitor (cycloheximide), mitochondrial protein synthesis inhibitor (chloramphenicol, erythromycin) and uncoupling agents (carbonylcyanide-*m*-chlorophenylhydrazone);

b) class II mutants, which are only resistant to oligomycin and rutamycin. These mutations show typical cytoplasmic inheritance and the determinants are located in the mitochondrial DNA.

Rank and Bech-Hansen (1973) isolated a strain carrying the *oli*^{PR}*1-1* mutation, eventually renamed *pdr1-1*, conferring resistance to oligomycin and chloramphenicol and showing cross-resistance to other unrelated drugs. This mutant also becomes sensitive to other unrelated inhibitors, paromomycin, neomycin, dequalinium chloride, ethidium chloride and acriflavin. The subsequent experiment showed such pleiotropic cross-resistance to result from mutations changing cytoplasmic membrane permeability (Rank *et al.*, 1975). However, the hypersensitivity of the mutants to other drugs remains unexplained. The second allele, *oliPR1-2* renamed *pdr1-2*, shows the same cross-resistance and sensitivity to various drugs as *pdr1-1;* additionally, this mutant fail to grow under physiological stress such as extreme temperatures and osmolalities (Rank *et al.*, 1976; Rank *et al.*, 1977). It has been proposed that these pleiotropic phenotypes could result from

alterations either in the plasma membrane or in the mitochondrial inner membrane, or in both.

Another allele, *pdr1-3,* described by Guerineau *et al.* (1974), exhibits cross-resistance to oligomycin, chloramphenicol, cycloheximide, triethylin and venturicidin, as well as hypersensitivity to paromomycin and ethidium bromide. It has been suggested that the genetic factors responsible for this drug-resistance were not located in the mitochondrial DNA, but rather in the nuclear DNA although some other episomal factors or plasmids might be involved.

Data obtained by Saunders and Rank (1982) have demonstrated all the above mutations to be alleles of the same locus, namely *PDR1,* which controls cell membrane permeability and located on chromosome VII. A further mutation, *smr3,* which is located on chromosome XV at locus *PDR2,* was described by Falco and Dumas (1985). It confers resistance to sulfometuron methyl and to unrelated drugs such as oligomycin and cycloheximide. The locus *PDR3*, a centromere-linked gene on chromosome II, is involved in resistance to mucidin, which is an inhibitor of the mitochondrial electron transport, and in cross-resistance to unrelated drugs (Subik *et al.,* 1986). Loci *PDR4* and *PDR5* are situated on chromosomes XIII and XV, respectively. These genes also confer resistance to various inhibitors upon amplification of the genes. The *PDR6* gene, located centromere-distal to *PDR1* on chromosome VII, participates in resistance to different cytoplasmic inhibitors such as cycloheximide and hygromycin B (Balzi and Goffeau, 1991).

Balzi *et al.* (1987) confirmed that the *pdr1-2* is a nuclear single-gene mutation which, in addition to drug resistance, confers a partial respiratory deficiency. The poor growth of this mutant on glycerol media was restored by the *PDR1* gene on a multicopy plasmid. They

suggested that this gene product might control the transcription of nuclear encoded mitochondrial components.

Membrane permeability is generally considered to be the barrier responsible for the transportation of certain substances into or out of the cell. Modification of membrane permeability can therefore alter the influx and efflux of substances, including drugs. Resistance or sensitivity to drugs might consequently be affected by modification of membrane permeability. Linnane *et al.* (1968) showed a decrease in cellular drug uptake in chloramphenicol and tetracyclin resistant mutants. Rank *et al.* (1975) noted a similar phenomenon. They found that modification of both plasma and mitochondrial membranes were associated with the *pdr1-2* mutation. Johnston and Coddington (1983) reported a reduction in the uptake of the drug and amino acids in cycloheximide mutants (*cyh3* and *cyh4* mutations) of *Schizosaccharomyces pombe.* Ulaszewski *et al.* (1983) found that modification of plasma-membrane ATPase in mutants of *S.cerevisiae* showed resistance to unrelated-drugs such as N,N-(p-xylylidene)-bis-aminoguanidine-HCl, ethidium bromide, vanadate and guanidine derivatives.

The above observations suggest that membrane permeability might be crucial in the mechanism of drug resistance.

1.6. Isolation of 4-quinolones resistant mutants in prokaryotes

Drug resistance in Gram-negative bacteria can be achieved by reducing drug-uptake (Hayes and Wolf, 1990; Borst, 1991). Such bacteria are surrounded by an outer membrane (OM) which acts as a permeability barrier. In *E.coli,* β-lactam antibiotics penetrate cells mostly through the porin pathway (Yoshimura and Nikaido, 1985) in

the outer membrane. Although 4-quinolones can also penetrate through the porin pathway (Hirai *et al.*, 1986a; Zweerink and Edison, 1986), hydrophobic quinolones may diffuse through the bilayer by disorganizing the membrane through the chelation of divalent cations (Chapman and Georgopapadakou, 1988) However, the mechanism of 4-quinolones resistance, regarding membrane permeability, presented a contradiction. Piddock and Wise (1986) found that the alteration of porin expression in porin-deficient mutants of *E.coli* K-12 produced no significant effects in the MICs of 4-quinolones. Hirai *et al.* (1986a) reported an increase, albeit only two to four fold higher in the MICs of 4-quinolones for the *OmpF* of *E.coli*.

A ciprofloxacin-resistant mutant of *P.aeruginosa* apparently developed a membrane alteration (Daikos *et al.*, 1988). This particular mutant lost the 31-32 kDa outer membrane protein, which is thought to correlate with the resistance phenotype. This resistance, however, was unstable; the mutant became susceptible to ciprofloxacin after being grown in drug-free medium for 5-15 generations. Acquisition of ciprofloxacin resistance was at least partially related to defects in lipopolysaccharide synthesis (Legakis *et al.*, 1989). The cross-resistance between 4-quinolones, β-lactam antibiotics, aminoglycosides and chloramphenicol was also reported (Robillard and Scarpa, 1988; Sanders *et al.*, 1984).

Alteration of the 4-quinolone target, DNA gyrase, is usually considered to be the major mechanism for this drug-resistance. Bourguignon *et al.*, (1973) described a *nalA* mutation, designated *gyrA*, which resulted in the quinolone-resistant DNA replication system of *E.coli* K-12. Subsequent research showed that this gene encodes the A subunit of DNA gyrase (Gellert *et al.*, 1977). Further studies in *E.coli* found resistance to be caused by a point mutation in

gyrA resulting in changes in amino acids at the region between 67-106. The amino acids changes are from Ala to Ser, Gly to Cys, Ser to Leu, Ser to Trp, Ala to Pro, Asp to Asn and Glu to His at position of 67, 81, 83, 83, 84, 87 and 106, respectively (Yoshida *et al.*, 1988, Yoshida *et al.*, 1990, Cullen *et al.*, 1989). These mutations are situated in the relatively hydrophilic regions of the GyrA polypeptide and close to Tyr at position 122, the residue thought to be the active site enzyme because of its covalent linkage to DNA (Horowitz and Wang, 1987). These mutations lead to an increase in the MIC varying from 2 to 100 times (Hooper and Wolfson, 1991). Additional data concerning the GyrA protein from *Klebsiella pneumoniae* (Dimri and Das, 1990) and *Staphylococcus aureus* (Hopewell *et al.*, 1990) showed a conservation of the active-site Tyr-122. Changes in amino acids at position 67, 83, 84, 87 and 106 in the GyrA polypeptide of *K.pneumoniae* and *S.aureus* with a conservative subtitution confer resistance to 4-quinolones. Other mutation in *gyrA* that confer resistance to 4-quinolones are *nfxA* (Hooper *et al.*, 1986), *norA* (Hirai *et al.*, 1986b), *cfxA* (Hooper *et al.*, 1987) and *ofxA* (Wolfson *et al.*, 1987).

Another mutation, *nalB*, described by Hane and Wood (1969), encodes resistance to low levels of nalidixic acid, approximately five times the MIC of the parent strain. This mutation is thought to confer resistance to the drug through the reduced uptake of the 4-quinolone (Bourguignon *et al.*, 1973). Two mutations in the *gyrB* encoding the subunit B of DNA gyrase have been identified (Yamagishi *et al.*, 1981, 1986). The alteration in these two 4-quinolone resistance-determining sites is due to a single nucleotide change in the gene resulting in amino acid changes from lysine to glutamic acid (at position 447) and aspartic acid to asparagine (at position 426), termed *nalC* and *nalD* mutations, respectively. The *nalC* mutation confers resistance to a low

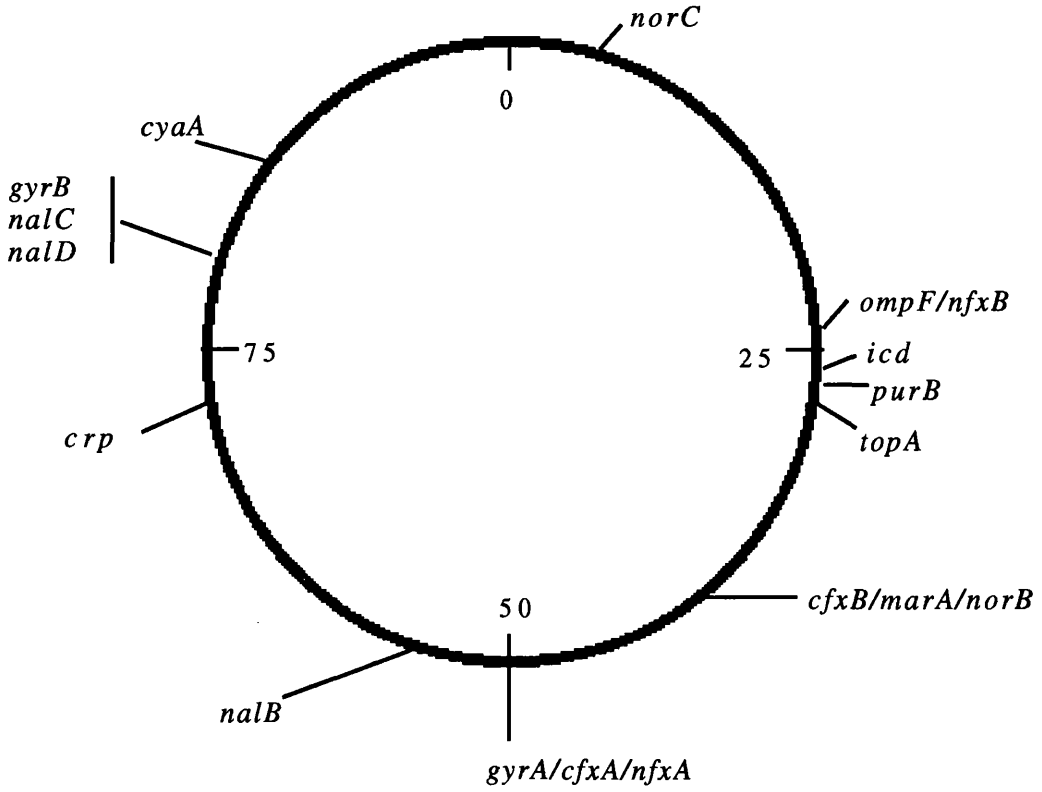


Figure 1.5: Chromosome map of 4-quinolone mutations in *E.coli*

level of acidic-quinolones with the hydrophobic group at position 7, such as nalidixic acid, oxolinic acid and cinoxacin but hypersensitive to amphoteric-quinolones with a positive charge at the corresponding position, such as pipemidic acid, ciprofloxacin, norfloxacin, enoxacin and ofloxaxin (Yoshida *et al.,* 1991). The *nalD* mutation is characterized by low level resistance to all 4-quinolones.

Further studies using the new generation of fluoroquinolones such as ciprofloxacin and norfloxacin revealed more detailed characterization of low level 4-quinolone resistance. Other mutant loci, in addition to those encoding DNA gyrase, include *nfxB, cfxB, norB* and *norC* mutations are shown in the chromosome map in figure 1.5. (Hirai *et al.,* 1986b, Hooper *et al.,* 1986, Crumplin, 1990). These mutations share common phenotypes, such as a low-level of resistance to 4-quinolones, also showing cross-resistance to other unrelated drugs, e.g. β−lactam antibiotics, tetracyclin and chloramphenicol. Further investigations revealed that *nfxB* and *cfx* mutations reduce the expression of the *OmpF* gene, resulting in the reduction of the OmpF protein in the membrane (Hooper *et al.,* 1989). In OmpF deletion mutants, however, the increase of the MIC level is not significant (Hirai *et al.,* 1986; Hooper *et al.,* 1989). This therefore suggests that the reduction in OmpF protein might not be responsible for resistance to the drugs in *nfxB* and *cfxB* mutants. *norC* differs from *nfxB* and *cfxB,* this mutations causes hypersensitivity to hydrophobic quinolones, whereas *norB* has the same characteristics as *cfxB* (Wolfson *et al.,* 1989).

Other 4-quinolone resistant mutants of *P aeruginosa* exhibited a reduction of outer membrane protein G (25.5 kDa) and the loss of a 40 kDa outer membrane protein (Chamberland *et al.,* 1989). The susceptibility of DNA synthesis to norfloxacin in these mutants is also

decreased. Similar results were found by Michea-Hamzehpour *et al.* (1991), who investigated the mechanism of resistance to pefloxacin in *P aeruginosa* strain. They suggested that resistance to pefloxacin is associated with:

a) altered DNA gyrase, increasing MIC by 16 fold;

b) altered permeability resulting from changes in LPS and from a decreased expression of the protein D2 (47 kDa);

c) both of the above mechanisms.

Further mutations in additional genes produce low-level resistance to nalidixic acid by as yet undefined mechanism include *crp* (Kumar, 1976), *cyaA, icd* (Kumar, 1980), *pur* (Helling and Adams, 1970, Kumar, 1980), *ctr* (Helling and Adams, 1970) and *marA* (George and Levy, 1983).

1.7. Multiple drug sensitivity in the yeast *S. cerevisiae*

Originally resistant to most drugs, the yeast *S.cerevisiae* can under certain conditions be sensitive to a wide variety of unrelated drugs. This might be due to mutations affecting membrane permeability. This phenomenon was described by Rank & Bech-Hansen (1973) and Rank *et al.* (1976), who isolated oligomycin resistant mutants. While the mutants are oligomycin resistant, they simultaneously become sensitive to other unrelated drugs. A similar phenomenon occurs in cycloheximide resistant mutants, which become hypersensitive to hygromycin B (McCusker and Haber, 1988a, 1988b).

Pocklington *et al.* (1990), also reported that one of novobiocin resistance mutants, namely *nbs1 (mds1)*, is sensitive to many unrelated drugs. It has been suggested that this mutant is a membrane permeability mutant. The phenotypes of the mutant

feature deficiency in mating ability and smaller cells, many of which are distorted.

Because of its sensitivity to most of drugs including nalidixic acid, this strain was chosen to isolate mutants which are resistant to 4-quinolones following the protocol for isolating mutants in prokaryotes.

1.8. *Saccharomyces cerevisiae* as a eukaryotic model

The yeast *Saccharomyces cerevisiae* has been successfully used as a model systems for understanding eukaryotic biology at the cellular and molecular level. As a unicellular microorganism, yeast shares both the simplicity and rapidity of growth of bacteria (its growth rate is roughly half of that *E.coli*). It is easily grown in laboratory media in large quantities, and consequently is suitable for preparation of large amount of protein for biochemical analyses.

Saccharomyces cerevisiae is a real eukaryote, as judged by many fundamental properties of cell biology, having a typical nuclear membrane, mitochondria, vacuoles, cytoskeleton, secretion machinery and chromosome organisation (Bostein and Fink, 1988). *Saccharomyces cerevisiae* can be grown either as haploid or diploid cells making it possible to isolate, analyse conditional lethal mutations and its effect on the cell.

The cell cycle of *Saccharomyces cerevisiae* (figure1.6) 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 stops when the majority of the cells have small buds, this indicates that it has been arrested at the beginning of the S phase, the DNA synthesis period (Pringle and Hartwell, 1982).

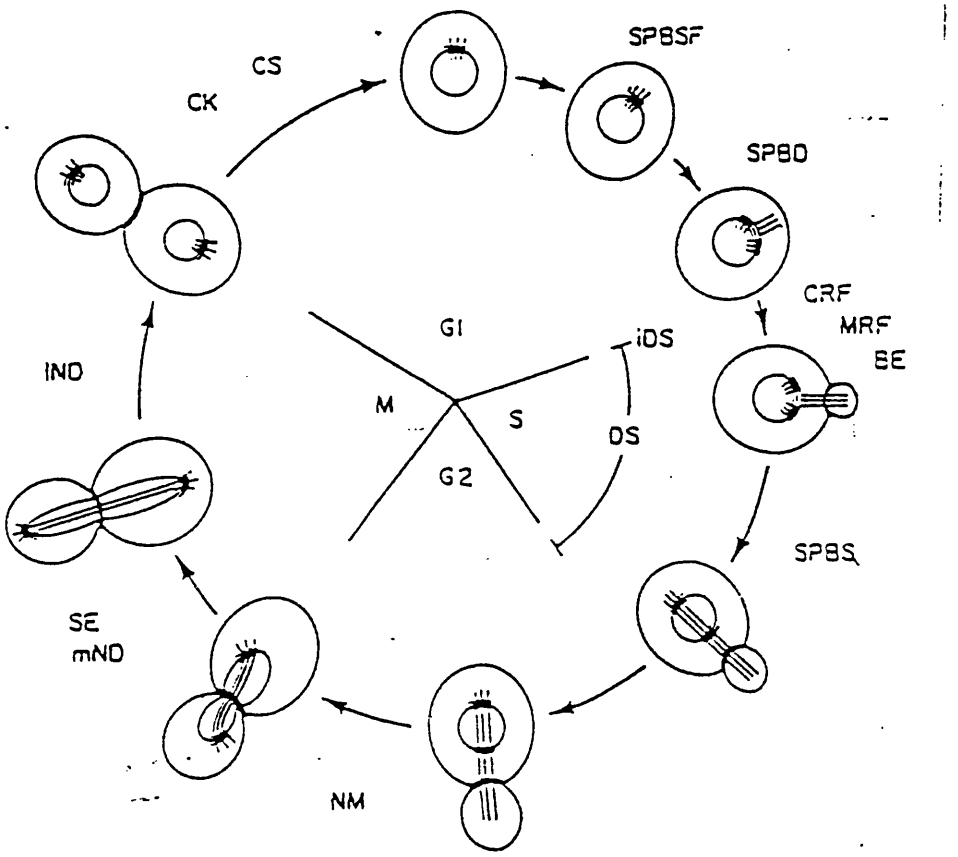


Figure 1.6: The cell cycle of *Saccharomyces cerevisiae*

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).

Classical genetic investigations are also possible. Many auxothropic mutants are available, it is easy to do chromosome mapping and the organism contains tightly packed genes with few introns on relatively little DNA. The availability of transformation systems in yeast makes it possible to clone genes and to manipulate them *in vitro.* Genes can be introduced into yeast either by plasmid transformation or by targeting them into chromosomes. In the latter case, they can subtitute a wild type gene.

The application of recombinant DNA technology has resulted in the demonstration that most eukaryotic proteins, including those of *S.cerevisiae,* are well conserved at the amino level (Botstein and Fink, 1988). Conservation is most extreme in ubiquitin and the cytoskeletal elements, although others (Table 1) still show significant (about 60% identity) homology.

Yeast or human protein	Identity (%) in amino acid sequence
Ubiquitin	96
Actin	89
β-tubilin	75
HMGCoA reductase	66
Cytochrome c	63
Citrate synthetase	62
RAS1/N-ras; RAS2/K-ras	60
Glucose transporter	25

Table 1: Degree of identity in amino acid sequence between corresponding protein of yeast (*Saccharomyces cerevisiae*) and humans (*Homo sapiens*). The data were generated from sequences in Genebank and other publish sources (Botstein and Fink, 1988).

This conservation is underlined by functional homologies. For example, mammalian *ras* genes complement yeast *ras* mutants (Kataoka *et al.*, 1984; Tatchel, *et al.*, 1984; Tamanoi, *et al.*, 1984). It has also been shown that chicken-yeast chimeric tubulin is incorporated in to mouse microtubules (Bond, 1986).

1.9. Aims of this study

The purpose of this study is to identify the genetical target of 4-quinolones in yeast. This will be achieved by the isolation of mutants which are simultaneously temperature sensitive and 4-quinolones resistant. The mutants will be characterised genetically and physiologically. The wild-type allele of the gene conferring the drug resistance will be cloned and its DNA sequence analysed. The product of the gene will be used to raise antibodies in order to localize the antigen in the cell.

Chapter 2 :Materials and methods

2.1. Abbreviations

Abbreviations listed below were used throughout

APS	Ammonium persulphate
Bisacrylamide	N',N'-methylene bisacrylamide
BSA	Bovine serum albumin
CIP	Calf intestinal phosphatase
CHO	Chinese hamster ovary
Cpm	Count per minute
DMF	Dimethylformamide
ddNTP	(di)deoxynucleoside triphosphate
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
DAPI	4',6'-diamidino-2-phenylindole
EDTA	Ethylene diamine tetra acetic acid
IMS	Industrial methylated spirit
kb	Kilobases
kDa	Kilodaltons
mds	Multiple drug sensitive
LPS	Lipopolysaccharide
MOPS	3-(N-morpholino) propanesulphonic acid
MIC	Minimum inhibitory concentration
NB	Nutrient broth
ORF	Open reading frame
POPOP	1,4-Bis-2-(4-methyl-5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole

IPTG	Isopropyl β-D-thiogalactopyranoside
TEMED	N,N,N',N' - tetramethylethylenediamine
TLA	Top layer agarose
Tris	Tris-(hydroxymethyl)-methylamine
Triton X-100	Octyl phenoxy polyethoxyethanol
ts	temperature sensitive
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

2.2. Media and chemicals

2.2.1.Media and buffer.

- YPD broth : 1% yeast extract, 2% peptone, and 2% glucose.
- YPD solid medium : 1% yeast extract, 2% peptone, 2% glucose, and 2% agar.
- Yeast minimal medium: 0.67% yeast nitrogen base w/o amino acids (Difco), 2% glucose and required amino acids.
- Yeast minimal solid medium: 0.67% yeast nitrogen base w/o amino acids (Difco), 2% glucose, the required amino acids and 2% agar.
- Pre-sporulation medium : 0.8% yeast extract, 0.3% peptone, 10% glucose, and 2% agar.
- Sporulation medium : 1% potassium acetate, 0.1% yeast extract, 0.05% glucose, and 2% agar.
- Nutrient broth: 2.5% nutrient broth.
- *E.coli* minimal medium : 10% of M9 (15.1% Na_2HPO_4, 3% KH_2PO_4, 0.5% NaCl, and 1% NH_4Cl), 4% glucose, and

1% CM (0.01 M $CaCl_2$, 0.1 M $MgSO_4.7H_2O$)

- Minimal medium for yeast transformation: 0.67 % yeast nitrogen base without amino acids (Difco), 2 % glucose, 100 µl 1% amino acids as required, 18.2 % sorbitol, and 2% agar.
- Top agar for yeast transformation: 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, 100 µl of 1% amino acids as required, 18.2 % sorbitol, and 2% agar.

-TE: 10 mM Tris pH8.0 and 1 mM EDTA

-TE': 10 mM Tris pH8.0 and 0.1 mM EDTA

2.2.2. Chemicals.

Nalidixic acid, pipemidic acid, enoxacin, econazole nitrate, clotrimazole, novobiocin, α-factor, TEMED, ribonuclease A, DTT, IPTG, protease K, ammonium persulphate, and bovine serum albumin were purchased from Sigma. Polyethyelene glycol 6000, phenol, formamide, urea were obtained from Fisons. Restriction endonucleases and their buffers were supplied primarily by Gibco BRL plc.

The sequencing kit, nested deletion kit, and T4 DNA polymerase were purchased from Pharmacia. Radioactive [α-^{35}S]ATP, [γ-^{32}P]CTP and [^{3}H]adenine were obtained from Amersham International.

2.3.Strains used

Saccharomyces cerevisiae and *Escherichia coli* strains used in this study are as listed in the tables below.

Table 2.1: ***Saccharomyces cerevisiae*** **strains.**

Strain	Genotype	Source
A37	*MATα, mds1, leu2, his7, tyr1, ura1*	M. Pocklington
DBY746	*MATα, leu2, trp1, his3, ura3*	D.Bostein
10C	*MATa, nov(ts), leu2, trp1, lys2*	M. Pocklington
10D	*MATα, nov(ts), leu2, trp1, lys2*	M. Pocklington
C1	*MATa, his4*	E. Orr
C6	*MATa, his4,*	E. Orr
A364	*MATa, ade1, ade2, ura1,tyr1,his7, lys2*	CSHL
483/1a	*MATα, ade2-1, his5-2, ura3-1, can1-100*	This laboratory
S288c	*MATa*	This laboratory
SD1.1	*MATα, top2(ts), ade1, ade2*	M. Pocklington
NI2D	*MATα, nal(ts)5, his4*	This study
NI3D	*MATα, nal(ts)5, ura3*	This study
NP5B	*MATα, nal(ts)1, leu2*	This study
NT56B	*MATα, nal(ts)5, leu2, ura3*	This study
NA528	*MATα, nal(ts)5, leu2, ura3, ade1, his trp1.*	This study
NA113	*MATa, nal(ts)5, leu2, ura3, ade*	This study
NT18C	*MATa, nal(ts)1, leu2, his, ura3*	This study
8HA-11-1	*MATa, trp1, ade1, leu2*	M. Pocklington
842D	*MATα/MATa, leu2/leu2, ade1/ade1, his /his, ura3/ura3, trp1/trp1*	K. Nasmyth

842	*MATa, ade1, his , leu2, ura3, trp1*	E. Orr
K382-23A	*MATa, spo11, ura3, can1, cyh2, ade2, his7, hom3*	CSHL
K382-19D	*MATα, spo11, ura3, can1, cyh2, ade2, his7, hom3, tyr1*	CSHL
K398-4D	*MATa, spo11, ura3, ade6, arg4, aro7, asp5, met14, lys2, pet17, trp1*	CSHL
K381-9D	*MATα, spo11, ura3, ade6, arg4, aro7, asp5, met14, lys2, pet17, trp1*	CSHL
K399-7D	*MATa, spo11, ura3, his2, leu1, lys1, met4, pet8*	CSHL
K393-35C	*MATα, spo11, ura3, his2, leu1, lys1, met4, pet8*	CSHL
K396-11A	*MATa, spo11, ura3, ade1, his, leu2, lys7, met3, trp5*	CSHL
K396-22B	*MATα, spo11, ura3, ade1, his, leu2, lys7, met3, trp5*	CSHL

CSHL= Cold Spring Harbor Laboratory

Table 2.2: *Escherichia coli* strains

Strain	**Genotype**	**Source**
DH5α	*F' phi80dlacZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17 (rk- mk+) deoR supE44 thi-1 gyrA96 reA1 λ-*	Hanahan (1983)

JA221	*F⁻, leu, trp, thi*	This laboratory
B15	*F⁻, ura, trp, thi*	This laboratory
5K	*F⁻thr-1, leu-B6, (r⁻,m⁺), thi, supE44 lacY1, tonA21*	This laboratory
BL21	*hsdS, gal(lcIts857 ind1 S*am7 *nin5 lac*UV5-T7 gene *1)*	Studier/Moffat (1986)

Table 2.3: Vectors used

Vectors	Genetic markers	Source
YEp13	*amp, tet, LEU2*	J. Broach (1979)
YEp24	*amp, URA3*	D. Botstein (1982)
pEMBL Yr25	*amp, TRP1*	M. Pocklington*
pEMBL Ye23	*amp, URA3*	M. Pocklington*
pEMBL Yi21	*amp, URA3*	M. Pocklington*
pYRG19	*amp*	P. Meacock*
pUC18	*amp*	This laboratory
pUC19	*amp*	This laboratory
pIC19H	*amp*	S. Mash (1984)
pIC20R	*amp*	S. Mash (1984)
pET3a1	*amp*	A.H. Rosenberg (1987)

* Personal communication

Table 2.4: Synthetic oligonucleotide primer

Oligonucleotide	Source
1. TCACCCGAATTACACGC	Dept. of Biochemistry, University of Leicester
2. GCTATGTTAATACCTGC	Dept. of Biochemistry, University of Leicester
3. GTAGAACCTATGATGCG	Dept. of Biochemistry, University of Leicester
4. TTTACCTACTCAGGCGC	Dept. of Biochemistry, University of Leicester
5. ATGTCCCATCTTTGACG	Dept. of Biochemistry, University of Leicester

2.4. General methods for genetic experiments.

2.4.1. Isolation of mutants.

A single colony of A37 strain (table 2.1) was cultured in YPD broth at 26°C with agitation overnight. 100 µl of the culture was spread on YPD plates containing nalidixic acid at final concentrations of 25, 50, 100, 200, 400, 800 and 1000 µg ml^{-1}. The cultures were then incubated at 26°C until colonies appeared.

Nalidixic acid-resistant colonies (about 100 colonies/plate) were picked up and transferred to minimal media plates containing leucine, tyrosine, histidine, uracil and nalidixic acid (200 µgml^{-1}). The plates were incubated at 26°C.

All purified colonies resistant to nalidixic acid were transferred to YPD plates and incubated at 26°C and 37°C. These colonies which grew at 26°C, but not at 37°C, were picked up and repurified on a YPD plate at 26°C.

2.4.2. Induction and selection of rho^0 mutants.

A single colony of the nalidixic acid resistant, temperature-sensitive *(nal)(ts)* mutant was grown in YPD overnight and subcultured in the morning. 100 µl of exponentially growing cells were spread on a YPD plate and 1µl of 10 mgml^{-1} Ethidium bromide was placed on the plate. The plate was incubated overnight at 30°C. Cells at the edge of inhibition zone were picked up and streaked out on YPD plate and incubated overnight. Rho^0 colonies were identified by their inability to grow in a medium supplemented with glycerol as a sole carbon source. The lack of mitochondrial DNA was confirmed through DAPI staining

2.4.3. Tetrad analysis.

The *nal(ts)* mutant was mated with a wild type yeast in YPD broth and placed on a YPD plate, which was incubated at 30°C overnight. The growing colonies were streaked out on a minimal-media plate containing only those amino acids required by both strains in order to obtain diploids. The diploids were repurified on the same medium containing those amino acids required by the diploids and incubated until the tetrads appeared.

The tetrads were suspended in 90µl distilled water and 10 µl of β-glucoronidase was added. The mixture was incubated at room temperature for 10 minutes and diluted to 1ml. Tetrads were dissected using a Zeiss Jena standard laboratory microscope with a micromanipulator attachment (Sherman, 1986).

2.4.4. Fixation of yeast cells.

Yeast cells grown to a log phase in YPD, were fixed either with 70% methanol or with 3% formaldehyde (final concentration) for 2-4 hours at room temperature. The fixed cells were then transferred to Eppendorf tubes, washed 5 times with PBS (100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 1 mM EDTA) and subsequently resuspended in 0.5 ml PBS and stored at -20°C.

2.4.5. Staining yeast nuclei.

Five µl of the fixed cells were spread on the microscope slide and fixed to the glass by gentle heating. They were stained with a drop of DAPI (2.5 $\mu g ml^{-1}$, final concentration) in glycerol containing antifade 10 $\mu g ml^{-1}$ p-phenylenediamine. Stained nuclei were visualized under UV light using a Zeiss Jena photomicroscope (Williamson and Fennel, 1975).

2.4.6. Complementation test of temperature sensitive mutants.

Single colonies of the original *nal(ts)* mutants were mated to an opposite mating type tester strain, in YPD. 10 µl of this mixture was plated on YPD and incubated at 26°C overnight. The diploids were then transferred to minimal media plates using replica velvets and incubated at 26°C and 37°C until colonies appeared.

2.4.7. Viability test.

A single colony of *nal(ts)* mutant was grown in YPD overnight. The culture was diluted to about 1x 10^6 cells ml^{-1} and incubated at 26°C in a water shaker for about 4 hours. 10 µl of the log phase culture was then taken out every hour and diluted to 1 ml. After four hours the culture was shifted from 26°C to 37°C. Again, 10 µl of samples were removed every hour and diluted to 1 ml. 100 µl of each diluted culture were subsequently plated on YPD plates. The colonies grown were counted after 2-3 days incubation at 26°C.

2.4.8. Assays for macromolecular synthesis.

A single colony of NA528 *nal(ts)* mutant was grown at 26°C overnight in 15 ml minimal medium containing 3.7 Mbq [^{3}H]adenine, 300 µg adenine and the required amino acids. The culture was diluted into the same medium so that the number of cells was about 2x10^6 cells ml^{-1}. The culture was then divided into two portions and incubated at 26°C. When the cell number reached approximately 2x10^7 cells ml^{-1}, one culture was shifted to 37°C. Samples were taken at different times during growth.

The procedure for determining the incorporation of [^{3}H]adenine into RNA was as follows: 20 µl samples of the mixture were applied on Whatman paper filters (Φ 2.5 cm). An equal volume of ice-cold 20% TCA was added to the filters. The filters were washed 4 times, each time in 200 ml of ice cold 10% TCA. All the filters were then washed in 100% ethanol, left on an ice-cold glass plate to dry and subsequently baked at 80°C for 30 minutes. Each dried filter was placed into a scintillation vial, containing 5 ml of scintillation liquid

(0.5 % PPO and 0.03 % POPOP in toluene). Radioactivity was counted in a Packard tri-carb liquid scintillation spectrometer.

The following procedure was used to determine the incorporation of [^{3}H] adenine into DNA: individual 400 µl samples of culture were mixed with 400 µl methanol in Eppendorf tubes. The samples were centrifuged using an Eppendorf centrifuge and the supernatant discarded. 50 µl of a stop mix solution (1M NaOH, 4 % N-laurylsarcosine, herring sperm DNA (Sigma, type III) 100 µg ml^{-1}, BSA 25 µgml^{-1}) was added to the pellet and the tubes were left at room temperature for about 18 hours. The mixture was then incubated at 80°C for 15 minutes, cooled and applied to Whatman GF/C filters. An equal volume of 20% ice cold TCA was added and the filters were further washed and counted as described in the RNA procedure above.

2.4.9. Spot test.

Mutant *mds1* and *nal ts* strains were grown in YPD overnight. The cultures were then subcultured so that the number of cells was about 1x10^8 cells ml^{-1}. 10 ml of the cultures was spun down and resuspended in 200 µl phosphate buffer. Approximately 10 ml of 0.5% low melting point agarose was added to the cells and spread on top of 3 YPD plates (3mls each). When the top agar layer was set, 1-5 µl of concentrated drug solutions was placed on the plates. The plates were further incubated at 30°C overnight and the inhibition zones measured.

2.4.10. Chromosome mapping by the recombination method (Falco and Botstein, 1983).

A construct, consisting of a cloned gene and an integration vector carrying the yeast *ura3* marker, was transformed into a *nal(ts) ura3*- mutant by electroporation. The transformants were checked by Southern blot to determine whether the integration was successful. The integrated transformants were mated with tester strains carrying assigned markers from chromosome I through to chromosome XVII. The diploids were purified on minimal media containing the amino acids required by diploids. Single colonies from different crossings were transferred to YPD broth and incubated at 30°C. The cell cycle was allowed to proceed for 25-50 generations. Small amounts of those cultures were taken out and streaked on YPD plates and incubated at 30°C for 1-2 days. 48 colonies from each different crossing were picked up and transferred to microtiter plates, containing approximately 200 µl of YPD broth, and incubated at 30°C overnight. The excess YPD broth was carefully discarded by aspiration and replaced with 100 µl sterile distilled water. All the colonies were replica-plated on minimal media containing the amino acids required. The chromosome loss was identified by the appearance of the auxothrophic marker corresponding to that chromosome.

2.4.11. Yeast chromosome separation using pulse field electrophoresis.

Wild type yeast cells were grown at 30°C to stationary phase in YPD media. The cells were then centrifuged at 3000 rpm in Omnispin

centrifuge for 20 minutes at 4°C. The pellet was resuspended in 0.05M EDTA, pH 8.0, at a ratio of 6 volumes EDTA solution to 4 volumes cells; 100 µl of 2mgml^{-1} Zymolyase 60,000 was added and incubated at 37°C for 20 minutes.

A 1.0% low melting temperature agarose solution (FMC) in 0.125 M EDTA, pH7.5, was prepared by melting agarose in a microwave oven and allowing the agarose solution to cool to 50°C. 0.9 ml melted agarose was mixed with 0.3 ml 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, 0.01M Tris pH7.5 and 7.5 % β-mercaptoethanol) and incubated at 37°C overnight.

The LET buffer was removed, replaced with NDS buffer (0.01M Tris, pH7.5, 0.5M EDTA, pH8.0, 1% N-laurylsarcosine and 1mgml^{-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 0.05M 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 sample wells at 1% base gel. Those agarose samples were then placed into sample wells; 0.5% 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 CHEF-DR^{TM}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 gel was 1% agarose in 0.5 x TBE.

When electrophoresis was completed, the gel was stained with 0.5 $\mu g ml^{-1}$ ethidium bromide for 15 minutes and destained twice with distilled water for a further 15 minutes each time. DNA fragments were visualized using an UV transilluminator. Finally, the DNA fragments were blotted on to Hybond-N filter and processed for Southern blot.

2.5. DNA preparation

2.5.1. 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 Sorval RC5B Refrigerated Superspeed, or an Omnispin Sorvall centrifuge. Ultracentrifugations were carried out in either a Beckman L5-65B or a Sorvall OTD65B ultracentrifuge.

2.5.2. Restriction endonuclease digestion of DNA.

a. Plasmid digests.

Plasmid or DNA molecules were digested by restriction endonuclease enzyme(s) using the appropriate buffer. The digestion was usually carried out at a volume of 10 µl and incubated at 37°C for 1 hour.

b. Partial digests.

Partial digest procedure was essentially as described by Maniatis *et al.*, (1982)

2.5.3. Phenol-chloroform extraction.

Aqueous solutions of DNA were extracted with equal volumes of phenol-chloroform consisting of phenol, chloroform and isoamylalcohol at a ratio of 25:24:1. This step was followed by an

extraction with an equal volume of chloroform to remove phenol traces.

2.5.4. Ethanol precipitation of DNA.

DNA was precipitated with 2 volumes of 100% ethanol in the presence of 3M Na acetate, pH6.0. The mixture was left on ice for 15 - 20 minutes. After centrifugation, the pellet was washed with 80% ethanol, centrifuged again, and the supernatant discarded. The pellet was dried in a vacuum chamber and resuspended in TE (10 mM Tris-HCl, pH8.0 and 1 mM EDTA) or sterile distilled water. The precipitation of small molecules of DNA could be accelerated by leaving the tubes in dry ice/IMS instead of ice.

2.5.5. Agarose gel electrophoresis

The percentage of agarose (w/v) in the gel was chosen to separate molecules of known or anticipated lengths efficiently. The agarose in 1 x TAE (40 mM Tris Acetate and 1 mM EDTA) or 1 x TBE (90 mM Tris, 90 mM Boric acid and 2.4 mM EDTA) was melted in a microwave oven, poured into a mould and polymerized at room temperature. DNA samples were loaded in 5 µl of sample buffer (TE pH7.5 containing 40% glycerol and 0.025 % bromophenol blue). Electrophoresis was performed in TAE or TBE buffers at 60-80V for 2-2.5 hours. When performing an experiment for Southern blot, electrophoresis was carried out at 20-25V overnight. DNA was visualized by staining with 0.25 $\mu g ml^{-1}$ ethidium bromide and destaining with several changes of distilled water.

2.5.6. Dephosphorylation of plasmid DNA.

Approximately 2 µg of plasmid DNA linearized by endonuclease was dissolved in a minimal volume of 10 mM Tris-HCl, pH8.0. The 50 µl reaction mixture contained 5 µl of 10 X CIP buffer (0.5 M Tris-HCl, pH9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$ and 10 mM spermidine), 1 unit of CIP and 48 µl sterile distilled water. The mixture was incubated at 37°C for 30 minutes, the same volume of CIP (1 unit) was added and incubation was continued for a further 30 minutes. Forty µl of sterile distilled water, 10 µl 10 X STE (10 mM Tris-HCl, pH8.0, 100 mM NaCl, 1 mM EDTA pH8.0) and 5 µl 1% SDS were added and the mixture was incubated at 65°C for 15 minutes. DNA was extracted twice with phenol-chloroform, followed by chloroform extraction and ethanol precipitation. The pellet was subsequently dissolved in sterile distilled water.

2.5.7. Ligation reaction.

Vector DNA was cut with the appropriate restriction enzyme(s) and, after incubation, the reaction was stopped by incubation at 65°C. The DNA was extracted with phenol/chloroform followed by chloroform extraction, and if necessary, dephosphorylated as described above.

Ligation reactions were set up with vector DNA and insert fragment at ratios of 1:1, 1:2, 2:1, 1:3 in reactions containing 1 X ligation buffer (20 mM Tris, pH7.5, 10 mM MgCl2, 10 mM DTT), 0.5 mM ATP and 0.1 U T4 ligase. Ligation reactions of "sticky-ended" fragments were incubated at 15°C overnight, whereas reactions of

"blunt-ended" fragments were incubated at room temperature for at least 2 hours.

2.5.8. Large scale plasmid preparation from *Escherichia coli.*

Plasmid containing *Escherichia coli* was inoculated in 1 liter of nutrient broth containing 0.2% glucose, 50 ml of minimal salt solution (50 mM NaCl, 10 mM Tris-HCl, pH7.5, 10 mM $MgCl_2$, and 1 mM Dithiothreitol) and 100 $\mu g\ ml^{-1}$ ampicillin, at 37°C overnight. The cells were harvested by centrifugation at 10,000 rpm for 20 minutes at 4°C. The supernatant was discarded leaving the pellet as dry as possible.

The pellet was resuspended in 10 ml ice cold lysis buffer (50 mM glucose, 25 mM Tris-HCl, pH8.0, and 10 mM EDTA), the total volume then being transferred to Corex SS34 tubes. After 10 minutes on ice, a mixture of 20 ml of freshly prepared solution of 0.2 N NaOH and 1% SDS was added and the tubes were left on ice for a further 10 minutes. Fifteen ml of ice-cold potassium acetate pH 4.8 were added to each tube and the tubes were left for 10 minutes on ice. They were centrifuged at 10,000 rpm in an SS34 rotor, using a Sorvall RC5B centrifuge at 4°C for 20 minutes. The supernatant was transferred to a fresh tube and mixed with 0.6 volume of isopropanol and left for 30 minutes on ice. Nucleic acid were pelleted at 15,000 rpm in an SS34 rotor for 30 minutes. The pellet was washed with 80% ethanol, spun down for 5 minutes, dried and dissolved in 8 ml TE (10mM Tris-HCl, pH8.0, 1 mM EDTA) at 65°C.

The plasmid was purified by equilibrium centrifugation in caesium chloride-ethidium bromide gradients (final density, n=

1,3860) in a 65VTi rotor using a Sorvall Ultracentrifuge OTD65B at 50,000 rpm. Ethidium bromide was removed by water-saturated butan-2-ol and the plasmid was ethanol-precipitated followed by overnight dialysis against TE buffer. The plasmid was again ethanol-precipitated and the pellet was resuspended in distilled water.

2.5.9. Mini-preparation of plasmid from *Escherichia coli.*

1.5 ml of an overnight culture was spun down in Eppendorf tubes. The supernatant was discarded and the pellet was resuspended in 100 µl lysis buffer as defined in the large-scale preparation above. The tube was left on ice for 5 minutes and 200 µl of lysis solution (0.2 N NaOH and 1% SDS) was added. The mixture was left on ice for a further 5 minutes. 150 µl of ice cold potassium acetate (pH4.8) solution, was added and the mixture was left on ice for 5 minutes. It was then spun down, the supernatant being transferred to a new Eppendorf tube. Proteins were removed by phenol-chloroform extraction and the nucleic acids were ethanol precipitated.

2.5.10. Preparation of high quality plasmid DNA by lithium chloride.

The following method was developed by Dr. Ian Eperon and colleagues at the University of Leicester, Department of Biochemistry.

Transformed bacteria were grown in 100 ml of NB containing 0.2% glucose, 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 100 µg ml^{-1} ampicillin at 37°C for 20 hours. The cells were harvested by centrifugation at 10,000 rpm using a Sorvall RC5B centrifuge at 15°C for 15 minutes.

Cells were lysed in alkali solution as described above in the large-scale plasmid preparation method. Plasmid DNA was precipitated in 0.6 volume of isopropanol and resuspended in 2 ml of TE' (10 mM Tris-HCl and 0.1 mM EDTA) buffer. Lithium chloride was then added to a final concentration of 2.4 M and the solution was incubated on ice for 60 minutes to precipitate high molecular weight DNA, RNA and proteins. The solution was centrifuged at 10,000 rpm for 15 minutes at 4°C. The DNA was ethanol-precipitated from the supernatant and resuspended in 400 µl of TE'. Pre-boiled RNaseA was added at 25 µg ml^{-1} and incubated at 65°C for 30 minutes. SDS and proteinase K, at final concentrations of 1 % and 50 µgml^{-1} respectively, were added to the solution and incubation was continued for a further 15 minutes.

The plasmid DNA was extracted twice with phenol-chloroform, once with chloroform, dialysed against TE buffer overnight, and then ethanol-precipitated. Finally, the plasmid DNA was resuspended in 50µl of distilled water.

2.5.11. Large scale yeast chromosomal DNA preparation.

Exponentially growing cells in YPD (1 liter) were pelleted and washed twice in buffer A (1.2 M sorbitol, 50 mM Tris-HCl, pH7.5, 20 mM EDTA and 1% β–mercaptoethanol). The pellet was resuspended in 2 ml of the same buffer containing 10 µgml^{-1} Zymolyase 60,000 and incubated at 37°C for 15 minutes to form spheroplasts. Spheroplasts were lysed in 10 ml of 100mM NaCl, 50 mM Tris-HCl, pH8.0, 20 mM EDTA, 1% SDS and 10 µgml^{-1} proteinase K. The mixture was incubated at 65°C for 90 minutes.

The lysates were extracted with phenol-chloroform and precipitated with 2 volumes of ethanol. The precipitate was pelleted at 6,000 rpm in an SS34 rotor using a Sorvall RC5B centrifuge for 15 minutes at 4°C. The pellet was then washed with 80% ethanol and dried in a vacuum chamber. The dried pellet was resuspended in 0.5 - 1 ml of TE buffer and incubated at 65°C with 10 $\mu g ml^{-1}$ RNaseA. Finally, 10 $\mu g ml^{-1}$ proteinase K and 1% 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 yeast DNA was ethanol precipitated in the presence of 0.2 M sodium acetate. The pellet was then resuspended in TE buffer.

2.5.12. Plasmid preparation from yeast.

Exponentially growing cells in 5 ml YPD were harvested by centrifugation. The pellet was washed with buffer A (as in 2.5.11). The cells were then resuspended in 0.5 ml of the same buffer containing 1 $\mu g ml^{-1}$ Zymolyase 60,000 and incubated at 37°C for 15 minutes. The spheroplasts were lysed in 200 µl of 0.5 N NaOH and 1% SDS and the mixture was left on ice for 10 minutes. 150 µl ice cold potassium acetate was added and the mixture was left on ice for a further 5 minutes.

The lysate was extracted with phenol-chloroform and the plasmid was ethanol precipitated and resuspended in TE buffer.

2.5.13. Preparation of *Escherichia coli* competent cells.

Exponentially growing cells in 50 ml of nutrient broth (OD_{600} ~ 0.3 -0.6) were spun down in sterile tubes, gently resuspended in 5 ml

of MR solution (10 mM MOPS, pH7.0 and 10 mM RbCl) and spun down immediately. The pellet was again resuspended in 2.5 ml of MRC (100mM MOPS, pH6.5, 10 mM RbCl and 50 mM $CaCl_2$) and left on ice for a minimum of 60 minutes. The competent cells are stored at -80°C.

2.5.14. Transformation in *Escherichia coli.*

150 µl-200 µl of competent cells were spun down in Eppendorf tubes. The pellets were resuspended in 150 µl of MRC. The appropriate DNA was added to each tube and left on ice for 60 minutes. Cells were heat-shocked by placing them at 55°C for exactly 35 seconds and then cooled on ice for 1 minute. 1 ml nutrient broth was added to each tube, which were incubated at 37°C without shaking for 60 minutes. The cultures were spun down and the pellets were gently resuspended in 200 µl of nutrient broth, plated on nutrient agar containing 100 $\mu g ml^{-1}$ ampicillin (final concentration). In case of blue-white selection, 25 $\mu g ml^{-1}$ X-gal and 25 $\mu g ml^{-1}$ IPTG were added to the plate. The cultures were incubated at 37°C overnight.

2.5.15. Transformation in yeast by chemical method.

Exponentially growing yeast cells in 40 ml of YPD were spun down at 3,500 rpm using an Omnispin centrifuge. Cells were washed twice with sterile distilled water. The pellet was resuspended in 40 ml of SED (1 M sorbitol, 25 mM EDTA, pH8.0, and 50 mM dithiothreitol), and incubated at 30°C for 10 minutes. The mixture was spun down and the pellet was washed once with 1 M sorbitol and resuspended in

10 ml of SCE (1M sorbitol, 0.1 M sodium citrate, pH5.8 and 0.01 M EDTA).

50-100 µl β-glucuronidase was added to the suspension of the cells, which were further incubated at 30°C with occasional gentle shaking. The formation of spheroplasts was determined by diluting 10 µl of cells in a drop of water on a microscope slide and looking for ghosts with a light microscope. The cells were spun down at 2,500 rpm and the pellet was very carefully washed twice in 10 ml 1M sorbitol and once in 10 ml of STC (1M sorbitol, 10 mM $CaCl_2$ and 10 mM Tris-Cl, pH7.5).

The pellet was resuspended in 1 ml of STC and divided into 100µl aliquots in 10 ml disposable tubes; 1-10 µl of DNA were added into each tube. The mixtures were left at room temperature for 10 minutes and 1 ml PEG 4000 (Fisons) was then added to each tube; they were then left for a further 10 minutes at room temperature. The cells were spun down at 2,500 rpm and carefully resuspended in 150 µl of SOS (10 ml 2M sorbitol, 6.7 ml YPD, 0.13 ml 1M $CaCl_2$, 27 µl 1% amino acid and 3.17 ml sterile distilled water). Cells were incubated at 30°C for 20 minutes.

3.5 ml of top agar which had been held at 45°C was added to each tube and immediately poured on minimal sorbitol agar plates. The plates were incubated at 30°C overnight before being transferred to 37°C and incubation was continued for 3-5 days, or until colonies appeared.

2.5.16. Transformation into yeast by electroporation.

100 ml culture of exponentially growing cells (OD_{600} ~ 0.5-0.6) was spun down at 3,000 rpm in an Omnispin centrifuge. The pellet was washed 5-6 times with sterile distilled water. The cells were resuspended in 1 ml sterile distilled water for electroporation.

DNA (1-10 µl) was added to 200 µl of the cell-suspension in an electroporation chamber. The instrument was set up at 5 $kVcm^{-1}$ and a capacitance of 25 mF. YPD (800 µl) was immediately added to the mixture and transferred to an Eppendorf tube. The cells were allowed to recover at 30°C for 30-60 minutes before being spun down. The pellets were resuspended in 100 µl of YPD, plated on selection media and incubated at 30°C until colonies appeared.

2.6. Double-stranded sequencing of plasmid DNA.

2.6.1. Creation of deletion clone.

The insert cloned DNA was cut with *Bam*HI and *Xba*I and inserted into *Xba*I-*Bam*HI sites of pUC18 and pUC19, thus creating two subclones in each of the two vectors. Sequential deletions were made using the Henikoff method (1984). Two enzymes, without recognition sites within the insert but which do cut the plasmid in the polylinker, were used to linearize the plasmid. A 5'- overhanging-end, susceptible to exonuclease III digestion was created with *Xba*I digestion. A 3'-overhang, resistant to exonuclease III, was produced by *Sph*I. The linear plasmid was then subjected to exonuclease III digestion,

keeping the enzyme concentration in molar excess, at 30°C without NaCl; aliquots were removed from the reaction at timed intervals.

Following the digestion of a single strand, the other strand was digested through incubation with S1 nuclease at room temperature for 30 minutes. The reaction was stopped by adding a stop buffer, and then incubated at 65°C for 15 minutes. Each timepoint aliquot was divided in two. Half of the reaction was analysed on an agarose gel to assess the course of deletion, the other half being recircularised in a blunt-ended ligation reaction. The circular DNA from each timepoint was transformed into *Escherichia coli* competent cells and transformants were selected on an ampicillin containing medium. Transformant colonies were analyzed using minipreparation of plasmid DNA. Subclones judged to overlap each other were amplified and their plasmid DNA were prepared by the lithium chloride procedure described above.

2.6.2. Sequencing of plasmid DNA.

The concentration of template DNA was adjusted so that 8 µl of the solution contained 1.5-2 µg of double-stranded plasmid DNA. The 8 µl solution of double-stranded plasmid DNA was then denatured by adding 2 µl of 2M NaOH and incubated at room temperature for 10 minutes. The mixture was neutralized with 3 µl 3M Na acetate pH4.8 and 7 µl distilled water. The single-stranded plasmid DNA was ethanol-precipitated with 60 µl of 100% ethanol and left on dry ice/methanol for 15 minutes. The pellet was collected by centrifuging for 10 minutes, washed with ice-cold 80% ethanol, recentrifuged for

10 minutes, dried briefly in a vacuum and resuspended in 10 µl of distilled water.

Primers used for sequencing reactions were the universal M13 "forward" and "reverse"primers, 5'-GTAAAACGACGGCCAT-3' and 5'-AACAGCTATGACCATG-3', respectively. The primers were adjusted to concentrations of 0.80 mM. The annealing reaction was set up as follows: in an Eppendorf tube, 10 µl of denatured template DNA from above was mixed with 2 µl of annealing buffer (280 mM Tris-HCl, pH7.5, 100 mM $MgCl_2$ and 350 mM NaCl) and 2 µl of primer solution. The mixture was incubated at 37°C for 20 minutes and then left at room temperature for at least 10 minutes before being centrifuged briefly.

Four Eppendorf tubes were labelled **'G', 'A', 'T', 'C'** respectively. 2.5 µl of sequencing mixture, **'G'** mix {N solution [150 mM (dATP, dCTP, dTTP and dGTP), 10mM MgCl2, 40 mM Tris-HCl/pH 7.5 and 50 mM NaCl] and 15 mM ddGTP}, **'A'** mix (N solution and 15 mM ddATP), **'T'** mix (N solution and 15 mM ddTTP), and **'C'** mix (N solution and 15 mM ddCTP) were pipetted respectively into the corresponding tubes.

Using ice-cold enzyme dilution buffer (10 mM Tris-HCl, pH8.0 and 1mM EDTA), the stock of T7 DNA polymerase was diluted to a concentration of 1.5 units ml^{-1}. The labelling reaction was performed as follows: in an Eppendorf tube, the annealed template and primer from the above reaction were mixed with 3 µl of labelling mixture (2 mM dGTP, 2 mM dCTP and 2 mM dTTP), 3.7 MBq [α-^{35}S]dATPαS, and 2 µl of 1.5 units μl^{-1} T7 DNA polymerase. The mixture was then incubated at room temperature for 5 minutes. 4.5 µl of this reaction mixture was transferred into each sequencing mixture, which had

been held for at least 1 minute at 37°C. The reaction was continued for a further 5 minutes and stopped by adding 5 µl of stop solution (10 mM EDTA, pH 8.0, 0.025% xylene cyanol and 0.025% bromophenol blue in deionised formamide). The mixtures were incubated immediately at 80°C for 2 minutes; if the sequencing is not performed in the same day then they may otherwise be stored at -20°C for up to one week.

2.6.3. Electrophoresis of DNA fragment from sequencing reaction

Sequencing gels were cast in a 20X50 cm mould comprising two thoroughly cleaned glass plates. The smaller plate was siliconised using 3ml of 2% dimethyldichlorosilane dissolved in 1,1,1,-trichloroethane. The 6 % gradient gel was prepared from the following stock solutions:

- 40% acrylamide stock (360 g acrylamide and 20 g bisacrylamide per liter double-distilled water) was filtered through Whatman no.1 paper and stored at 4°C in the dark;
- 10X TBE pH8.3 (109 g Tris, 55 g boric acid and 9.3 g EDTA) was filtered as above and stored at room temperature.

Working solutions were as follows:

- 0.5 x stock solution (6% acrylamide, 0.5 X TBE, and 50% urea) was filtered and stored at 4°C in the dark.
- 2.5 X stock solution (6% acrylamide, 2.5 X TBE, 50% urea, 5% sucrose and 0.025% bromophenol blue) was also filtered and stored at 4°C in the dark,

The gel was cast following the addition of 0.03% APS and 0.03% TEMED to 12.5 ml and 75 ml of the 2.5 X and 0.5 X stock solutions, respectively. 8 ml of the 0.5 X stock was mixed in a 25 ml pipette with 12ml of 2.5 X stock by introducing an air bubble. The gradient was poured down one edge of the mould and levelled before the rest of the 0.5 X stock solution was added, avoiding the formation of any air bubbles in the gel. Inverted sharktooth combs were placed at the top of the gel to ensure a level air-gel interphase and were removed following polymerisation and inverted in such a way that they barely deformed the surface of the gel. The top reservoir of a BRL (model S2) tank was filled with 0.5 X TBE, whereas the bottom was filled with 2.5X TBE. The samples were loaded following 2 minute incubation at 75-80°C. Electrophoresis was performed at 52 W for 3-4 hours. The gel, supported on a larger plate, was then fixed with 10% glacial acetic acid and 10% methanol for 15 minutes at room temperature, dried under vacuum at 80°C for 2 hours and exposed to Fuji-XR X-ray film overnight at room temperature before being developed.

2.6.4. Analysis of sequencing data.

All DNA sequences and peptide manipulation data were carried out using the suite of computer programmes written by the University of Wisconsin Genetics Computer Group run on the Vaxcluster computer system.

2.7. Southern blotting.

Southern blotting was essentially carried out as described by Perbal *et al.*(1988).

2.7.1. Radiolabelling probe DNA.

Feinberg and Vogelstein (1983)

Fragments of DNA for used as a probe were recovered from an agarose gel electrophoresis by electroelution onto dialysis membrane (the "death wish" method). Approximately 20 ng of the probe DNA was denatured by boiling at 100°C for 3 minutes and was immediately added to the labelling reaction mixture containing 30% OLB (100 mM ATP, 100 mM TTP, 100 mM dGTP, 250 mM Tris, pH8.0, 25 mM MgCl2, 52 mM β-mercaptoethanol, 1M HEPES, pH6.6, and 540 $mgml^{-1}$ hexadeoxyribonuleiotide), 0.5 $mgml^{-1}$ DNAase-free BSA, 3.7 MBq [α-^{32}P]dCTP and 0.5 unit Klenow fragment of DNA polymerase I. The reaction was incubated at room temperature for about 5 hours; the labelled DNA was denatured by boiling prior to the hybridisation reaction.

2.7.2 Prehybridisation and hybridisation of Hybond-N filter.

This method was described by Church and Gilbert (1984)

The filter carrying fixed DNA was placed in a 200 ml hybridisation bottle containing 7 ml hybridisation buffer (0.5 M Na_2HPO_4, pH7.5, 1 mM EDTA and 7% SDS) and prehybridised at 65°C in a Banchofer Rotisserie oven for at least 60 minutes. The solution was then replaced with 7 ml fresh hybridisation buffer, which had been held at 65°C, and 10-50 ng of single-stranded probe DNA. Incubation was continued at 65°C overnight.

2.7.3. High stringency washing of the filter.

The filter was washed twice for 30 minutes, each time in 200 ml of solution I (100 mM Na_2HPO_4 and 1% SDS) and twice for 30 minutes, each time in 200 ml of solution II (40 mM Na_2HPO_4 and 1% SDS) at 65°C. The filter was dried and wrapped in Saran film before being exposed to Fuji-XR X-ray film at room temperature.

2.8. Isolation of the wild-type *NAL* gene.

Approximately 20 µg of YEp13 vector was digested with *Bam*HI and dephosphorylated as described above. The pellet was resuspended in 10 µl distilled water.

100 µg of wild-type yeast genomic DNA was partially digested with *Sau*3A enzyme as described in Maniatis *et al.* (1982), and separated using an electrophoresis gel. Fragments, 2 to 10 kb, were selected, and isolated by the "death wish" method. These were then ethanol-precipitated and the pellet was resuspended in distilled water.

Ligation reactions, consisting of linear YEp13 vector, wild-type yeast genomic DNA fragments, ligation buffer, ATP and T4 DNA ligase, were set up as described in the ligation reaction method above.

The ligation mixtures were transformed into competent cells of *E.coli* 5K and incubated at 37°C overnight. Twelve transformants were picked up at random and their DNA isolated using the miniprep method so as to determine their insert sizes. All of the transformants were eventually pooled and transferred into 2 liter NB containing

0.2% glucose, 100 ml of M9 and 100 $\mu g ml^{-1}$ ampicillin. The plasmid library was isolated as described in large scale plasmid preparation.

Approximately 100 µg of the plasmid library was transformed into *nal(ts)* mutant of *S.cerevisiae;* transformants were selected in minimal medium plates without leucine at 30°C. The following day, some plates were transferred to 37°C, whereas others were left at 30°C as a control. Incubation was further continued until colonies appeared.

Colonies that grew at 37°C were picked up, all their markers checked and the plasmids isolated, as described in the plasmid preparation of yeast. The plasmids were re-transformed into *E.coli* competent cells and subsequently re-isolated from the transformants, after which the insert sizes were determined. The purified plasmids were then retransformed into the *nal(ts)* mutant. Those plasmids which still restored the temperature-sensitive phenotype were further analysed.

2.9. Preparing Nal protein

2.9.1. Preparing a construct for overproducing the Nal protein

The PET3a1 vector for overproducing protein was used. This vector was digested with an endonuclease enzyme *Bam*HI at the cloning site. The linear vector was then dephosphorylated. The cloned gene was digested with endonuclease enzyme *Bam*HI within the coding region. The digestion mixture was separated on an agarose gel and the fragments isolated using the "death wish" method. The

fragments of the cloned gene were ligated to the linear PET3a1 vector. The ligation mixture was transformed into *E.coli* BL21 strain (Table 2.2) and the cells plated on ampicillin-containing nutrient agar and incubated at 37°C overnight. A dozen transformants were randomly picked up so as to determine their insert sizes and orientations. The proper construct was then amplified and purified.

2.9.2. Overproducing the Nal protein

The transformant containing the above-mentioned proper construct was grown in ampicillin-containing nutrient broth at 37°C. The inducer IPTG was added at a final concentration of 25 $mgml^{-1}$ when the optical density of the culture was 0.1-0.15 at ~600. Incubation was continued for 3-4 hours. Inclusion bodies were observed using a light microscope.

2.9.3. Isolation of inclusion bodies

Ten ml of induced-cell culture (see above) was spun down and resuspended in 1ml 10 mM Tris-HCl, pH8.0. The suspension was spun down again, resuspended in 500 µl 10 mM Tris-HCl, pH8.0 and frozen in dry-ice/methanol for 10 minutes. The frozen cells were defrosted. Twenty µl of 15 $mgml^{-1}$ lysozyme in 50 mM EDTA pH8.0 was added and incubated for 30 minutes at room temperature. Another 10 µl of lysozyme solution was added and incubated for a further 15 minutes. The cell lysis was observed under microscope. Deoxycholate was added to a final concentration of 0.1% and the mixture was centrifuged in a microfuge for 10 minutes.

The pellet was washed twice with 0.1% deoxycholate, 3-5 times with 0.1% Triton and twice with distilled water. Finally, the pellet was resuspended in 50-100 µl of sample buffer (0.5M Tris-HCl, pH6.8, 25% glycerol, 6% SDS, 0.14M β-mercaptoethanol and 0.025% bromophenol blue). The sample was incubated at 90°C for 5 minutes prior to loading on polyacrylamide gels.

2.9.4. Mini Polyacrylamide Gel Electrophoresis

Two clean glass plates were used to make a cassette by aligning spacers on three sides of the plates. Bulldog clips were used to clamp the mould. The mould was then sealed by pouring molten 1% agarose down three sides of the cassette. The separating gel (10% acrylamide, 0.375M Tris-HCl, pH8.8, 0.1% SDS, 0.025% APS and 0.002% TEMED) was cast in the mould. Butan-2-ol was pipetted onto the surface of the gel before it polymerised to ensure a level interphase. The butan-2-ol was removed after the separating gel had polymerised. The stacking gel (5% acrylamide, 0.125M Tris-HCl, pH6.8, 0.1% SDS, 0.3% APS and 0.002% TEMED) was cast, the comb inserted and left for 15 minutes until polymerisation was complete.

The mould was placed in the electrophoresis tank containing running buffer (14.2g glycine and 3,03g Tris per liter at pH8.8) and 0.15% SDS. The comb was removed before the samples were loaded into the wells and electrophoresis was carried out at 275V for 30 minutes. The minigel was stained in 0.25% (w/v) Coomasie Blue R, 50% methanol and 10% glacial acetic acid for 10-15 minutes followed

by destaining using 10% glacial acetic acid and 20% methanol until the background staining was removed.

2.9.5. Preparing an antigen to raise polyclonal antibodies in chickens

The protein from the stained acrylamide (see above) was sliced using scalpel. Destaining was continued until the minigel was free from Coomasie Blue R. The excised gel, was washed with 30% ethanol overnight, followed by sterile TBS.

300 µl of sterile TBS were added to an Eppendorf tube containing the excised gel which was sonicated until no acrylamide particles were visible. The antigen was diluted in sterile TBS to a final concentration of 500 µl and emulsified with an equal volume of complete Freund's adjuvant. The emulsion was then injected at two sites into the chicken pectoral muscle. Further injections of the protein were given to the hen 12 and 20 days later. The eggs were collected daily, marked and stored at 4°C until needed.

2.9.6. Preparing an antigen for raising polyclonal antibodies in rabbits

Protein samples were run on a 15% acrylamide gel for 1 hour. The gel was electroblotted onto a nitrocellulose filter at 300mA for 30 minutes, the filter stained with Ponceau S for 1-2 minutes and washed 3-4 times with distilled water. The desired protein was cut and washed in sterile TBS to remove the remaining Ponceau S. The strip filter was incubated in sterile TBS at 4°C overnight.

So as to prepare the protein-containing filter for injection, the filter was immersed in liquid nitrogen and grounded in a mortar. Finally, 500µl of sterile TBS were added to the nitrocellulose powder to make the protein suspension ready for injection. Primary immunisation was followed by a secondary one after 4 weeks. Subsequent injections were carried out at fortnightly intervals for six weeks. Test bleeding was taken from the ear (1 ml serum). Injections were carried out until the titre peaked, at which point serum was collected.

2.9.7. Extraction of antibodies from eggs

The yolk was carefully separated from the egg white, washed briefly with deionised water and its yolk skin removed. The egg yolk suspension was made up to 30 ml with buffer A (10 mM potassium phosphate pH7.2 and 100 mM NaCl) and mixed with 30 ml of a 7 % (w/v) PEG solution dissolved in buffer A. The suspension was centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatant was filtered through 2 milk filters and solid PEG was added to a final concentration of 12% (w/v). The mixture was stirred until all the PEG was dissolved.

The solution was centrifuged at 14,000 x g for 10 minutes at 4°C, the pellet containing IgY was resuspended in 20 ml of buffer A and mixed with an equal volume of a 24% (w/v) PEG solution in buffer A. The suspension was centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatant was carefully removed and the pellet was resuspended in 10 ml of buffer A and dialyzed against the same

buffer overnight. The solution was once again centrifuged at 14,000 x g for 10 minutes at 4°C to remove floating precipitation.

2.9.8. Affinity purification of antibodies using Western blot

Approximately 4 µg of antigen protein per lane was run on a 10% polyacrylamide minigel. The proteins were then blotted onto nitrocellulose filter and stained with 0.5 % Ponceau S. The protein-containing band was cut and washed several times with TBS buffer.

The nitrocellulose strip was blocked in TBS buffer containing 1% Marvel and 3% BSA at 4°C overnight. The following day, the nitrocellulose strip was washed with TBS buffer three times, each time for 5 minutes. This protein-containing strip was incubated by gentle shaking in 0.5-1 ml antiserum containing 10 mM sodium azide at 4°C overnight.

The nitrocellulose strip was then washed three times with TBS buffer containing 0.05% tween20. The antibody was eluted off by shaking gently with 500 µl of 0.2M glycine-HCl, pH2.2 at 4°C for 5 minutes. The nitrocellulose was taken out and the solution was neutralised with a predetermined volume of 2M Tris buffer to pH8.0. Bovine serum albumin was added at a concentration of 1%. Finally the antibody solution was dialysed against TBS buffer overnight. Sodium azide at a final concentration of 10mM was added and the antibody solution stored at 4°C or -70°C.

2.9.9. Testing antibodies on Western blot

The protein on a polyacrylamide gel was blotted onto a nitrocellulose filter and stained with Ponceau S as described above.

The filter was stripped along the lanes. The strips were blocked in blocking solution (TBS buffer, 0.05% tween 20, 3% BSA and 1% Casein) overnight. They were washed twice in TBS buffer containing 0.05% tween 20 and twice in TBS buffer. They were incubated with the chicken antibodies or rabbit antiserum appropriately diluted in TBS buffer containing 1% BSA for 2 hours at 37°C. The strips were washed four times in TBS buffer containing 0.05% tween 20 and incubated with goat anti-chicken or goat anti-rabbit antibodies (1:500) diluted in TBS containing 1% BSA for 1 hour at 37°C.

At the end of the incubation, the strips were washed twice in TBS containing 0.05% tween20, twice in TBS, and incubated in rabbit anti-goat peroxidase (1:1000) diluted in TBS for 1 hour in the dark at room temperature. The peroxidase conjugated antibodies were washed twice with TBS containing 0.05% tween20 as described above.

Finally, the strips were stained in developer solution (40 mg 4-chloronaphthol in 10 ml methanol, 30 ml TBS and 40 ml H_2O_2) until the purple colour appeared. When staining was sufficient, the reaction was stopped by adding deionised water.

2.9.10. Fixation and immunoflourescence of *Saccharomyces cerevisiae*

Fifty ml of an exponentially growing yeast culture was fixed by adding formaldehyde at a final concentration of 3%, and the cells were left for 2 hours at room temperature. These were spun down, washed in TBS and once again fixed with 3% formaldehyde for 1 hour.

The cells were washed 5 times in TBS buffer and resuspended in 0.5ml of buffer A (see 2.5.11) in an Eppendorf tube. Five µl β-

mercaptoethanol and 10 µl of 5mgml^{-1} Zymolyase 60,000 were then added to the cell suspension. The cells were incubated at 30°C for 10 to 30 minutes. The formation of spheroplasts was observed using a light microscrope. Incubation was stopped when 50% of the cells had become spheroplasts. The cells were then spun down in low speed centrifugation (2000 rpm in an Eppendorf microcentrifuge).

The cells were washed 4 times in TBS and resuspended in 0.5ml TBS and 50µl 38% formaldehyde. The cell suspension was incubated at room temperature for 30 minutes. The cells were then washed 3 times in TBS, resuspended in 0.5ml TBS containing 0.5% (final concentration) Triton X100 at room temperature for 15 minutes.

The cells were washed 3 times in 1.2M sorbitol and resuspended in 0.5ml sorbitol. In this state, the cells can be stored in the freezer for several weeks.

Fifty µl of the cell suspension was taken, washed twice in TBS buffer and resuspended in 50 µl TBS. The primary antibody with an appropriate dilution was added and incubated for 2 hours at 37°C. The suspension was washed 3 times in 200µl TBS and resuspended in 50µl. The secondary antibody, coupled to flourescence dye, with 1:50 dilution was added and incubated for 1 hour in the dark to prevent bleaching.

The cells were washed 3 times in TBS and resuspended in small amount of TBS. Three microliters of the cells were dropped on a microscope slide, spread out and allow to dry. A drop of antifade (100mg p-phenylenediamine in 10 ml TBS buffer and 9 ml glycerol) was added to the dry cells, covered with a coverslip and pressed gently together with a piece of tissue so as to absorb excess antifade.

The coverslip was then sealed with nail polish to prevent the cells from drying out. The cells were examined using a fluorescence microscope.

Chapter III : Isolation and characterization of *nal(ts)* mutants

3.1. Introduction.

Antibacterial agents, particularly antibiotics often have a highly selective action upon biochemical processes. However, the basis of selectivity can vary from one to another. Therefore any given antibiotics or chemical agents can affect both prokaryotic and eukaryotic cells. These drugs may interfere directly or indirectly with a single or multiple targets in the cell. The target of these drugs can be biosynthetic enzymes, ribosomal function or structural proteins (Gale, 1981).

In order to study the mode of action of an antimicrobial agent, we can start by isolating a mutant with resistance to the drug. Such resistant mutants are investigated and any changes to its morphology or impairement to its physiology noted. When the inhibited biochemical system has been identified in intact cells, further analysis can be carried out. This may involve the isolation of suspected targets such as genes, enzymes and other proteins (Franklin and Snow, 1989).

Another method to study the mode of action of antimicrobial agents is by a biochemical approach. This involves isolation of the target of the drug by exploiting its affinity to the drug. Isolation of suspected target can be achieved by running cell extract in an affinity column containing the drug. Proteins which are bound to the drug are then separated by eluting with different buffers, and the individual protein can be further analysed.

As has been mentioned in chapter I, quinolones inhibit the cell cycle of *E.coli* by interfering with DNA gyrase. The effect of nalidixic

acid on a wild-type strain of the yeast *Saccharomyces cerevisiae* is a transient G1 arrest (Singer and Johnston, 1979).

In the following experiments, the genetic approach was chosen as the starting point and was aimed at generating mutants that would be resistant to 4-quinolones. The first problem that had to be overcome, however, was the relatively high resistance of the wild type strain to most drugs including 4-quinolones. Consequently, we used mutant strains of *Saccharomyces cerevisiae* that are permeable to most drugs (Pocklington *et al.,* 1990), to isolate 4-quinolone resistant mutants.

In the following experiments, the *mds* strain (*mds* = multiple drug sensitive) (Table 2.1) of *Saccharomyces cerevisiae* was used rather than that of a wild-type strain.

In preliminary observation, it was established that *mds* strains were sensitive to nalidixic acid at much lower concentrations than the wild-type, although the inhibitory effect of other 4-quinolones on the former strain depended on very high concentrations. This was the main reason for choosing nalidixic acid as a representative of 4-quinolones.

3.2. Isolation of mutants.

Spontaneous nalidixic acid resistant mutants of *Saccharomyces cerevisiae* were obtained on YPD plates containing nalidixic acid at final concentrations varying from 200 $\mu g ml^{-1}$ to 1000 $\mu g ml^{-1}$. Colonies were picked up and screened for their ability to grow at 26°C and 37°C in YPD and minimal media containing the required amino acids. Fifteen independent *temperature sensitive (ts)* mutants were isolated. In YPD culture, the mutant cells tended to clump together and to grow very slowly, the doubling time being approximately 2.5 to 3 hours.

3.3. Tetrad analysis.

Tetrad analysis experiments were carried out for the purpose of:

a) providing a genetic explanation as to whether nalidixic acid resistance (Nal^r) and Ts phenotypes are the result of a single mutation;

b) determining whether the *nal(ts)* mutation was a reversion of the original *mds* mutation;

c) moving the *ts* mutation from its original background.

Ideally, the experiment should be carried out by mating *mds* strain and *mds(ts)* mutant to see if the nal^r and *ts* are separable. The result would show whether nal^r and *ts* are conferred by a single gene. Unfortunately, this experiment is unfeasible, due to the mds phenotype; mds diploids cannot be made, consequently the A37 *nal(ts)* (Table 2.1) strain was crossed with the wild-type (wt) strain DBY746 and sporulated. The result showed that the auxotrophic markers (*trp* and *tyr*) are determined by single genes as they segregate in a 2:2 mode (Table 3.3.1). The Ts phenotype also segregates in a 2:2 mode, proving this phenotype to be determined by a single nuclear gene. It is shown that some of the haploids are *mds* strain. This proves that the *nal(ts)* mutation is not a revertant of *mds* mutation, although it does not rule out the possibility that the mutant is a result of suppression. So as to determine whether the mutation is suppressor, second crossing between the *nal(ts)* mutants was carried out. Tetrad analysis shows that all haploids are temperature sensitive (Table 3.3.2) and the auxothropic markers also segregate in a 2:2 mode. This proves that the mutation is not a result of suppression.

The third experiment was to mate the *mds* strain with wt *nal(ts)* mutant (table 3.3.3). As well as showing that all the markers such as *ts*, *tyr* and the mating type, were represented by a single genes, this

clearly demonstrated that the *ts* and *nal*^{r} were inseparable, as all temperature-sensitive clones are nalidixic acid resistant. The *nal*^{r}, however, did not segregate in a 2:2 mode, as would be expected due to the presence of the wild-type MDS gene in one half of the progeny.

Tetrad	YPD 37°C	leu	his	ura	tyr	trp	*mat*	nal.acid 400µgml^{-1}
1 A	+	-	+	+	+	+	*a*	+
B	-	-	-	+	-	-	α	+
C	-	-	-	-	-	+	*a*	+
D	+	-	-	-	+	-	α	-
2 A	.-	-	-	-	-	-	α	+
B	-	-	-	+	+	+	α	+
C	+	-	-	-	-	-	*a*	+
D	+	-	+	+	+	+	*a*	+
3 A	-	-	+	+	+	+	*a*	+
B	-	-	-	-	-	-	α	+
C	+	-	-	-	+	+	*a*	-
D	+	-	-	+	-	-	α	+

Table 3.3.1: A selection of tetrads (3 of 80) dissected from the first cross between A37 *mds1, nal(ts)5* mutant and DBY746. (+) indicates growth; (-), a non-growth.
Number of tetrads:
Parental ditype (PD)= 22;
Non parental ditype (NPD)= 0
Tetratype (T)= 58

Tetrad		37°C	leu-	his-	mating type
1	A	-	+	-	α
	B	-	-	+	a
	C	-	+	-	a
	D	-	-	+	α
2	A	-	-	+	a
	B	-	+	-	a
	C	-	-	-	α
	D	-	+	+	α
3	A	-	+	-	a
	B	-	-	+	α
	C	-	+	-	a
	D	-	-	+	α
4	A	-	-	+	α
	B	-	-	+	a
	C	-	+	-	α
	D	-	+	-	a
5	A	-	+	-	α
	B	-	+	-	a
	C	-	-	+	a
	D	-	-	+	α
6	A	-	+	-	a
	B	-	-	+	α
	C	-	+	-	a
	D	-	-	+	α

Table 3.3.2: A selection of tetrads (6 of 24) from a cross between NI2D *MATa nal(ts)5* and NP5B *MATα, nal(ts)1*. All haploid produced are temperature sensitive and all other the markers segregate in a 2:2 mode. (+) indicates growth; (-), a non-growth.

Tetrad	YPD 37°C	tyr	*mat*	nal acid 400 (μgml^{-1})	nal acid 800 (μgml^{-1})
1 A	.-	+	α	+	+
B	+	-	α	+	+
C	.+	-	*a*	-	-
D	-	+	*a*	+	+
2 A	+	+	*a*	+	+
B	+	-	α	-	-
C	-	+	*a*	+	+
D	-	-	α	+	+
3 A	-	-	*a*	+	+
B	+	+	α	+	+
C	+	-	*a*	-	-
D	-	+	α	+	+

Table 3.3.3: A selection of tetrads (3 of 12) from a cross between A37 *MAT*α, *mds1* strain and NT18C *MATa*, *nal(ts)1* mutant. Ts phenotype, mating type and tyr- segregate in a 2:2 mode, whereas nalidixic acid resistant does not. All progeny were auxotrophic for the leucine, histidine and uracil requirements. (+) indicates growth; (-), a non-growth.

3.4. Complementation test.

Complementation tests were carried out in order to determine whether all *nal(ts)* mutants are allelic. Two *nal(ts)* mutants NI3D *MAT*α *nal(ts)5* and NI2D *MATa* *nal(ts)5* (Table 2.1), derived from A37 *nal(ts)5* were used as tester strains. All the original fifteen A37 *nal ts* strains were crossed with the tester strains. The resulting diploids grew at 26°C, but not at 37°C, strongly indicating that all *nal(ts)* mutants are allelic (Table 3.4.1). The *nal(ts)* strains were also crossed with a *type II topoisomerase (ts)* mutant and with A37 (the parental

Strains	26°C		37°C	
	*a*NI2D	*α*NI3D	*a*NI2D	*α*NI3D
a A37 *nal ts1*		+		-
a A37 *nal ts2*		+		-
a A37 *nal ts3*		+		-
a A37 *nal ts4*		+		-
a A37 *nal ts5*		+		-
a A37 *nal ts6*		+		-
a A37 *nal ts7*		+		-
a A37 *nal ts8*		+		-
a A37 *nal ts9*		+		-
a A37 *nal ts10*		+		-
a A37 *nal ts11*		+		-
a A37 *nal ts12*		+		-
a A37 *nal ts13*		+		-
a A37 *nal ts14*		+		-
a A37 *nal ts15*		+		-
α 10D *nov ts*	+		+	
a 10C *nov ts*		+		+
α SD1.1 *top2 ts*	+		+	
α A37 *mds*	+		+	

Table 3.4.1. : Complementation test. The diploids were grown on minimal medium. (+) indicates growth and (-) non-growth.

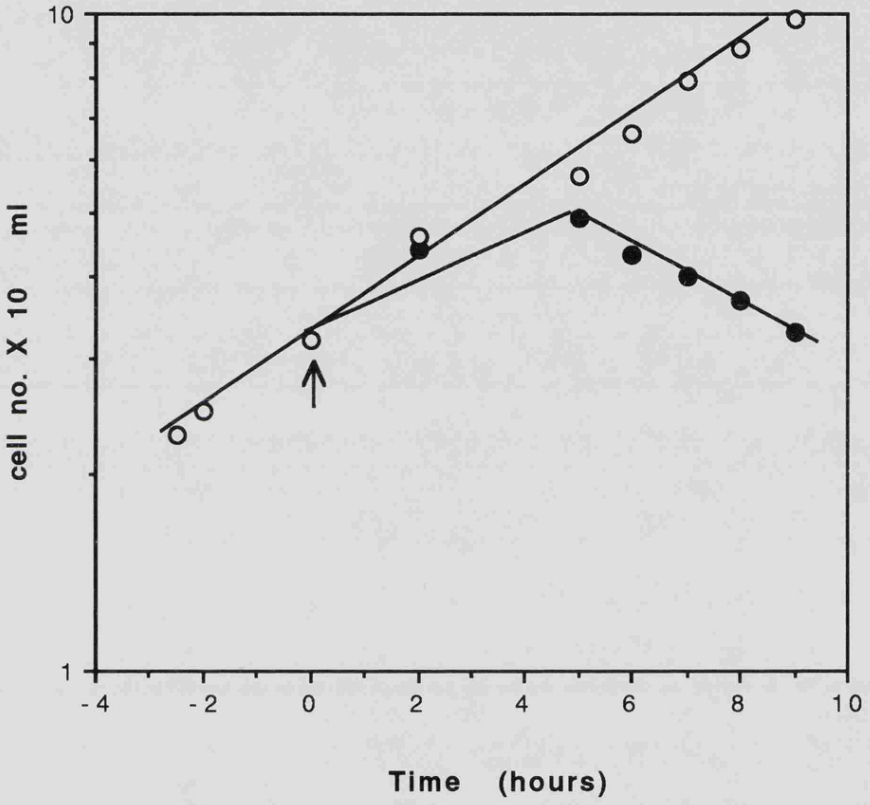


Figure 3.1: Lethality curve of the *nal(ts)5* mutant
Lethality curve of *nal(ts)5* following a temperature shift to 37°C during exponential growth in YPD medium. Aliquots of the culture were sonicated and diluted in fresh YPD medium, and plated onto YPD plates.
(o) *nal(ts)5* mutant at 26°C, (●) *nal(ts)5* mutant at 37°C.
The arrow indicates at the time the culture was shifted to 37°C.

strain), as well as with the *novobiocin temperature sensitive nov(ts)* mutants (Pocklington *et al.*, 1990). All the diploids grew at 26°C as well as at 37°C (Table 3.4.1) showing that the *nal(ts)* mutation does not identify these previously isolated genes.

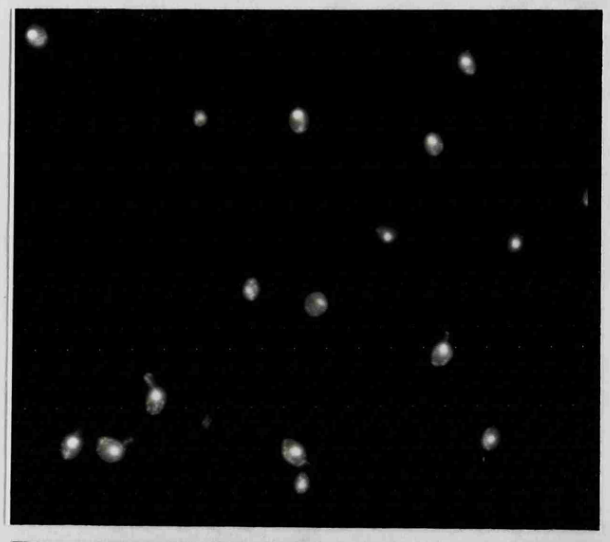
3.5. Viability test.

A viability test was carried out to determine how quickly the *nal(ts)* mutant dies at the non-permissive temperature. The NP5B *nal(ts)5* strain was used to study this phenotype, Cells were grown in YPD broth and incubated at 26°C for several hours before being shifted to 37°C. After a temperature shift from 26°C to 37°C, the cells continued growing with a slower rate for five hours. Further incubation at non-permissive temperature, however, caused a rapid decline in the number of viable cells. Very few cells survived after 24 hours of incubation at 37°C (figure 3.1).

3.6. Mutant morphology

Samples from growing wild type and mutant strain were taken 8 hours after temperature shift and after being kept overnight at the non-permissive temperature. These strains were then fixed with 3% (final concentration) formaldehyde, and the fixed cells were stained with DAPI. The morphology of the *nal(ts)5* mutants is similar to that of the wild type at 26°C (figure 3.2). The majority of the population consists of single cells, each with a single nucleus. The number of budded cells is roughly equal to the number of single cells. However, at 37°C the majority of the cells (>80%) have small buds, and each has a single nucleus in the mother cell. Incubation at 37°C for periods longer than 8 hours did not change the appearance of the cells. This

A)



B)

Figure 3.2: Fluorescence microscopy of DAPI-stained cells of the *nal(ts)5* mutant at 26°C (A), and at 37°C (B). At the non-permissive temperature, the majority of the cells have a small bud. (1000 X)

result suggests that, at the non-permissive temperature, the cell cycle was arrested at or around the S phase.

So as to investigate the phenotype of the *mds* strain in the presence of nalidixic acid, a further experiment was carried out, in which nalidixic acid was directly applied to an A37 *mds1* strain culture. Nalidixic acid at a final concentration of 100 $\mu g ml^{-1}$ and 400 $\mu g ml^{-1}$ was added to the cells of A37 *mds1* strain growing at 30°C. The cell cycle was arrested 2 hours after the additon of the drug at G1 phase where the majority of the cell population were single cells without buds. The cell cycle failed to recover following further incubation. This result differs from previous work carried out by Singer and Johnston (1979) who reported the cell cycle to be arrested at the G1 phase transiently, then recovering within 1 hour.

The phenotypic differences between the *nal(ts)* mutant at the non-permissive temperature and A37 *mds1* in the presence of nalidixic acid, suggest that there could be several targets of nalidixic acid in the yeast *Saccharomyces cerevisiae.*

3.7. Macromolecular synthesis

The terminal phenotype of strains carrying the *nal(ts)* mutation at the non-permissive temperature suggests that an event related to DNA replication might be affected in these strains. For this reason, the synthesis of macromolecules (RNA and DNA) under various growth conditions was investigated. The results demonstrate that neither DNA nor RNA synthesis of an asynchronous culture was inhibited immediately after the shift to the restrictive temperature, but rather continued for approximately 5 hours. About 6 hours after the temperature shift both DNA and RNA synthesis stopped almost simultaneously (figure 3.3).

Figure 3.3: Relative incorporation of [^{3}H]adenine into RNA (A) and DNA (B) into *nal(ts)5* mutant before and after a shift from 26°C to 37°C. (o), DNA or RNA synthesis at 26°C; (•), DNA or RNA synthesis at 37°C. The arrow indicates at the point when the cultures were shifted.

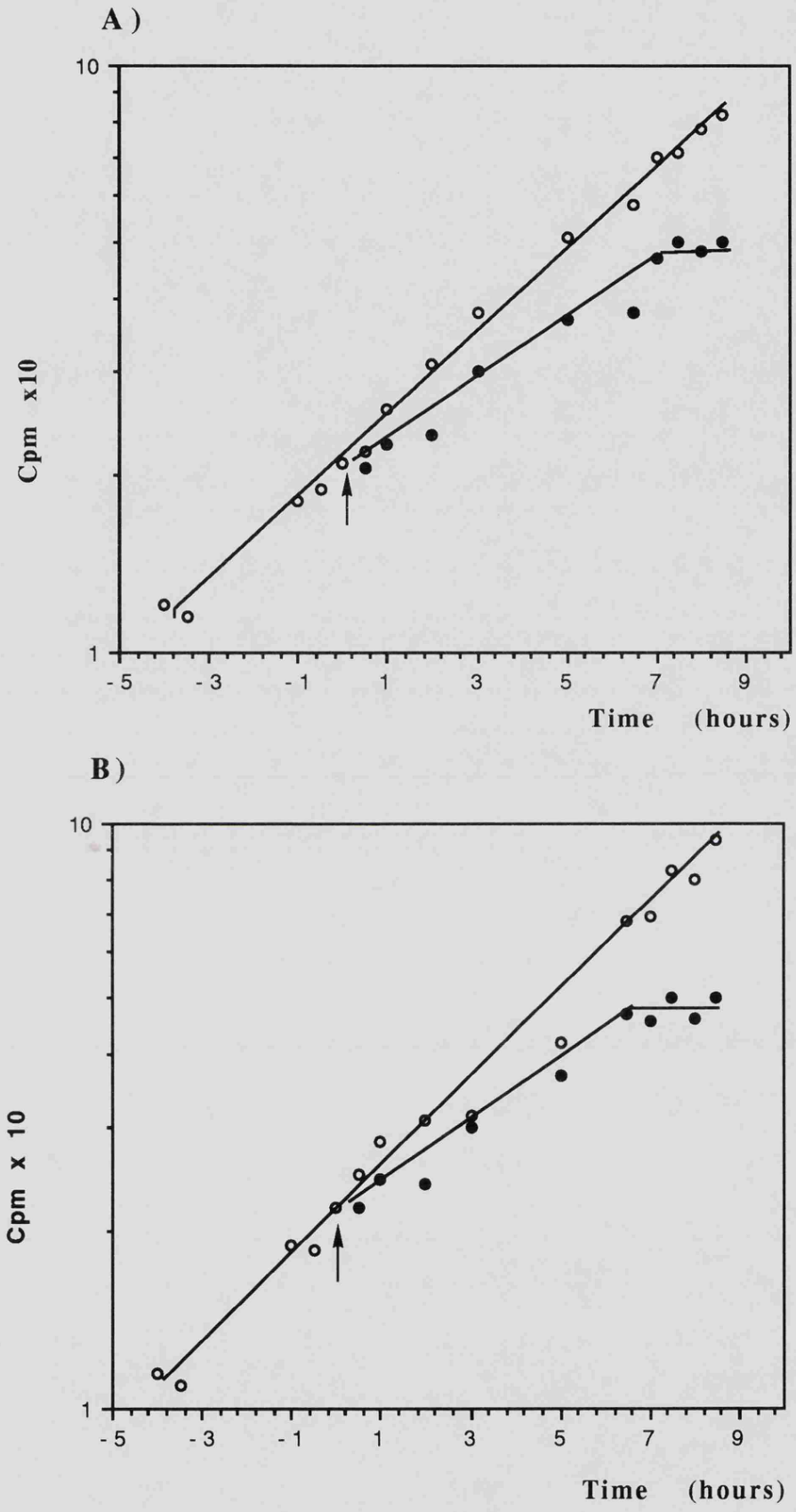

A)
10
1
Cpm x10
-5
-3
-1
1
3
5
7
9
Time (hours)
B)
10
1
Cpm x 10
-5
-3
-1
1
3
5
7
9
Time (hours)

Figure: 3.3

Figure 3.4: Relative incorporation of [^{3}H]adenine into RNA (A) and DNA (B) into *nal(ts)5* mutant before and after a shift from 26°C to 37°C. The cultures were synchronized using the α mating type pheromone at a concentration of 0.5 $\mu g ml^{-1}$ for approximately 2 hours. The pheromone was washed and the cultures were incubated in the fresh media at 26°C and 37°C. (o), DNA or RNA synthesis at 26°C; (•), DNA or RNA synthesis at 37°C. The arrow indicates at the point when the cultures were shifted.

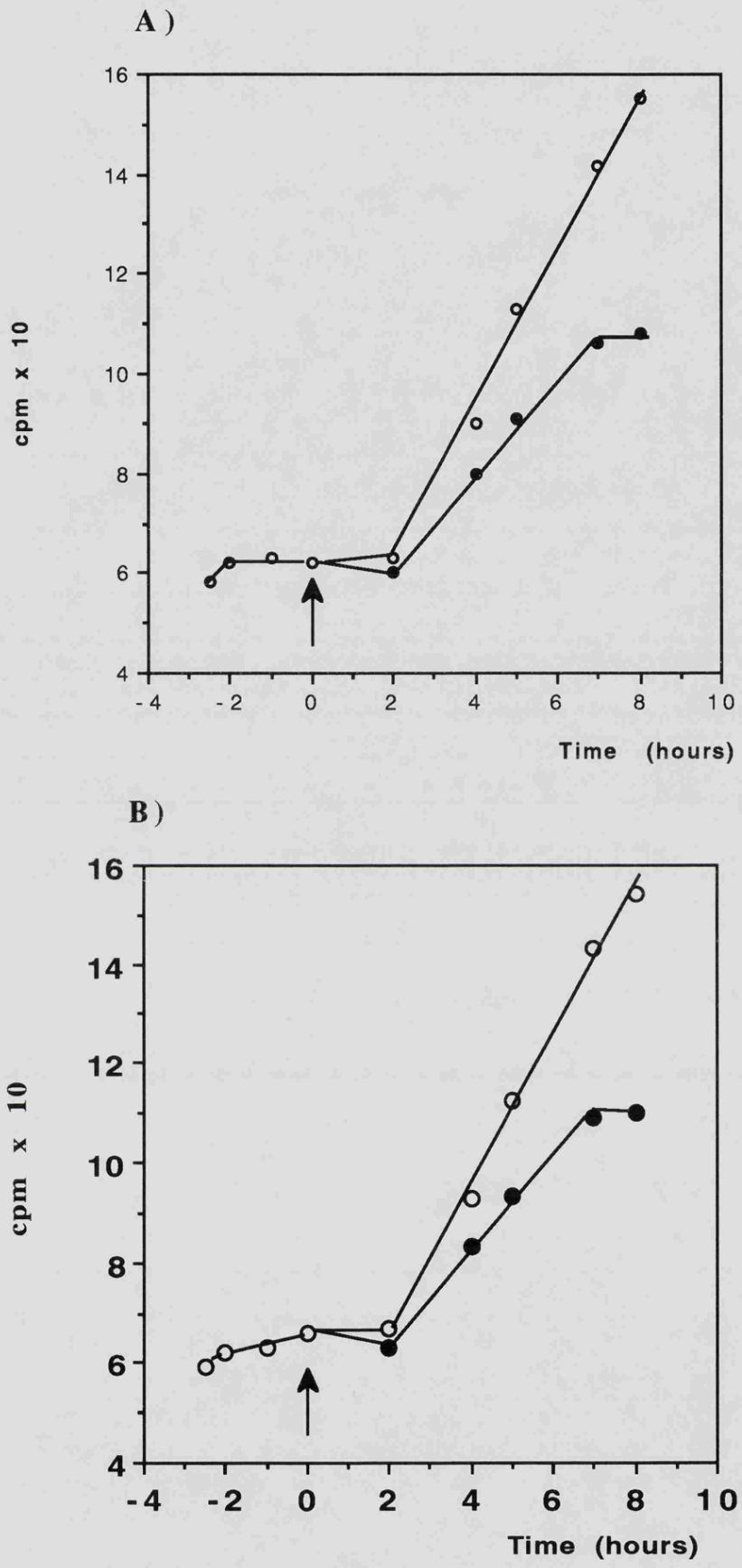

A)
16
14
12
10
8
6
4
cpm x 10
-4
-2
0
2
4
6
8
10
Time (hours)
B)
16
14
12
10
8
6
4
cpm x 10
-4
-2
0
2
4
6
8
10
Time (hours)

Figure: 3.4

DNA synthesis could stop at either the initiation of the process, the elongation step or at both. So as to obtain additional information that could clarify such possibilities, a similar experiment was carried out with synchronized cell cultures. For the purpose of synchronizing cell growth, α mating pheromone (factor) at a concentration of 0.5 $\mu g ml^{-1}$ was added to the cultures to stop the cell cycle at G1 phase. The cell cycle was arrested at the G1 phase, 2 hours after the addition of pheromone. The α factor was washed away, and the cells recovered their growth rate at the non-permissive temperature. RNA and DNA synthesis stopped after 5-6 hours at the non-permissive temperature, as did cell division, similar to the results observed for asynchronous cultures (figure 3.4).

It is evident that, in both experiments, the amounts of both DNA and RNA have doubled, suggesting that the arrest of the cell cycle at the restrictive temperature was not immediate.

3.8. Spot test experiments

It is broadly accepted that the cell membrane can act as a barrier toward harmful chemical subtances. Resistance of the *nal(ts)* mutants to nalidixic acid could thus result from changes in their cell envelope. A possible way to monitor such changes is by studying the sensitivity of these strains to various drugs and detergents (Table 3.7.1). A number of antibiotics and other drugs demonstrate that the *nal(ts)* mutants:

a) are more resistant to some agents;

b) like the A37 *mds1* strain are still sensitive to some agents

c) are hypersensitive to agents that affect the cytoplasmic membrane, i.e polyene antibiotics (nystatin and amphotericin

Table 3.7.1: Spot test experiment

10 ml of exponentially growing yeast cells were spun down and resuspended in 200 µl phosphate buffer. Approximately 10 ml of 0.5% low melting point agarose were added to the cells and spread on top of 3 YPD plates. When the top agar layer was set, 1-5 µl of concentrated drug solutions were placed on the plates. The plates were incubated at 30°C overnight and the inhibition zones were measured.

_ : indicating no inhibition.

(.): non-complete inhibition.

A37 - parental strain

NT5 - *nal(ts)5*mutant in the Mds background

NI2D - *nal(ts)5* mutant in the wild-type background

DBY746 - wild-type strain

TABLE 3.7.1 : Spot test

Agents used	Φ of inhibition zones (mm)			
	A37	NT5	NI2D	DBY746
Fusaric acid 60 $mgml^{-1}$	10	9	9	9
Hygromycin B 100 μgml^{-1}	19	28	9	10
Kanamycin 100 $mgml^{-1}$	11	8	-	-
Cycloheximide 30 μgml^{-1}	21	13	16	8
Nystatin 20 μgml^{-1}	19	26	27	13
Paromomycin 5 $mgml^{-1}$	6	5	-	-
Chlorpromazine 100 μgml^{-1}	21	32	21	16
Verapamil 100 μgml^{-1}	7	5	6	6
Novobiocin 100 μgml^{-1}	6	5	5	3
48/80 200 $mgml^{-1}$	16	19	10	8
Neomycin 50 $mgml^{-1}$	9	8	-	-
EtBr 10 μgml^{-1}	4 (16)	6	(15)	(16)
Daunomycin 100 $mgml^{-1}$	7	5	5	6
Vinblastine 100 $mgml^{-1}$	-	-	-	-
Actinomycin D 100 $mgml^{-1}$	16	8	-	-
Gramicidine 25 $mgml^{-1}$	-	-	-	-
Clotrimazol 30 $mgml^{-1}$	16	33	28	14
Saponin 8%	-	7	8	-
Nalidixic acid 100 mgml-1	7	2	-	-
SDS 10%	5	6	7	6

Table 3.7.2: Spot test experiment using drugs and agents affecting the plasma membrane.

10 ml of exponentially growing yeast cells were spun down and resuspended in 200 µl phosphate buffer. Approximately 10 ml of 0.5% low melting point agarose were added to the cells and spread on top of 3 YPD plates. When the top agar layer was set, 1-5 µl of concentrated drug solutions were placed on the plates. The plates were incubated at 30°C overnight and the inhibition zones were measured.

_ : indicating no inhibition.

A37 - parental strain

NT5 - *nal(ts)5* mutant in the Mds background

NP5B - *nal(ts)5* mutant in the wild-type background

Table 3.7.2: Spot test

Agents used	Φ of inhibition zones (mm)		
	A37 (mds)	NT5	NP5B
Nystatin 20 $mgml^{-1}$	12	20	15
Amphothericin B 30 $mgml^{-1}$	8	16	12
Clotrimazole 50 $mgml^{-1}$	16	33	24
Econazole 50 $mgml^{-1}$	26	35	31
SDS 5%	6	8	7
Triton X-100	-	9	10
Sarcosyl 8%	6	9	9
Saponin	-	6	8

B), azole (clotrimazole and econazole), and non-ionic detergents (Table 3.7.2);

d) are resistant to other 4-quinolones tested, such as pipemidic acid, cinoxacin and oxolinic acid.

Unlike *E.coli* which become hypersensitive to SDS after nalidixic acid treatment, the *nal(ts)* mutants of *Saccharomyces cerevisiae* are slightly sensitive to SDS. Sphingosine and gramicidin, antibiotics that are thought affect the cytoplasmic membrane, do not affect the *nal(ts)* mutants. These results support the idea that the *(ts)* mutation affects the cytoplasmic membrane specifically.

3.9. Conclusion

The fifteen *nal(ts)* mutants were isolated from different cultures with various concentrations of nalidixic acid. The results of the complementation test, however, show these mutations to be functionally allelic. As the probability of getting a single spontaneous mutation is very low (estimated around 10^{-9}), and obtaining an independent double mutant is even lower, it is most likely that the Nal^R and Ts phenotypes are the result of a mutation in a single gene.

The *nal(ts)* gene is different from the *nov(ts)* gene, and the *top2(ts)* gene, as it complements the other two mutants. It is therefore unlikely that the Nal protein is the yeast type II topoisomerase or acts similarly to the Nov protein.

The fact that the *nal(ts)* mutants had small buds after several hours of incubation at the non-permissive temperature suggests that *nal(ts)* mutations arrest cellular growth at the S phase of the cell cycle, perhaps interfering with DNA synthesis. The increase in cell number, however, demonstrated that the mutants could grow even five hours after being transferred to the non-permissive temperature. The

growth test further confirmed that the cells were still alive These results are in agreement with observations of the synthesis of RNA and DNA which completely stopped approximately 5-6 hours after the shift to the non-permissive temperature. Following the temperature shift, the amount of DNA and RNA approximately doubled prior to the cessation of growth. At this point, cell viability dropped rapidly.

The spot test experiment demonstrated that *nal(ts)* mutants became hypersensitive to polyene antibiotics, clotrimazole, econazole and non-ionic detergents. However, gramicidin, an antibiotic that leads to the formation of pores in the cytoplasmic membrane (Harold and Baarda, 1967) produces no effect at all. It seems that antibiotics and agents that interact with the phospholipid membrane or sterols produce severe effects in *nal(ts)* mutants. This result is in agreement with the possibility that the *nal(ts)* mutations alter the composition of the cytoplasmic membrane making the cell resistant to nalidixic acid but hypersensitive to polyene antibiotics, azole agents and non-ionic detergents. The exact nature of this alteration remains unclear.

Chapter IV: Cloning the *NAL* gene

4.1. Introduction

Determining the target of nalidixic acid in *Saccharomyces cerevisiae* requires the isolation of the *NAL* gene. Of the various techniques used to isolate genes in yeast, the easiest is to obtain the *NAL* gene through complementing for its function. This cloning method affords two options:

a) selection of mutant gene for nalidixic acid resistance, conferred by the *nal(ts)* gene, in a *mds* background.

b) selection for growth at the non-permissive temperature, carried by the wild-type gene, in a *nal(ts)* background.

Option b) was the easist, due to the relative ease of transformation of the *nal(ts)* strain compared to the *mds* strain. Accordingly, wild-type yeast genomic DNA was used to construct a library, and the *nal(ts)* mutant was chosen as the host, to screen the library by complementation.

4.2. The choice of vector

The vector chosen was YEp13 (Table 2.3). This vector is based on pBR322 and contains the selectable yeast marker *LEU2*, as well as the autonomous replication origin of the 2μm circle, a *Saccharomyces cerevisiae* native plasmid (figure 4.1). The YEp13 vector has several advantages for cloning:

a) it provides a suitable cloning site (*Bam*HI) in the *tetracycline* resistance gene;

b) it transformes yeast at high frequency;

c) it replicates autonomously in yeast at a high copy number.

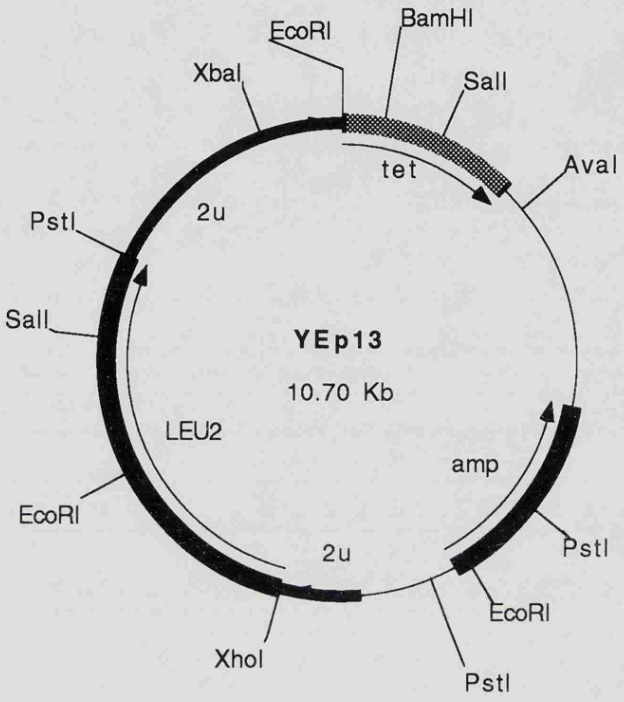

EcoRI
BamHI
XbaI
SalI
tet
AvaI
PstI
2u
SalI
YEp13
10.70 Kb
LEU2
amp
EcoRI
PstI
2u
EcoRI
XhoI
PstI

Figure: 4.1

A high copy number can nonetheless have drawbacks; for example, a gene product, lethal at multiple copies, will kill the cells, or an unrelated gene at high copy number can lead to complementation by suppressing the proper gene.

4.3. Construction of genomic library

The protocol of the construction of genomic library and the cloning of the *NAL* gene was summarised in figure 4.2.

In order to generate random sizes of genomic DNA fragments for cloning, wild-type yeast genomic DNA was partially digested with the restriction endonuclease *Sau*3A. The best results were obtained through the digestion of 20 μg DNA with 0.2 units of *Sau*3A at 37°C for 1 hour. The fragments of 3-10 kb were then isolated from an agarose gel by the "death wish" method.

These fragments were ligated with the *Bam*HI-digested and dephosphorylated YEp13 plasmid DNA. The ligation mixtures were then transformed into *Escherichia coli* 5K strain under selection of ampicillin. The highest transformation frequency was obtained using the ligation mixture with a 2:1 ratio of the genomic DNA fragments to that of the vector DNA.

So as to determine the sizes of the insert in the library, several transformants were randomly picked up and the insert sizes were analysed. The majority of the transformants had an insert of 3-5 kb. The transformants collected were about 8×10^4 colonies, such that the size of the DNA library had reached 400 Mb. As the size of the yeast genome is approximately 16 Mb, the size of the library was more than 15 times that of the yeast genomic DNA.

All the transformants were pooled and amplified, the library DNA was then extracted and purified in CsCl-EtBr gradient.

Figure 4.2 : Cloning the *NAL* gene.

Wild-type yeast genomic DNA was partially digested with the restriction endonuclease *Sau*3A. The fragments were ligated with the *Bam*HI-digested and dephosphorylated YEp13 plasmid DNA. The ligation mixture was transformed into *E.coli* 5K on nutrient agar containing 100 $\mu g ml^{-1}$ ampicillin and incubated at 37°C. The transformants were pooled, amplified and their recombinant plasmids were extracted and purified on CsCl-EtBr gradient. These recombinant plasmids were transformed into yeast *nal(ts)* mutant using the spheroplast method (see chapter II: 2.5.15). Cells were plated on agar minimal media containing the required amino acids and incubated at 30°C overnight before being transferred to 37°C. Plasmid from the transformants which grew at 37°C were isolated using the yeast miniprep method (see chapter II: 2.5.12). Recombinant plasmids were transformed into *E.coli*5K to be amplified. After isolation and purification, the recombinant plasmids were re-transformed into yeast *nal(ts)* mutant to determine whether the transformants still grew at 37°C.

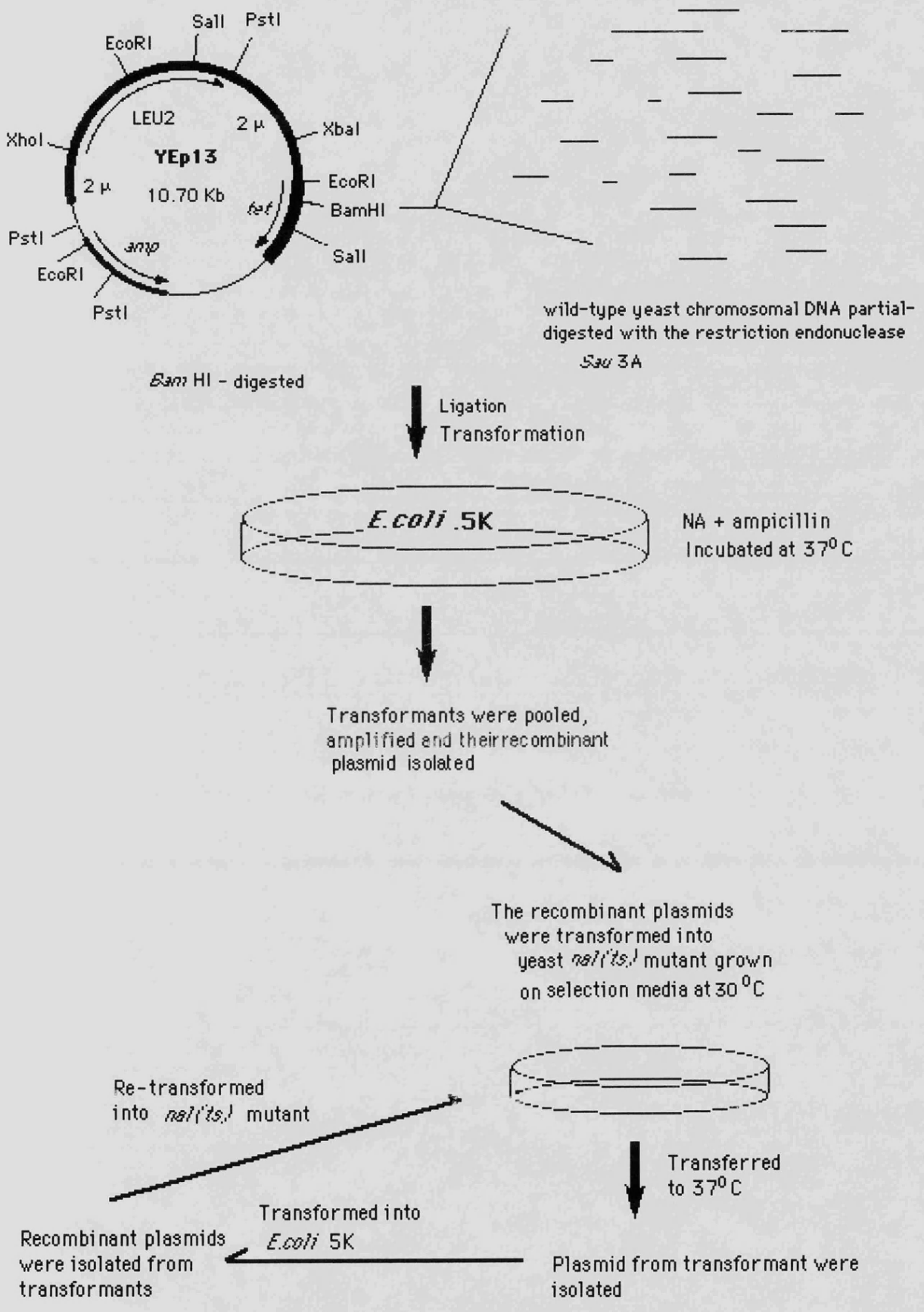

SalI
PstI
EcoRI
LEU2
2 μ
XbaI
XhoI
YEp13
10.70 Kb
2 μ
EcoRI
BamHI
tet
PstI
amp
SalI
EcoRI
PstI
wild-type yeast chromosomal DNA partial-digested with the restriction endonuclease Sau 3A
Bam HI - digested
Ligation
Transformation
E.coli 5K
NA + ampicillin
Incubated at 37°C
Transformants were pooled, amplified and their recombinant plasmid isolated
The recombinant plasmids were transformed into yeast rad(ts) mutant grown on selection media at 30°C
Re-transformed into rad(ts) mutant
Transferred to 37°C
Transformed into E.coli 5K
Recombinant plasmids were isolated from transformants
Plasmid from transformant were isolated

Figure: 4.2

4.4. Cloning the *NAL* gene

The library DNA was transformed into the mutant strain NT56B *nal(ts), leu2, ura3* using the spheroplast and electroporation methods. The yeast cells were plated on selection medium without leucine, and incubated at 30°C overnight before being shifted (except for a few control plates) to 37°C. Incubation at 37°C was continued until colonies appeared. A considerable number of transformants (several hundreds) were obtained when the amount of the library DNA used was increased to about 25-50 μg per transformation mixture.

Three transformant colonies (using the chemical-transformation method) eventually grew at 37°C. The auxotrophic markers of the transformant colonies indicated that they were not contaminants. The insert-containing vectors were further recovered from the transformed mutant using the yeast miniprep method. These cloned DNA (pNAL1, pNAL2, pNAL3) were transformed into *Escherichia coli* 5K, amplified and retransformed into the NT56B *nal(ts) leu2 ura3* mutant to determine whether they were genuinely complementing clones. The resulting failure of the pNAL1-containing mutant to grow at 37°C suggested that pNAL1 did not complement the *nal(ts)* gene. The other two clones, pNAL2 and pNAL3, still complemented the temperature-sensitive phenotype of the NT56B *nal(ts)* mutant at the non-permissive temperature. Consequently, pNAL2 and pNAL3 clones were amplified for further analysis.

4.5. Restriction mapping of pNAL2 and pNAL3

Restriction mapping of pNAL2 and pNAL3 was carried out in order to identify their sizes and restriction sites. The subsequent result indicated that the size of pNAL2 was 4.3 kb, whereas that of

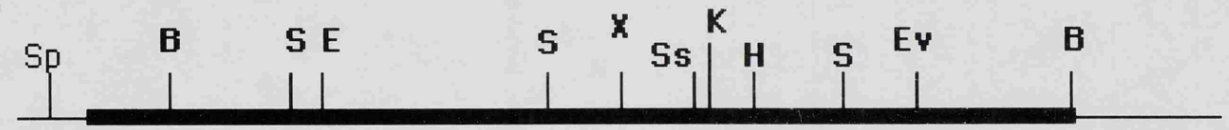
Figure 4.3 : Restriction maps of cloned-genes pNAL2 and pNAL3.

pNAL2 and pNAL3 have an identical pattern except that pNAL3 is approximately 1 kb longer.

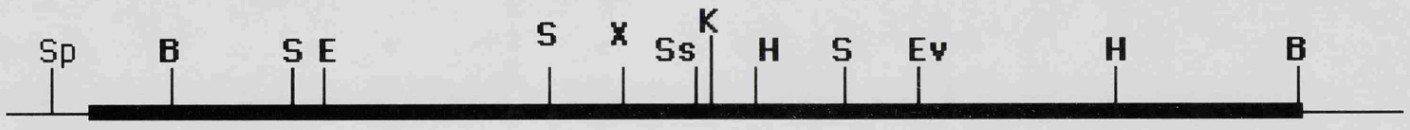
B = *Bam*HI S = *Sal*I E = *Eco*RI X = *Xba*I

K = *Kpn*I Ev = *Eco*RV

H = *Hin*dIII Ss = *Sst*I



pNAL2



pNAL3

1 kb

Figure: 4.3

pNAL3 was 5.4kb. Except for the 1 kb difference in size, the restriction mapping pattern of these two clones is identical (figure 4.3).

As these two clones were obtained by partially digesting yeast genomic DNA with the endonuclease *Sau*3A and ligated at the *Bam*HI site, it is possible to generate different fragment sizes of the same clone. The *Bam*HI site at the 3'-end of pNAL2 seems not to be the real *Bam*HI site, most likely being created by chance, due to the adjacent nucleotides.

4.6. Subcloning *NAL* gene

The inserts in pNAL2 and pNAL3 have been subcloned to different, purpose-specific vectors, such as pUC18, pUC19, pEMBL Ye23 and pEMBL Yi21 (Table 2.3) to enhance further studies of the *nal(ts)* gene.

4.6.1. Subcloning the *NAL* gene into pEMBL Ye23

The complementing inserts of pNAL2 and pNAL3 were subcloned into pEMBL Ye23 (Table 2.3) so as to identify the smallest fragment that complemented the temperature-sensitive phenotype of the *nal(ts)* mutant at the non-permissive temperature.

pEMBL Ye23 contains an *ampicillin-resistance* (*amp*) gene for selection and the *lacZα* gene for detection of recombinant plasmid in *Escherichia coli*. This plasmid has cloning sites at the polylinker downstream the *lacZα* promoter. When pEMBL Ye23 is transformed into *E.coli* DH5α strain lacking of the *lacZα* gene in the presence both of an inducer IPTG and of the chromophore substance X-Gal, then the plasmid-containing colonies become blue. The *E.coli* DH5α is otherwise a white colony. The change in colour from white to blue results from

Figure 4.4.1 : Subcloning of the cloned-gene pNAL2 in pEMBLYe23

The pNAL2 was digested with the restriction endonuclease *Bam*HI. The 3.8 kb *Bam*HI fragment was isolated from the agarose gel using the "death wish" method. The fragment was ligated with the *Bam*HI-digested pEMBLYe23. The ligation mixture was transformed into *E.coli* DH5α, plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37^{o}C overnight. The white colonies were picked up and examined for recombinant plasmids. The recombinant plasmid is designated pEMBL232

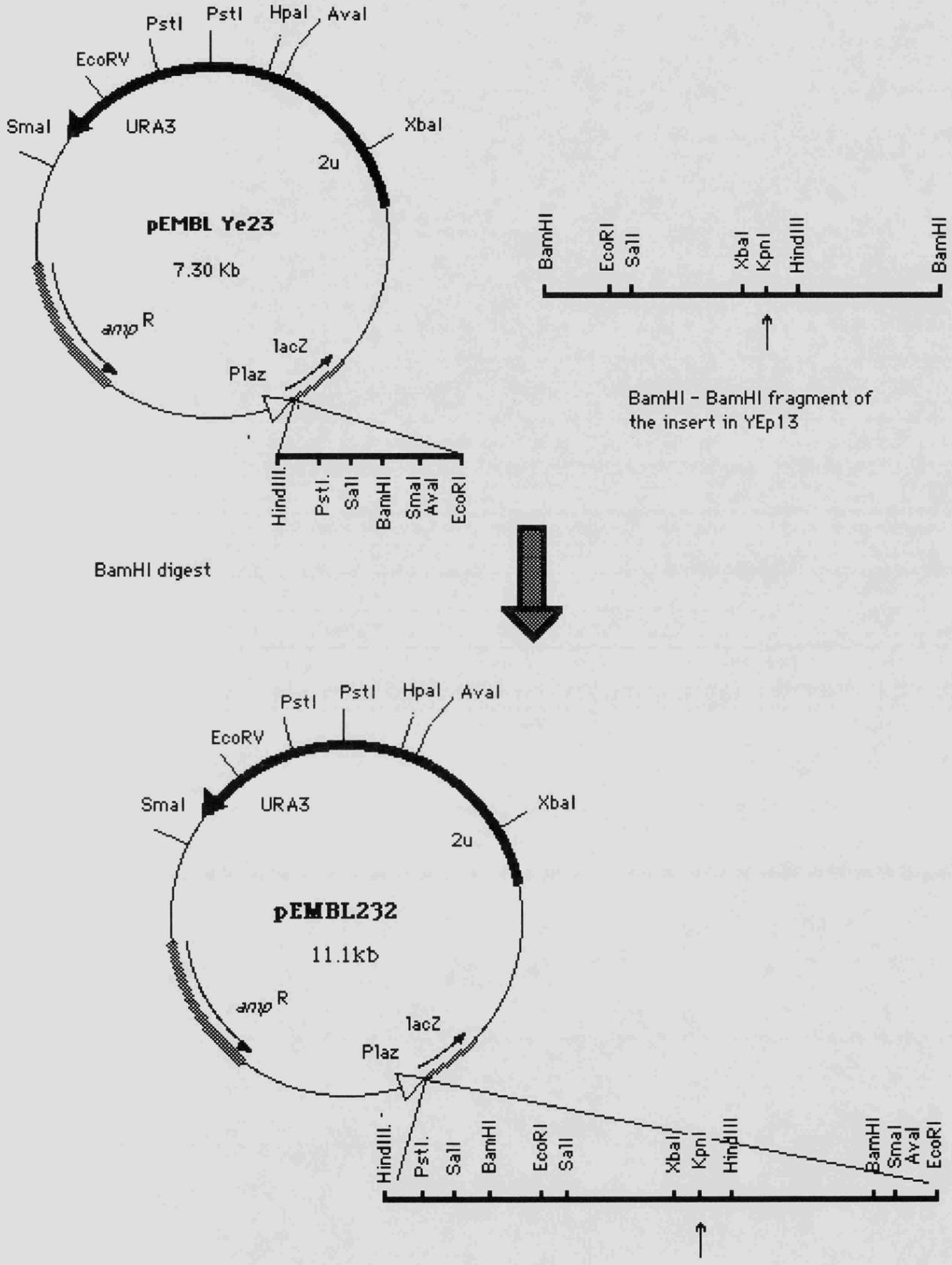

PstI
PstI
HpaI
AvaI
EcoRV
SmaI
URA3
XbaI
2u
pEMBL Ye23
7.30 Kb
amp R
lacZ
Plaz
HindIII.
PstI.
SalI
BamHI
SmaI
AvaI
EcoRI
BamHI
EcoRI
SalI
XbaI
KpnI
HindIII
BamHI
BamHI - BamHI fragment of
the insert in YEp13
BamHI digest
PstI
PstI
HpaI
AvaI
EcoRV
SmaI
URA3
XbaI
2u
pEMBL232
11.1kb
amp R
lacZ
Plaz
HindIII.
PstI.
SalI
BamHI
EcoRI
SalI
XbaI
KpnI
HindIII
BamHI
SmaI
AvaI
EcoRI

Figure: 4.4.1

Figure 4.4.2 : Subcloning of the cloned-gene pNAL3 in pEMBLYe23

The pNAL3 was digested with the restriction endonuclease *Bam*HI. The 4.9 kb *Bam*HI fragment was isolated from the agarose gel using the "death wish" method. The fragment was ligated with the *Bam*HI-digested pEMBLYe23. The ligation mixture was transformed into *E.coli* DH5α, plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37^{o}C overnight. The white colonies were picked up and examined for recombinant plasmids. The recombinant plasmid is designated pEMBL233

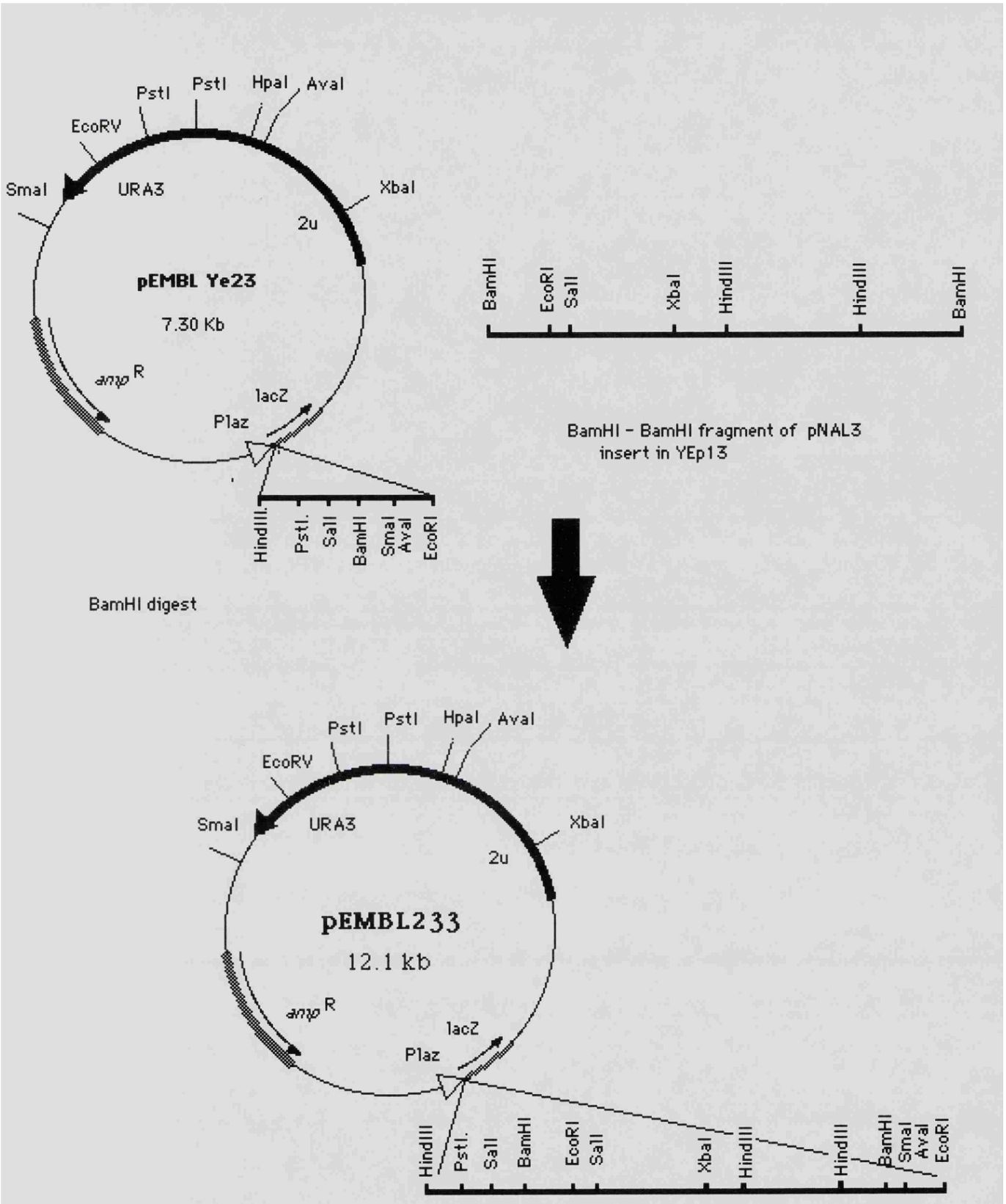

PstI
PstI
HpaI
AvaI
EcoRV
SmaI
URA3
XbaI
2u
pEMBL Ye23
7.30 Kb
amp R
lacZ
Plaz
HindIII
PstI.
SalI
BamHI
SmaI
AvaI
EcoRI
BamHI
EcoRI
SalI
XbaI
HindIII
HindIII
BamHI
BamHI - BamHI fragment of pNAL3
insert in YEp13
BamHI digest
PstI
PstI
HpaI
AvaI
EcoRV
SmaI
URA3
XbaI
2u
pEMBL233
12.1 kb
amp R
lacZ
Plaz
HindIII
PstI.
SalI
BamHI
EcoRI
SalI
XbaI
HindIII
HindIII
BamHI
SmaI
AvaI
EcoRI

Figure: 4.4.2

the cleavage of X-Gal by β-galactosidase enzyme, which is produced by the *lacZα* gene in the presence of an inducer IPTG. Successful subclones in the cloning site at the polylinker will disrupt the *lacZα* gene, rendering it incapable of producing the β-galactosidase enzyme. The chromophore substance X-Gal hence remains intact and the *E.coli* DH5α colony remains white. The change in colour can therefore be used to screen the ligation products for recombinant plasmid.

pEMBL Ye23 also contains the 2μ plasmid and *E.coli* replicating origins, thus enabling its propagation either in the yeast *Saccharomyces cerevisiae* or *E.coli*. This plasmid contains the yeast *URA3* gene which can be used as a selective marker in *Saccharomyces cerevisiae* as well as in *Echerichia coli.*

pNAL2 and pNAL3 were digested with *Bam*HI to remove the 3.8kb and 4.9kb inserts, respectively, from the cloned plasmid. The insert fragments were isolated from agarose gel following electrophoresis using the " death wish " method. The fragments were ligated to the *Bam*HI-digested pEMBL Ye23 plasmid. The ligation mixtures were transformed into *E.coli* DH5α and plated on nutrient agar containing ampicillin. After incubation overnight at 37°C a dozen recombinant plasmid-containing colonies (white colonies), were picked up to examine their inserts and those with the correct inserts were identified.

Both recombinant plasmids, pNAL232 and pNAL233, respective subclones of pNAL2 and pNAL3 in pEMBL Ye23 (figure 4.4.1 and 4.4.2), were transformed into an NA528 *nal(ts)* mutant, using the spheroplast method. The cells were plated on minimal medium containing the required amino acids, except for uracil, and incubated at 30°C for 3-4 days. The colonies that grew on uracil-minus medium were picked up and purified on the same medium and the other

Figure 4.5: Subcloning of the *Bam*HI-*Bam*HI fragment of th *NAL* gene into pEMBLYe23

The 3.8 kb *Bam*HI-*Bam*HI fragment of the *NAL* gene was ligated to the *Bam*HI-digested pEMBLYe23. The ligation mixture was transformed into *E.coli* DH5α. Cells were plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37°C overnight. The white colonies were picked up and examined for recombinant plasmids. The correct recombinant plasmid, designated pNAL232A was chosen to be amplified and purified.

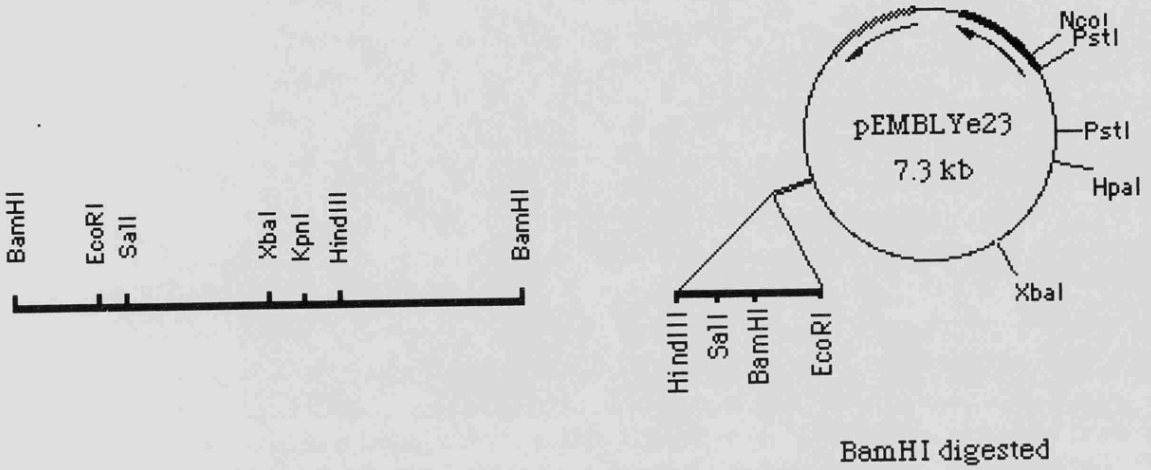
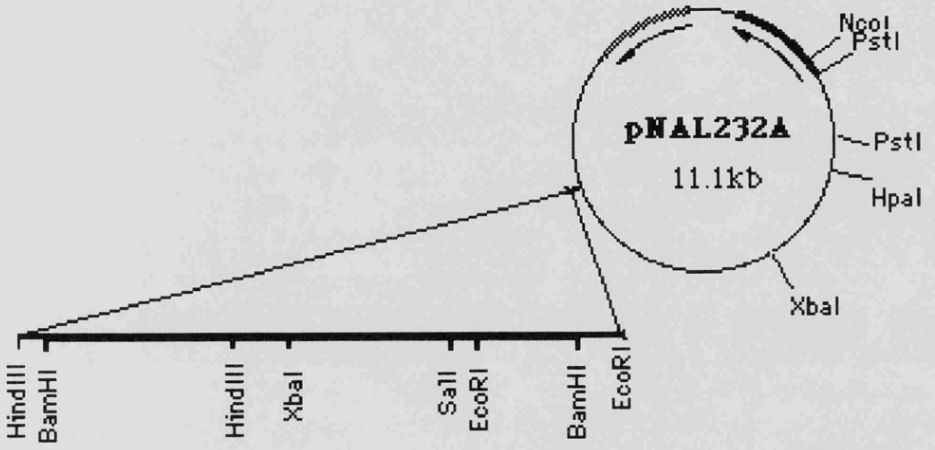

BamHI
EcoRI
SalI
XbaI
KpnI
HindIII
BamHI
NcoI
PstI
pEMBLYe23
7.3 kb
PstI
HpaI
XbaI
HindIII
SalI
BamHI
EcoRI
BamHI digested
Ligation
NcoI
PstI
pNAL232A
11.1kb
PstI
HpaI
XbaI
HindIII
BamHI
HindIII
KpnI
XbaI
SalI
EcoRI
BamHI
EcoRI

Figure: 4.5

Figure 4.6: Subcloning of the *Bam*HI-*Hin*dIII fragment into pEMBLYe23

pNAL232A was digested with restriction endonuclease *Hin*dIII. The 9.5 kb fragment was isolated and re-ligated. The ligation mixture was transformed into *E.coli* DH5α. Cells were plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37°C overnight. The white colonies were picked up and examined for recombinant plasmids. The corect recombinant plasmid, designated pNAL232H, was amplified and purified.



Digested with HindIII

Isolate the 9.5 kb fragment and

re-ligated

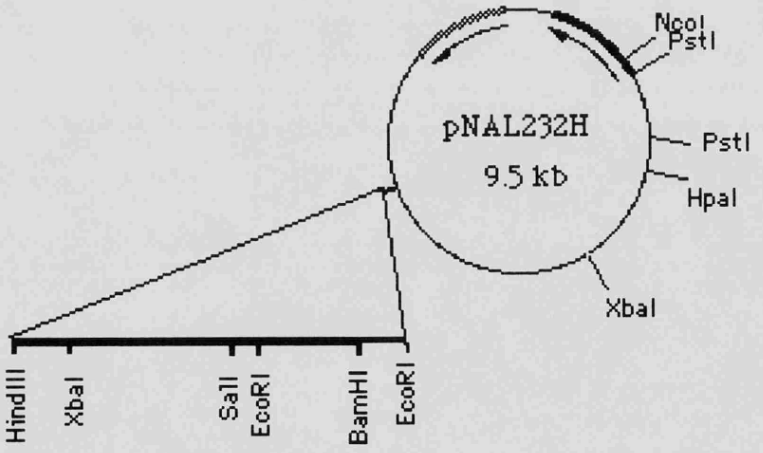


Figure: 4.6

required auxotrophic markers were checked to determine whether these colonies were the original transformants. These pNAL232 and pNAL233-containing transformants were streaked out on YPD plates and incubated at 30°C and 37°C.

All of the pNAL232 and pNAL233-containing transformants grew at 37°C as well as at 30°C, indicating that both subclones complemented the temperature-sensitive phenotype at non-permissive temperature. As the previous result showed that both subclones behaved the same, the subclone containing the smaller insert, pNAL232, was henceforth used for further studies.

4.6.2. Further analysis of subclone pNAL232

The insert size of the pNAL232, *Bam*HI - *Bam*HI fragment, was 3.8kb. Further experiments concerning the smallest insert fragment that still complemented the temperature-sensitive phenotype at non-permissive temperatures were carried out by manipulating the *NAL* gene even further.

The pNAL232A plasmid described in figure 4.5 was digested with the restriction endonuclease *Hin*dIII to remove the 1.3kb fragment. The digested plasmid was run on an agarose gel. Of the two bands of 9.8kb and 1.3kb, the former was recovered from the agarose gel by the "death wish" method. This fragment was religated and transformed into *E.coli* DH5α and the sizes of transformant plasmids were determined. The correct plasmid size was 9.8kb. This plasmid, pNAL232H (fig. 4.6), was amplified and purified.

The pNAL232H plasmid was then transformed into the NA528 *nal(ts)* mutant. The cells were plated on uracil-minus synthetic medium. Transformants were streaked out on YPD plates and incubated at 30°C and 37°C overnight. All of the transformants grew

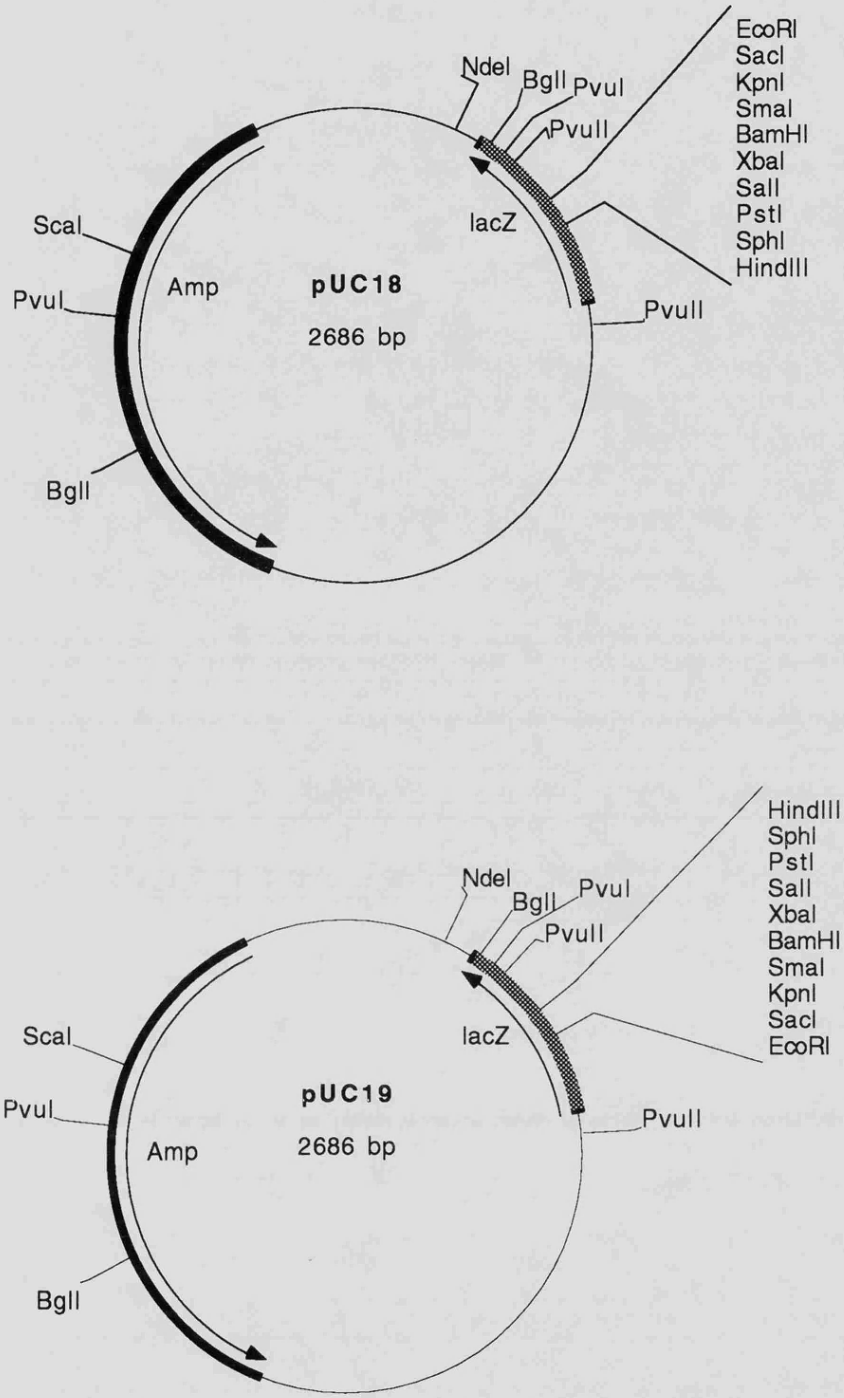
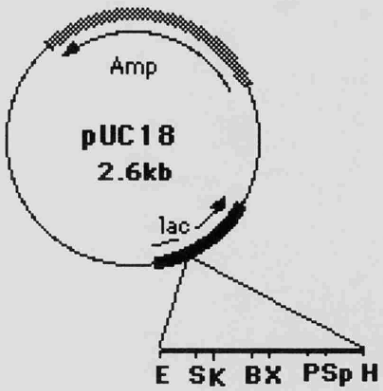
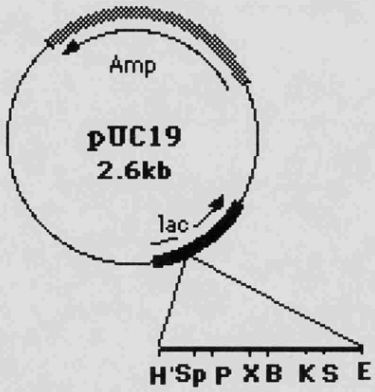

EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
NdeI
BglI
PvuI
PvuII
lacZ
ScaI
PvuI
Amp
pUC18
2686 bp
PvuII
BglI
HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
NdeI
BglI
PvuI
PvuII
lacZ
ScaI
PvuI
pUC19
Amp
2686 bp
PvuII
BglI

Figure: 4.7

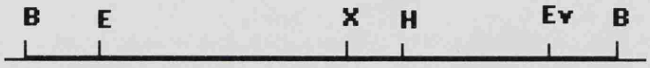
Figure 4.8: Subcloning the *NAL* gene in pUC18 and pUC19
*Bam*HI fragments of the *NAL* gene were ligated with the *Bam*HI-digested pUC18 and *Bam*HI-digested pUC19. The ligation mixtures were transformed into *E.coli* DH5α. Cells were plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37°C overnight. The white colonies were picked up and examined for recombinant plasmids. Recombinant plasmids in pUC18 and pUC19 are designated pNUC182B and pNUC192B, respectively.



Bam HI-digested



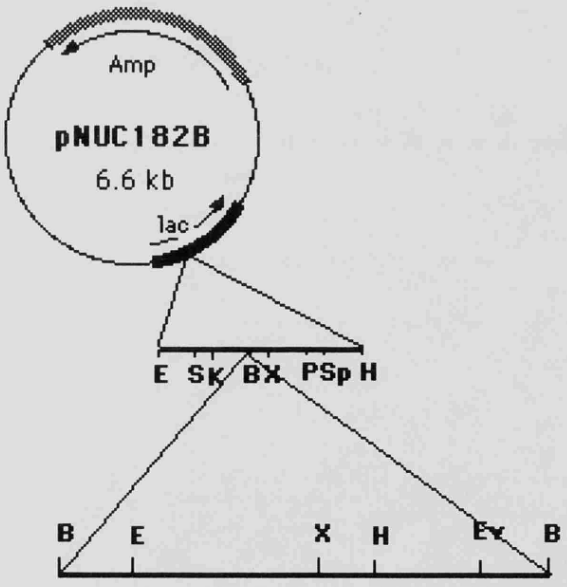
Bam HI-digested



MAL fragment in YEp13

digested with restriction enzyme *Bam*HI

Ligation



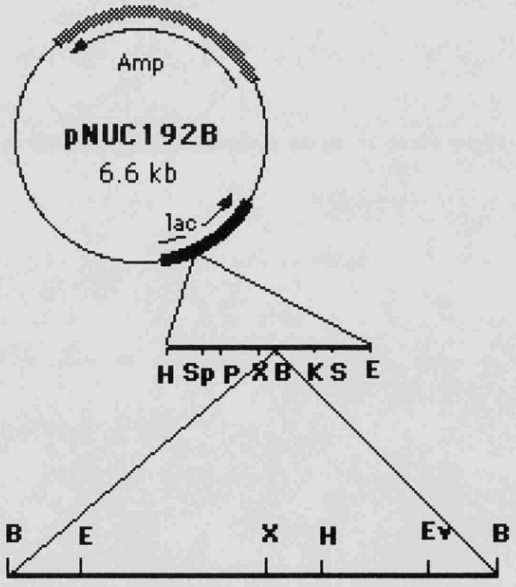


Figure: 4.8

Figure 4.9: Subcloning the *Eco*RI-*Bam*HI fragment of the *NA* gene in pUC19

pNUC192B plasmid was digested with the restriction endonuclease *Eco*RI. The 3.4 kb fragment was isolated and ligated with the *Eco*RI-digested pUC19. The ligation mixture was transformed into *E.coli* DH5α. Cells were plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37°C overnight. The white colonies were picked up and examined for recombinant plasmids. The recombinant plasmid is designated pNUC192BE.

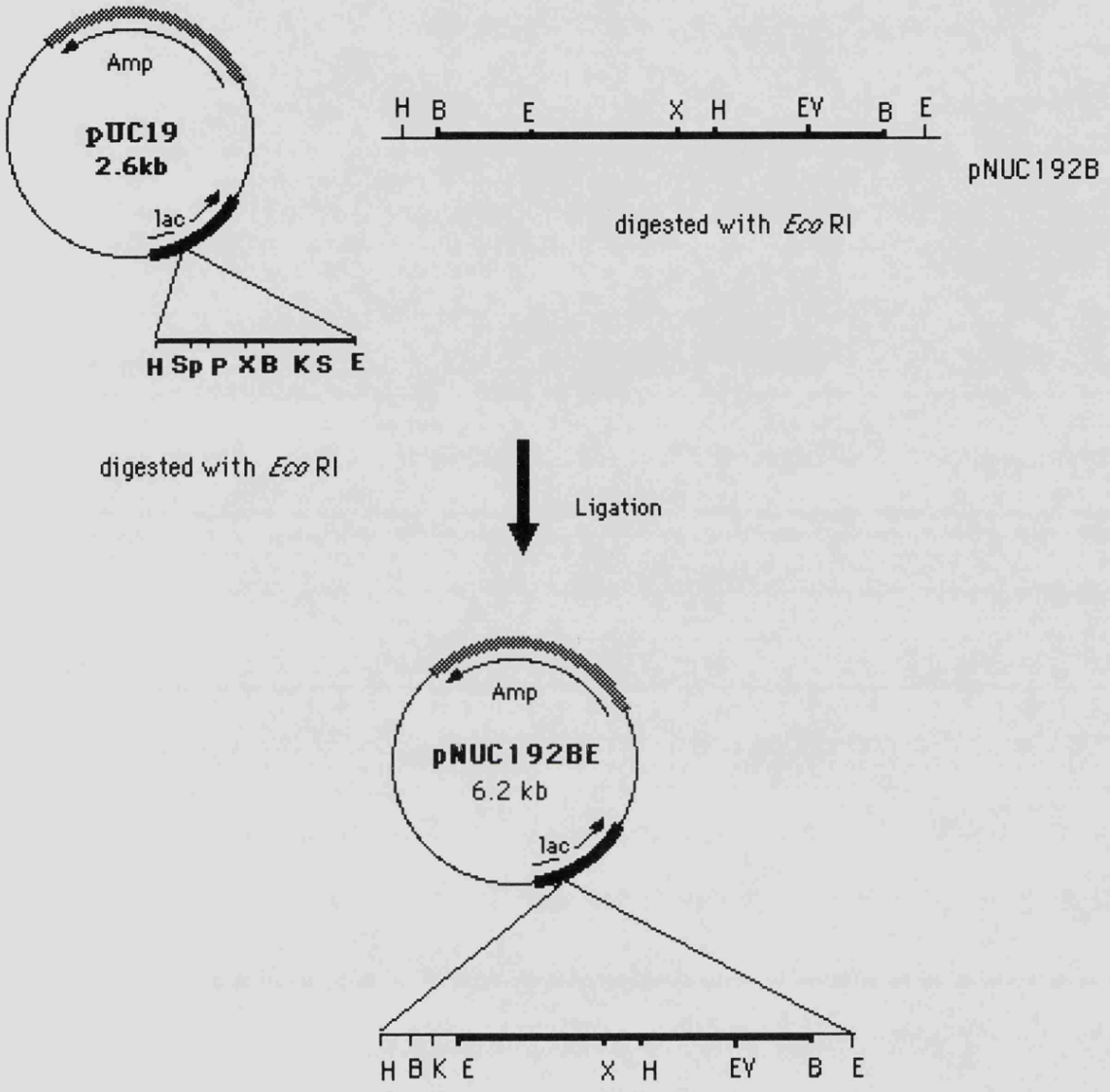

Amp
pUC19
2.6kb
lac
H Sp P XB KS E
digested with Eco RI
H B E X H EV B E
pNUC192B
digested with Eco RI
Ligation
Amp
pNUC192BE
6.2 kb
lac
H B K E X H EV B E

Figure: 4.9

at 30°C, but not at 37°C. These results indicate that the 2.6kb *NAL* fragment in pNAL232H did not rescue the temperature-sensitive phenotype of the *nal(ts)* mutant at the non-permissive temperature. The 1.3kb *Hin*dIII-*Bam*HI fragment of the clone was essential and therefore contained part of the *NAL* gene.

The next experiment involved the subcloning of the 3.4kb *Eco*RI-*Bam*HI fragment of the *NAL* gene. Due to a lack of vectors with convinent sites for direct subcloning, this fragment was subcloned indirectly.

First, the *Bam*HI-*Bam*HI fragment of the *NAL* gene was subcloned in pUC18 and pUC19 (figure 4.7). These vectors are identical except for the orientation of their cloning sites. The 3.8kb *Bam*HI-*Bam*HI fragment was ligated to the *Bam*HI-digested pUC18 and pUC19. The transformant colonies from both transformations were picked up. The sizes and orientation of the inserts were examined. The correct recombinant constructs in pUC18 and pUC19 were amplified and purified. They were designated as pNUC182B and pNUC192B (figure 4.8), respectively.

The pNUC192B, with the insert orientation as described in figure 4.8, digested with restriction endonuclease *Eco*RI on agarose gel electrophoresis, was shown to contain two bands corresponding to the 3.4kb and 3.1kb fragments. The former were recovered from the agarose gel using the "death wish" method. These fragments were then ligated to the *Eco*RI-digested pUC19 and the ligation mixture was transformed into *E.coli* DH5α. The white transformants were picked up and the recombinant plasmids analysed. The recombinant plasmid with the insert orientation as described in figure 4.9 was amplified and designated as pNUC192BE.

Figure 4.10 : Subcloning of *Eco*RI-*Bam*HI fragment of the *NAL* gene in pEMBLYe23

pNUC192BE plasmid was digested with the restriction endonuclease *Bam*HI. The 3.4 kb fragment was isolated and ligated with the *Bam*HI-digested pEMBLYe23. The ligation mixture was transformed into *E.coli* DH5α. Cells were plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37°C overnight. The white colonies were picked up and examined for recombinant plasmids. The recombinant plasmid is designated pEMBL23BE.

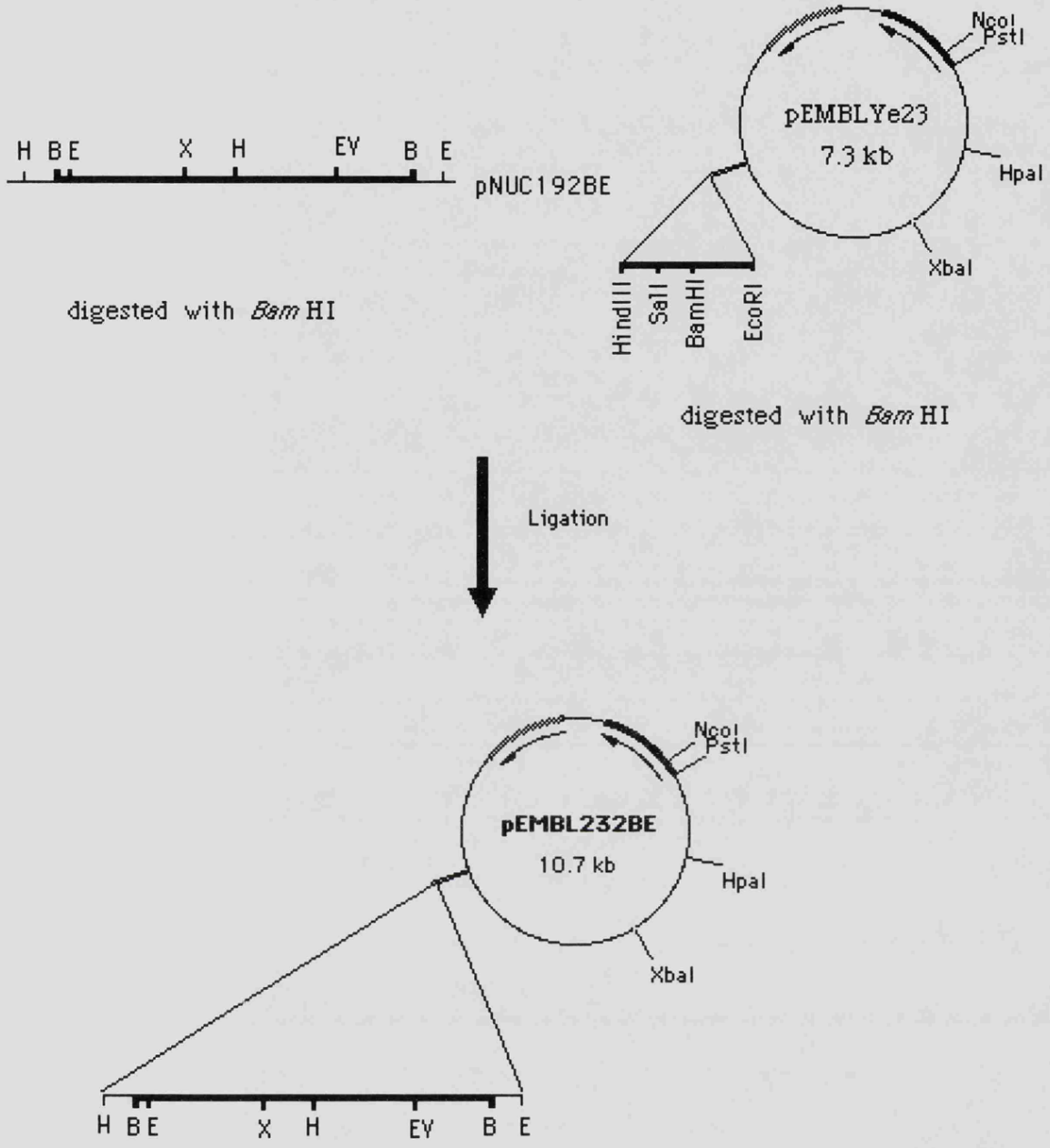

H B E X H EV B E
pNUC192BE
digested with *Bam* HI
pEMBLYe23
7.3 kb
NcoI
PstI
HpaI
XbaI
HindIII
SalI
BamHI
EcoRI
digested with *Bam* HI
Ligation
pEMBL232BE
10.7 kb
NcoI
PstI
HpaI
XbaI
H B E X H EV B E

Figure: 4.10

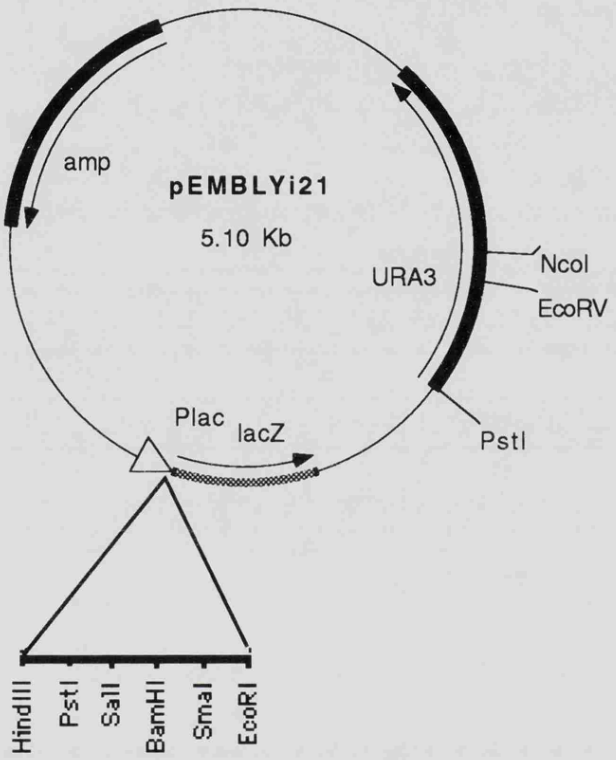


Figure 4.11: pEMBLYi21

The pNUC192BE was digested with restriction endonuclease *Bam*HI and the fragments were run on an agarose gel. There were two bands corresponding to 3.4kb and 2.6kb. The 3.4kb fragment of the *NAL* gene was isolated from the agarose gel and ligated to the *Bam*HI-digested pEMBL Ye23. The ligation mixture was transformed into *E.coli* DH5α and the cells were plated on nutrient agar containing IPTG, X-Gal and ampicillin. The white transformant colonies were picked up and their recombinant plasmids were analysed. The recombinant plasmid with a 3.4kb insert as described in figure 4.10, was amplified and purified in CsCl-EtBr gradient. This recombinant plasmid was named pEMBL232BE.

Afterwards, the pEMBL232BE was transformed into the NA528 *nal(ts)* mutant and the cells were plated on uracil-minus synthetic medium. The transformants that grew on uracil-minus medium were purified on the same medium, and the other auxotrophic markers were determined. Several of the positive transformants were streaked out on YPD and incubated at 30°C and 37°C. All of the transformants grew at 37°C as well as at 30°C, indicating that the 3.4kb *Eco*RI-*Bam*HI fragment of the *NAL* gene could rescue the temperature-sensitive phenotype of the *nal(ts)* mutant at non-permissive temperatures.

4.7. Subcloning of the *NAL* gene into pEMBL Yi21

The pEMBL Yi21 vector is an integrative vector (fig. 4.11). Unlike an episomal vector such as pEMBL Ye23, pEMBL Yi21 lacks an element for replication in *Saccharomyces cerevisiae*, and is therefore unable to replicate on its own. It will thus integrate into the chromosome DNA through homologous recombination. This vector contains the *ampicillin-resistance* gene for selection and the *lacZ* gene, for detection, of recombinant plasmids in *E.coli*. This vector also contains

Figure 4.12: Subcloning of the *NAL* gene in pEMBLYi21.
The *Bam*HI-*Bam*HI fragment of the *NAL* gene was ligated with the *Bam*HI-digested pEMBLYe21 vector. The ligation mixture was transformed into *E.coli* DH5α. Cells were plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37°C overnight. The white colonies were picked up and examined for recombinant plasmids. The recombinant plasmid is designated pEMBL212.

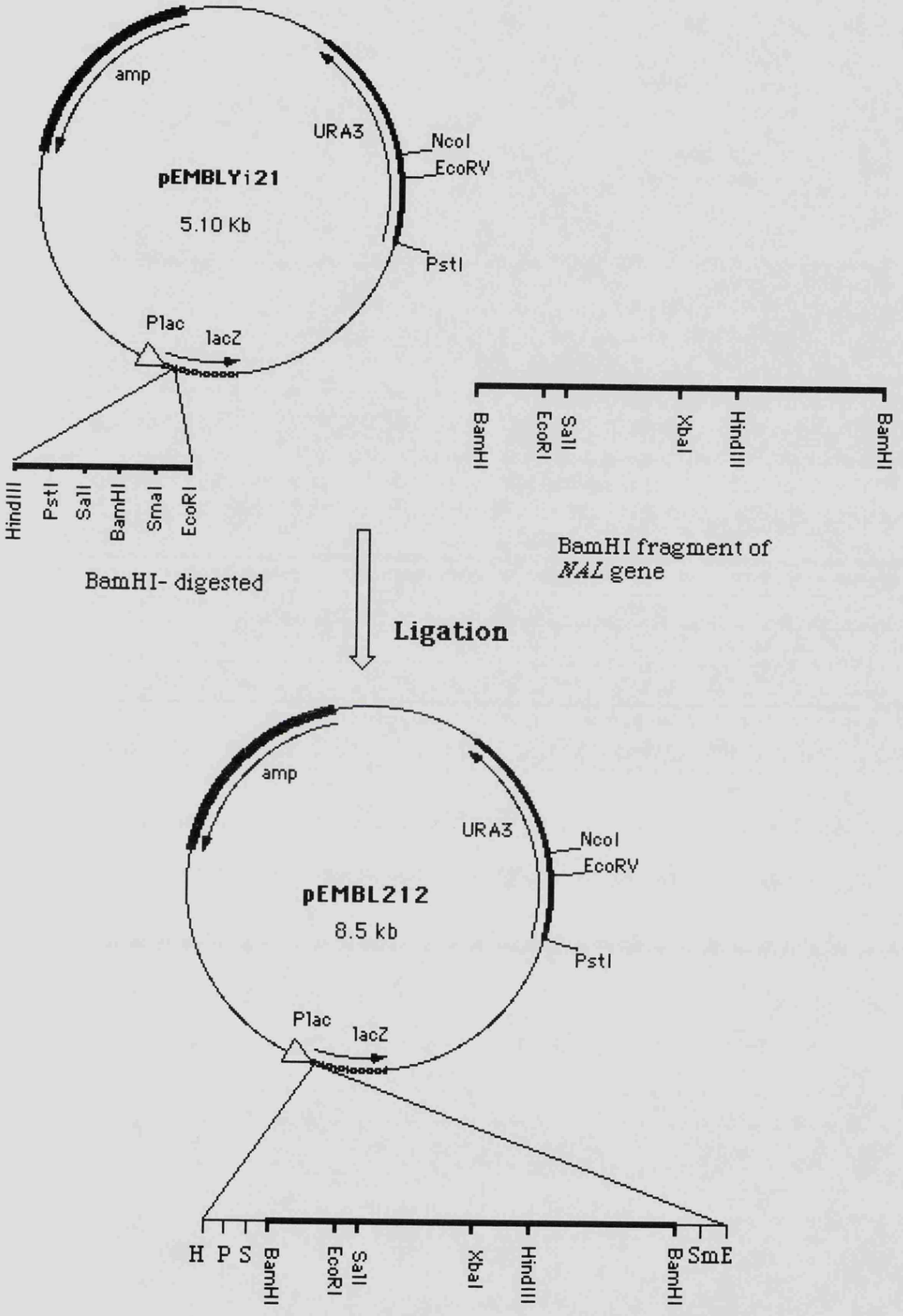

amp
URA3
NcoI
EcoRV
pEMBLYi21
5.10 Kb
PstI
Plac
lacZ
HindIII
PstI
SalI
BamHI
SmaI
EcoRI
BamHI- digested
BamHI
EcoRI
SalI
XbaI
HindIII
BamHI
BamHI fragment of
NAL gene
Ligation
amp
URA3
NcoI
EcoRV
pEMBL212
8.5 kb
PstI
Plac
lacZ
H P S
BamHI
EcoRI
SalI
XbaI
HindIII
BamHI
SmE

Figure: 4.12

the *URA3* gene of *Saccharomyces cerevisiae* as a selection marker for *S.cerevisiae* and *E.coli*.

The 3.8kb *Bam*HI-*Bam*HI fragment of the *NAL* gene isolated from pNAL232 was ligated to the *Bam*HI-digested pEMBL Yi21. The ligation mixture was transformed into *E.coli* DH5α and the cells were plated on nutrient agar containing IPTG, X-Gal and ampicillin. The white colony transformants were picked up for further analysis. The correct recombinant plasmid should contain a 3.8kb *Bam*HI-*Bam*HI fragment of the *NAL* gene insert. Such a recombinant plasmid was found, designated pNALI212 (figure 4.12), amplified and purified.

The cloned gene can integrate at its homologous site in the genome. So as to increase integration efficiency, the circular plasmid must be linearized through digestion at a unique site within the gene. For this purpose, pNALI212 was digested by the restriction endonuclease *Xba*I. The linear pNALI212 was transformed into the NA528 *nal(ts)* mutant strain by the spheroplast and electroporation methods. Cells were plated on uracil-minus synthetic medium. Again, the transformation frequency was very low in both methods, fewer than 20 transformants per 1 μg plasmid DNA. The transformants were purified on the same medium, streaked out on YPD plates and incubated at 30°C and 37°C. They all grew at both temperatures. This result demonstrated that the cloned gene, even at a single copy, could rescue the temperature-sensitive phenotype at the non-permissive temperature, suggesting that it is unlikely to be a suppressor.

The integration transformant was crossed with the *nal(ts)* mutant to determine whether the integration of the cloned gene was at the right site, i.e. at the chromosomal *NAL* locus. The diploid colonies were sporulated, and the tetrads were dissected and analysed. The result showed that the temperature-sensitive phenotype segregated in a 2:2

Tetrad		37°C	leu-	ura-	trp-	his-	ade-	mating type
1	A	+	-	+	+	-	-	a
	B	+	-	+	+	-	-	α
	C	-	-	-	-	+	-	α
	D	-	-	-	-	+	-	a
2	A	-	-	-	-	-	-	α
	B	+	-	+	+	-	-	a
	C	-	-	-	-	+	-	a
	D	+	-	+	+	+	-	α
3	A	+	-	+	-	+	-	α
	B	-	-	-	-	+	-	α
	C	+	-	+	+	-	-	a
	D	-	-	-	+	-	-	a

Table 4.1: A selection of tetrads (3 of 12) from a cross between NA528IN and NT18C *nal(ts)5*. NA528IN is a transformant that contains *NAL* gene integrated on the chromosome. The Ts phenotype is segregated in a 2:2 mode along with the uracil minus marker.

mode, always along with the uracil-minus marker (Table 4.1). This strongly suggests that the cloned gene was integrated at the same locus as its homologous counterpart.

4.8. Gene mapping in the chromosome

4.8.1. Gene mapping by generating chromosome loss

First developed by Falco and Botstein, (1983), this method consists of the introduction of a 2 micron plasmid into the chromosome, which renders it unstable and eventually leads to its degradation. In order to integrate the cloned gene with a 2 micron plasmid into the *NAL* gene in the chromosome, the plasmid pEMBL232 (figure 4.4.1) was digested with the restriction endonuclease enzyme *Kpn*I, which uniquely cut the *NAL* gene, thus linearising the plasmid. The linear pEMBL232 was then transformed into the NA528 *nal(ts)*5 mutant, and the cells were plated on uracil-minus synthetic medium. The successful transformants were purified on the same medium.

A number of the transformants were crossed to other *nal(ts)5* mutants and the resulting diploids sporulated so as to confirm the integration of the cloned gene into the chromosomal *NAL* gene. The tetrads were dissected and analysed. The results showed the temperature-sensitive phenotype and the uracil-auxotrophic marker to be segregated in a 2:2 mode. The temperature-sensitive phenotype always segregated along with the uracil-minus marker, strongly suggesting that the cloned gene was integrated at the proper site.

This integrant strain was subsequently mated to a series of spo11 strains as these strains have auxotrophic markers corresponding to each of the yeast chromosomes. The diploids were selected on synthetic media containing the amino acid required by the diploids.

Single colonies of each diploid were incubated in YPD broths up to 50 generations. Small amounts of the cultures were streaked out on YPD plates to obtain single colonies. This procedure was repeated 3 times. Forty eight single colonies of each diploid were picked up, transferred to YPD-containing microtiter plates and then incubated at 30°C overnight. The YPD broth was discarded and replaced with sterile distilled water to starve the cells. The cells were replica-plated on synthetic media lacking single amino acid and incubated at 30°C overnight. The resulting loss of the chromosome III on the part of these colonies suggested the presence of the *NAL* gene on this particular chromosome.

The *nal(ts)5* mutant was mated to strains carrying auxotrophic markers for chromosome III, i.e. *his4*, *leu2*, *MATα* and *MATa*, to confirm and to locate the *NAL* gene on chromosome III. This procedure regrettably failed to provided conclusive evidence that the *NAL* gene was on chromosome III.

4.8.2. Gene mapping by hybridisation

This procedure was basically a Southern blot of yeast chromosomes. The yeast chromosomes were separated using pulse-field electrophoresis as described in chapter II. The separated chromosomes were transferred onto Hybond-N filter by blotting. The filter was hybridised with a labelled-*Xba*I-*Bam*HI fragment of the *NAL* gene as a probe. The result of this experiment suggested that the *NAL* gene was on the chromosome XII.

So as to confirm this finding, the same experiment was carried out using fragments of the *sst2* gene which is known to be on chromosome XII. The result shows that the *sst2* gene fragment picked up the same chromosome as the *NAL* gene (figure 4.13.).

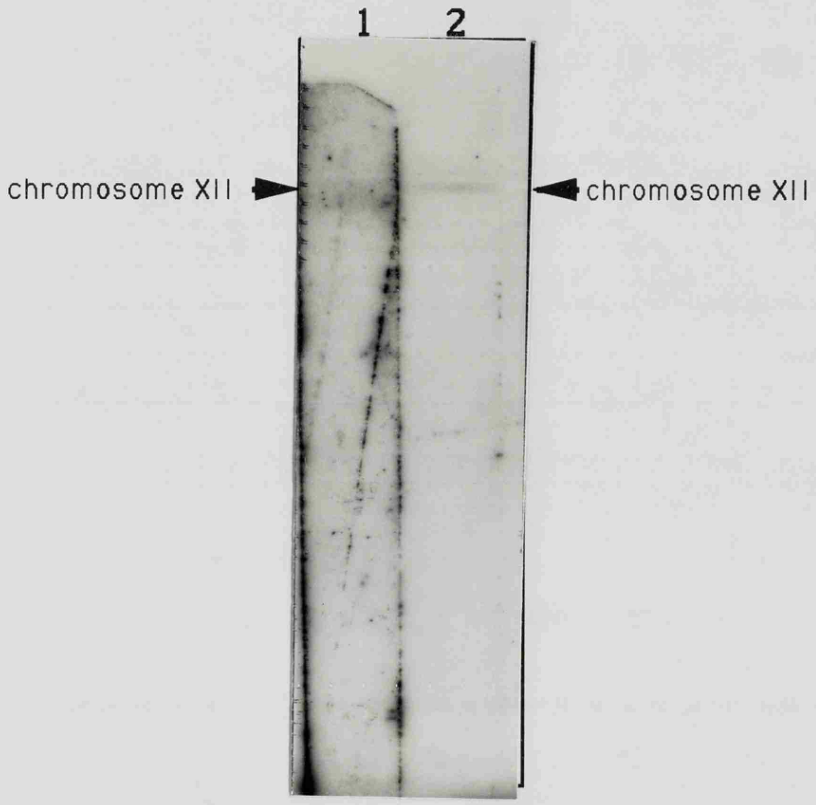


Figure 4.13: Southern blot of the chromosome mapping.
Lane 1, the chromosome was probed using fragments of the *sst2* gene.
Lane 2, the chromosome was probed using fragments of the *NAL* gene

Tetrad		37°C	asp-	met-	lys-	his-	leu-	arg-
1	A	+	-	-	+	-	-	+
	B	+	+	-	-	+	-	+
	C	-	-	+	+	-	+	-
	D	-	+	+	-	+	+	-
2	A	+	-	-	-	+	-	+
	B	-	+	-	+	+	-	+
	C	-	+	+	-	-	+	-
	D	+	-	+	+	-	+	-
3	A	-	-	+	-	+	-	-
	B	+	-	-	+	-	+	+
	C	+	+	-	+	+	-	-
	D	-	+	+	-	-	+	+
4	A	+	+	-	-	+	-	+
	B	-	-	+	-	+	-	+
	C	-	-	-	+	-	+	-
	D	+	+	+	+	-	+	-
5	A	+	-	+	-	-	-	-
	B	+	+	-	+	-	+	+
	C	-	+	-	+	+	+	-
	D	-	-	+	-	+	-	+
6	A	+	-	+	-	-	+	+
	B	-	-	+	+	+	+	+
	C	+	+	-	-	+	-	-
	D	-	+	-	+	-	-	-

Table 4.2: A selection of tetrads (6 of 12) from a cross between NA528 *nal(ts)5* and K398-4D strains. The Ts phenotype and the aspartic acid marker segregates in 2:2 mode. The ratio of parental ditype (PD) : Non-parental ditype (NPD) : tetratype(T) is 1:1:4. This is an independent assortment, indicating that there is no linkage between the *NAL* and *ASP5* gene. (+) indicates growth and (-) non-growth.
Number of tetrads:
Parental ditype (PD)= 2;
Non-parental ditype (NPD)= 2;
Tetratype (T)= 8

Tetrad		37°C	ade-	leu-	trp-	ura-	his	mating type
1	A	-	-	-	-	+	+	α
	B	+	-	-	-	+	-	a
	C	+	-	-	-	-	-	α
	D	-	-	-	-	-	+	a
2	A	+	-	-	-	+	-	α
	B	+	-	-	-	-	+	α
	C	-	-	-	-	+	-	a
	D	-	-	-	-	-	+	a
3	A	-	-	-	-	-	+	α
	B	-	-	-	-	-	-	a
	C	+	-	-	-	+	-	a
	D	+	-	-	-	+	+	α
4	A	+	-	-	-	-	-	a
	B	-	-	-	-	+	-	α
	C	+	-	-	-	+	+	α
	D	-	-	-	-	-	+	a
5	A	-	-	-	-	+	-	α
	B	-	-	-	-	+	-	a
	C	+	-	-	-	-	+	α
	D	+	-	-	-	-	+	a
6	A	+	-	-	-	+	-	α
	B	-	-	-	-	-	+	a
	C	+	-	-	-	-	+	α
	D	-	-	-	-	+	-	a

Table 4.3: A selection of tetrads (6 of 12) from a cross between NA528 *nal(ts)5* and 8HA-11-1 strains. 8HA-11-1 strain is a mutant which has a *URA3* gene inserted at the *SST2* gene. The Ts phenotype and the uracil marker segregates in 2:2 mode. The ratio of parental ditype (PD) : Non-parental ditype (NPD) : tetratype (T) is 1:1:4. This is an independent assortment, indicating that there is no linkage between the *NAL* and *SST2* gene. (+) indicates growth and (-) non-growth.
Number of tetrads:
Parental ditype (PD)= 2;
Non-parental ditype (NPD)= 2;
Tetratype (T)= 8

A recombination experiment was performed to find out the precise location of the *NAL* gene on chromosome XII. The *nal(ts)5* mutant strain was crossed against strains carrying auxotrophic markers on chromosome XII, i.e. *asp5* and *URA3* in the *sst2*gene. The resulting diploids were sporulated and the spores were dissected. Tetrad analysis showed the *NAL* gene to have no close-linkage to the *asp5* (Table 4.2) or the *sst2* genes (Table 4.3).

4.9. Conclusion

In the attempt to isolate the wild-type *NAL* gene, we encountered difficulty in transforming library clones into *nal(ts)* mutants. Data from chapter III suggest that the *nal(ts)* mutation may lead to alteration of the plasma membrane. Such alteration might reduce the transformation frequency, thus explaining the difficulty in transforming the library clones.

Two clones of the wild-type *NAL* gene were eventually isolated. The restriction endonuclease maps of these clones suggested that they were derived from the same chromosomal locus. Further analysis showed that the 3.4 kb *Eco*RI-*Bam*HI fragment is sufficient to rescue the Ts phenotype of the *nal(ts)* mutants. A single copy of the *NAL* gene, introduced by integrating the cloned gene at the *nal(ts)* locus, is capable of rescuing the Ts phenotype, suggesting that the cloned gene is not a suppressor.

Finally, it was shown that the *NAL* gene is located on chromosome XII, although we have as yet been unable to show its precise location, due to a lack of available markers for chromosome XII.

Chapter V : Sequencing the *NAL* gene

5.1.Introduction

The sequencing of the *NAL* gene was carried out using the double-stranded sequencing method (Chen and Seeburg, 1985). The *NAL* fragment was subcloned in pUC18 (see chapter IV). Obtaining subclones with approximately 300 bp differences required a series of deletions in the cloned gene in pUC18, achieved through the double-stranded nested deletion method (Henikoff, 1984). This method includes an exonuclease III digestion starting from a 5'-overhang end or from a blunt-end, while a 3'-overhang end, resistant to exonuclease digestion, protects the fragment from being digested from both directions. As there was not a unique restriction site within the cloned gene in plasmid pNUC182B (figure 4.8) from which the deletion could be started, two parts of the cloned gene were subcloned separately. Thus, both the 2.2 kb *Bam*HI-*Xba*I and the 1.6 kb *Xba*I-*Bam*H I fragments were subcloned in pUC18.

5.2. Subcloning the *Bam*HI-*Xba*I fragment into pUC18

The plasmid pNUC182B was digested with the restriction endonuclease *Bam*HI for 1 hour followed by *Xba*I digestion. The fragments were separated on a 1.5% agarose gel. The two lower bands, corresponding to 2.2 kb *Bam*HI-*Xba*I and 1.6 kb *Xba*I-*Bam*H I fragments of the *NAL* gene, were recovered from the agarose by the "death wish" method.

These fragments were ligated to *Bam*HI and *Xba*I double-digested pUC18 plasmids. The ligation mixtures were transformed into *E.coli* DH5α. The white colonies were randomly picked up and their plasmid

Figure 5.1: Subcloning of *Bam*HI-*Xba*I fragments in pUC18. The pNUC182B was double-digested with restriction endonucleases *Bam*HI and *Eco*RI. The 2.2 kb and 1.6 kb *Bam*HI-*Xba*I fragments were isolated and ligated with *Bam*HI-*Xba*I-pUC18 vector. The ligation mixtures were transformed into *E.coli* DH5α. Cells were plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37°C overnight. The white colonies were picked up and examined for recombinant plasmids. The respective recombinant plasmids of 2.2 kb and 1.6 kb *Bam*HI-*Xba*I fragments are designated pNUC18BXL and pNUC18BXS.

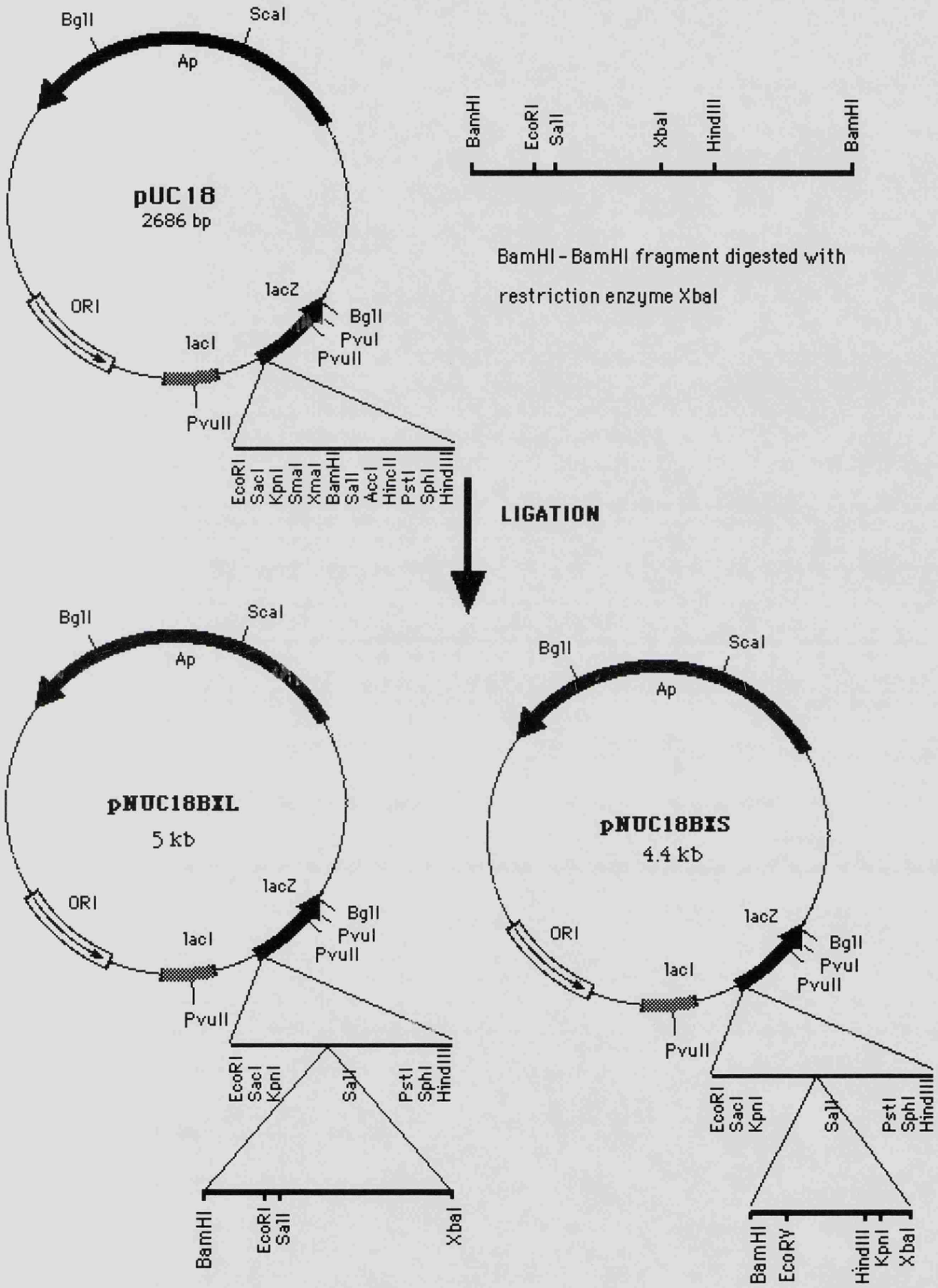

BglI
ScaI
Ap
pUC18
2686 bp
ORI
lacZ
lacI
BglI
PvuI
PvuII
PvuII
EcoRI SacI KpnI SmaI XmaI BamHI SalI AccI HincII PstI SphI HindIII
BamHI EcoRI SalI XbaI HindIII BamHI
BamHI - BamHI fragment digested with restriction enzyme XbaI
LIGATION
pNUC18BXL
5 kb
pNUC18BXS
4.4 kb
EcoRI SacI KpnI
SalI
PstI SphI HindIII
BamHI EcoRI SalI XbaI
EcoRY
HindIII KpnI XbaI

Figure: 5.1

DNA examined to determine whether the construct plasmids were successful. The correct constructs were respectively named pNUC18BXL and pNUC18BXS corresponding to the 2.2 kb *Bam*HI-*Xba*I and the 1.6 kb *Xba*I-*Bam*HI fragment insert in pUC18 (figure 5.1).

5.3. Double-stranded nested deletion of pNUC18BXL and pNUC18BXS

Both pNUC18BXL and pNUC18BXS (figure 5.1) were digested with the restriction endonuclease *Sph*I to generate a 3'-overhang end resistant to exonuclease III digestion. The linear plasmids were digested with *Bam*HI to obtain a 5'-overhang from which the exonuclease III digestion starts.

Treatment with the exonuclease III resulted in the removal of nucleotides from one strand of the target sequence, creating a single-stranded region which could be removed using S1 nuclease. The *exo*III digestion was performed at 30°C, samples being removed at timed intervals, followed by treatment with S1 nuclease to remove single-stranded regions generated by the *exo*III. Analysis of the deletions was carried out by agarose gel electrophoresis (figure 5.2). The results of the deletion analysis were used in the selection of samples to be recircularised. These were subsequently transformed into *E.coli* competent cells.

So as to determine the sizes of the deleted-DNA, this library was screened by electrophoretic analysis of miniprep DNA (figure 5.3). The DNA of subclones with appropriate deletions was amplified and were purified in CsCl-EtBr gradient for sequencing.

Figure 5.2: Analysis of double-stranded nested deletion of the *NAL* subclone in pUC18.

Lane 1 is a λ marker, lane 2-13 are *NAL* subclones which are deleted with *exo*III at 3 minute intervals at 30 C.

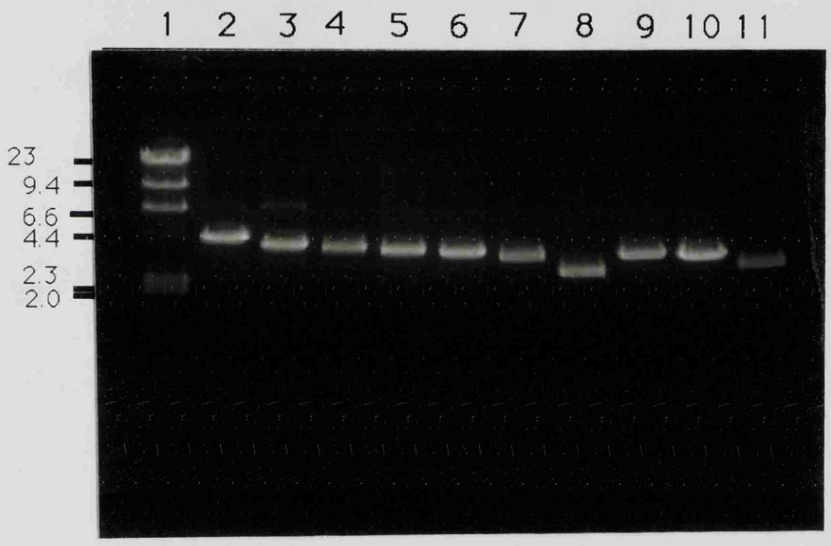


Figure 5.3: Analysis of double-stranded deletion subclones of the *NAL* gene in pUC18.

Lane 1 is a λ marker, lane 2-10 are pUC18 subclones with different size insert and lane11 is a linear pUC18 vector.

5.4. DNA sequencing

Sequencing of the subclones described above was carried out using the double-stranded sequencing method, the protocol of which has been described in chapter II. The rationale behind deletion cloning is to obtain overlaps in sequence information from successive clones. T7 DNA polymerase was used in the dideoxy chain termination reaction. In the termination mixture, 7-deazaGTP was used instead of deoxyGTP to minimize double-banding on the sequencing gel due to compression caused by the formation of stable secondary structures (Mizusawa *et al.*, 1986).

The deletion subclones from pNUC182BS were sequenced using a "forward" and a "reverse" universal M13 primer. Eventually, a 17-mer oligonucleotide primer obtained from Dept. of Biochemistry, University of Leicester (Table 2.4) was used to verify the sequence. The other serial deletion subclones from pNUC182BL were also sequenced using the same primers as mentioned above, with the exception of three oligonucleotide primers (Table 2.4) used to fill three gaps in the sequence. The strategy of sequencing is illustrated in figure 5.3a. The complete cloned sequence is shown in Appendix A.

Assembled sequence data were analysed using the Genetics Computer Group (GCG) computer programmes. Analysis of the NAL sequence showed it to contain three open reading frames (ORF) on the same strand (figure 5.4). The first of these run through the 5'-end with the size of approximately 110 amino acid residues. The second ORF begins from basepairs no.300, with a predicted length of 264 amino acid residues (~24 kDa). Finally, the third ORF with the predicted length of 563 amino acids residues (~63 kDa), is flanked by the non-coding region of the second ORF.

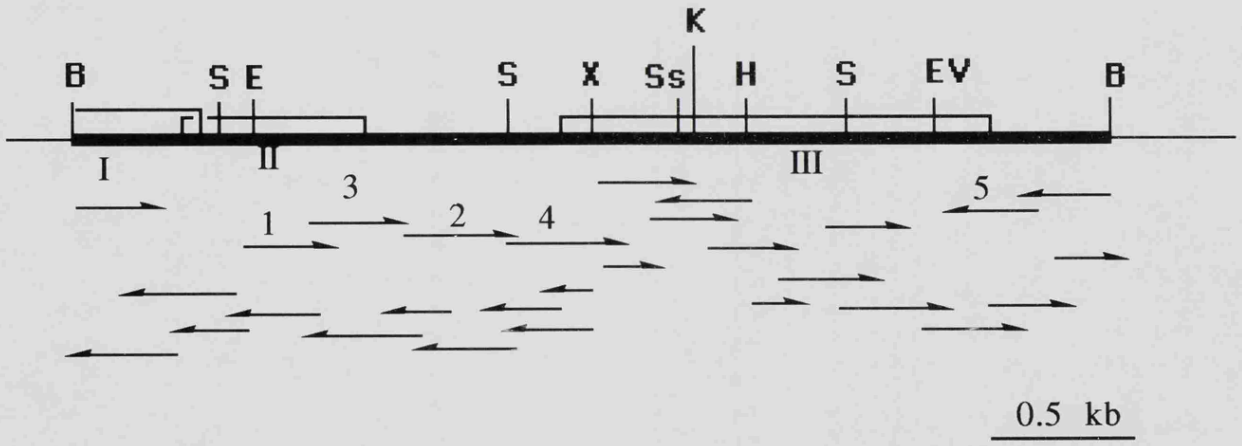


Figure 5.3a: Restriction map of the cloned gene, indicating regions sequenced using dideoxy chain termination method. Bar indicates ~0.5 kb.
B= *Bam*HI, S= *Sal*I, E= *Eco*RI, X= *Xba*I, Ss= *Sst*I, K= *Kpn*I, H= *Hin*dI, Ev= *Eco*RV
1, 2, 3, 4, 5= synthetic oligonucleotides were used as primers
I, II, III = open reading frames

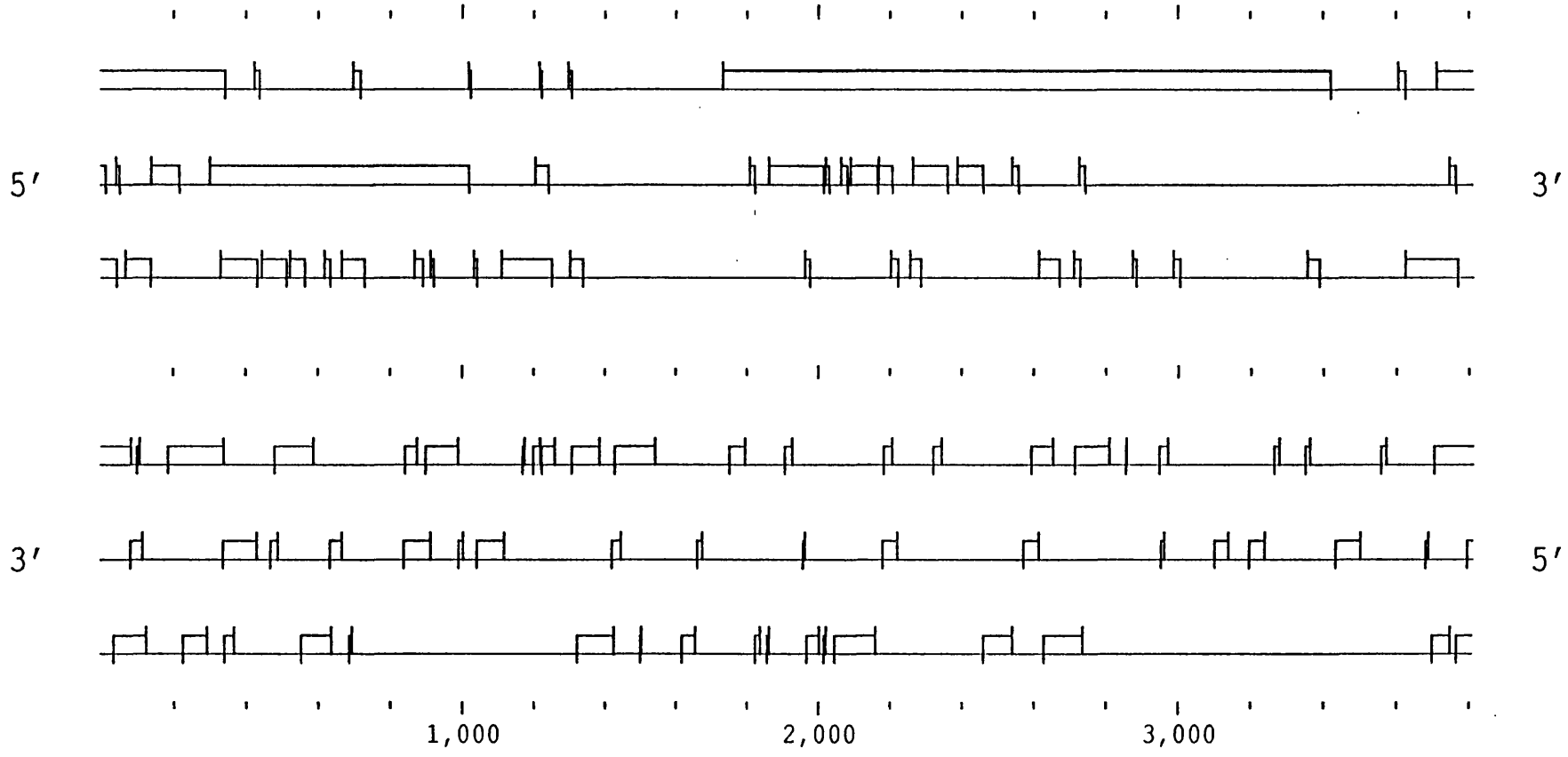


Figure 5.4: Open reading frames of the cloned sequence.
Analysis of the cloned sequence shows it to contain three open reading frames (ORFs). The first is approximately 110 amino acid residues, the second is 264 amino acid residues and the third is 563 amino acids residues.

The possibility of these three ORFs being in fact just one required investigation. The lack of mistakes in the sequence of the first ORF suggests that the first and the second ORFs are separate. In the yeast *S.cerevisiae*, a highly conserved consensus sequence, known as the TACTAAC box, is located at or near the 3'-end of an intron. This sequence is believed to be a splicing recognition signal identifying a TACTAAC box containing sequence as an intron. The absence of the TACTAAC box in the non-coding region flanking the second and the third ORFs indicates that this non-coding region is not an intron. The possible assignment of the gene product of the first and the second ORFs should prove an interesting topic for further investigation.

The results mentioned in chapter IV demonstrate that the 3.4 kb *Eco*RI-*Bam*HI fragment of *NAL* gene complements the temperature sensitive phenotype. As the *Eco*RI site is in the middle of the second ORF, removing the 0.5 kb *Bam*HI-*Eco*RI fragment would therefore disrupt this ORF. Nevertheless, the 3.3 kb *Eco*RI-*Bam*HI fragment did prove sufficient in complementing the temperature-sensitive phenotype, suggesting that those first and second ORFs are not the complementing genes.

The ORF of the *NAL* gene starts at approximately 1.6 kb from the 5'-end of the insert and extends for 1.7 kb.

5.5. Analysis of the *NAL* gene sequence

5.5.1. The upstream 5' non-coding region

The length of the non-translated region prior to the presumed initiation codon varies among organisms.

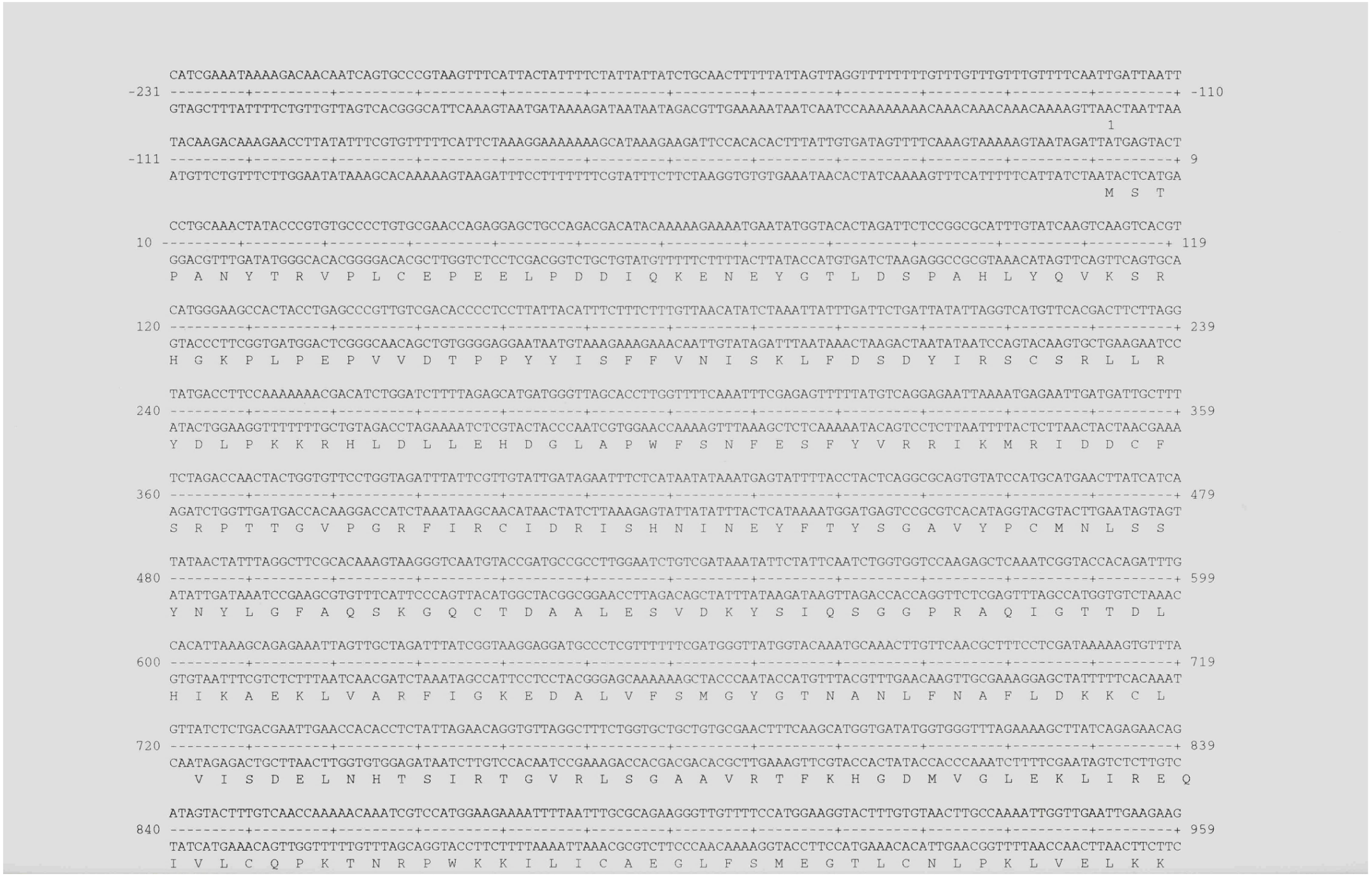
Kozak (1984) has suggested that the sequence environment of the translation initiation codon (AUG) modulates the efficiency with which

the 40S ribosomal subunit binds to the 5'-end of the message. The selection of the correct AUG is thought to depend, to some degree, on the sequence around the AUG. It is known in 205 out of 211 cases that a consensus for the AUG environment is highly conserved, in which a purine at -3 is usually represented by an adenine (79%) (Ballance, 1986).

Dobson *et al.*, (1982), have shown the same pattern to be present in yeast. Furthermore, they noticed that eleven of the seventeen compared have a purine at +4, mostly G, although it was suggested that efficient translation of the gene does not depend on purine at this position. It has subsequently been established that the presence of a pyrimidine, usually U, at position +6, is even more important than that of purine at +4. Dobson *et al.*, (1982) found the hexanucleotide CACACA to be close to the initiation codon in several (8/17) yeast genes, although they did not find any obvious correlation between the abundance of a gene product and the presence of this sequence.

In eukaryotes, at least two other features are believed to be important for transcription initiation. The first of these sequences is an AT-rich region, named the TATA box (Gannon *et al.*, 1979). In the yeast *Saccharomyces cerevisiae*, it is known that the TATA box location varies from 50 to 200 bases upstream of the translation initiation site, ATG, (Darnell, 1989).

The second feature is the CAAT box with the canonical sequence GC(C/T)CAATCT (Benoist *et al.*, 1980). The sequence has been found in several eukaryotic genes at about 80 basepairs upstream of transcription initiation site. Although the function of this element is not well defined, it is nevertheless thought to be involved as a recognition sequence for transcriptional factors (Kingsman *et al.*, 1983). A sequence related to the CAAT box has also been described in


CATCGAAATAAAAGACAACAATCAGTGCCCGTAAGTTTCATTACTATTTTCTATTATTATCTGCAACTTTTTATTAGTTAGGTTTTTTTTGTTTGTTTGTTTGTTTTCAATTGATTAATT
-231 -110
GTAGCTTTATTTTCTGTTGTTAGTCACGGGCATTCAAAGTAATGATAAAAGATAATAATAGACGTTGAAAAATAATCAATCCAAAAAAAACAAACAAACAAACAAAAGTTAACTAATTAA
1
TACAAGACAAAGAACCTTATATTTCGTGTTTTTCATTCTAAAGGAAAAAAAGCATAAAGAAGATTCCACACACTTTATTGTGATAGTTTTCAAAGTAAAAAGTAATAGATTATGAGTACT
-111 9
ATGTTCTGTTTCTTGGAATATAAAGCACAAAAAGTAAGATTTCCTTTTTTTTCGTATTTCTTCTAAGGTGTGTGAAATAACACTATCAAAAGTTTCATTTTTCATTATCTAATACTCATGA
M S T
CCTGCAAACTATACCCGTGTGCCCCTGTGCGAACCAGAGGAGCTGCCAGACGACATACAAAAAGAAAATGAATATGGTACACTAGATTCTCCGGCGCATTTGTATCAAGTCAAGTCACGT
10 119
GGACGTTTGATATGGGCACACGGGGACACGCTTGGTCTCCTCGACGGTCTGCTGTATGTTTTTCTTTTACTTATACCATGTGATCTAAGAGGCCGCGTAAACATAGTTCAGTTCAGTGCA
P A N Y T R V P L C E P E E L P D D I Q K E N E Y G T L D S P A H L Y Q V K S R
CATGGGAAGCCACTACCTGAGCCCGTTGTCGACACCCCTCCTTATTACATTTCTTTCTTTGTTAACATATCTAAATTATTTGATTCTGATTATATTAGGTCATGTTCACGACTTCTTAGG
120 239
GTACCCTTCGGTGATGGACTCGGGCAACAGCTGTGGGGAGGAATAATGTAAAGAAAGAAACAATTGTATAGATTTAATAAACTAAGACTAATATAATCCAGTACAAGTGCTGAAGAATCC
H G K P L P E P V V D T P P Y Y I S F F V N I S K L F D S D Y I R S C S R L L R
TATGACCTTCCAAAAAAACGACATCTGGATCTTTTAGAGCATGATGGGTTAGCACCTTGGTTTTCAAATTTCGAGAGTTTTTATGTCAGGAGAATTAAAATGAGAATTGATGATTGCTTT
240 359
ATACTGGAAGGTTTTTTTTGCTGTAGACCTAGAAAATCTCGTACTACCCAATCGTGGAACCAAAAGTTTAAAGCTCTCAAAAATACAGTCCTCTTAATTTTACTCTTAACTACTAACGAAA
Y D L P K K R H L D L L E H D G L A P W F S N F E S F Y V R R I K M R I D D C F
TCTAGACCAACTACTGGTGTTCCTGGTAGATTTATTCGTTGTATTGATAGAATTTCTCATAATATAAATGAGTATTTTACCTACTCAGGCGCAGTGTATCCATGCATGAACTTATCATCA
360 479
AGATCTGGTTGATGACCACAAGGACCATCTAAATAAGCAACATAACTATCTTAAAGAGTATTATATTTACTCATAAAATGGATGAGTCCGCGTCACATAGGTACGTACTTGAATAGTAGT
S R P T T G V P G R F I R C I D R I S H N I N E Y F T Y S G A V Y P C M N L S S
TATAACTATTTAGGCTTCGCACAAAGTAAGGGTCAATGTACCGATGCCGCCTTGGAATCTGTCGATAAATATTCTATTCAATCTGGTGGTCCAAGAGCTCAAATCGGTACCACAGATTTG
480 599
ATATTGATAAATCCGAAGCGTGTTTCATTCCCAGTTACATGGCTACGGCGGAACCTTAGACAGCTATTTATAAGATAAGTTAGACCACCAGGTTCTCGAGTTTAGCCATGGTGTCTAAAC
Y N Y L G F A Q S K G Q C T D A A L E S V D K Y S I Q S G G P R A Q I G T T D L
CACATTAAAGCAGAGAAATTAGTTGCTAGATTTATCGGTAAGGAGGATGCCCTCGTTTTTTCGATGGGTTATGGTACAAATGCAAACTTGTTCAACGCTTTCCTCGATAAAAAGTGTTTA
600 719
GTGTAATTTCGTCTCTTTAATCAACGATCTAAATAGCCATTCCTCCTACGGGAGCAAAAAAGCTACCCAATACCATGTTTACGTTTGAACAAGTTGCGAAAGGAGCTATTTTTCACAAAT
H I K A E K L V A R F I G K E D A L V F S M G Y G T N A N L F N A F L D K K C L
GTTATCTCTGACGAATTGAACCACACCTCTATTAGAACAGGTGTTAGGCTTTCTGGTGCTGCTGTGCGAACTTTCAAGCATGGTGATATGGTGGGTTTAGAAAAGCTTATCAGAGAACAG
720 839
CAATAGAGACTGCTTAACTTGGTGTGGAGATAATCTTGTCCACAATCCGAAAGACCACGACGACACGCTTGAAAGTTCGTACCACTATACCACCCAAATCTTTTCGAATAGTCTCTTGTC
V I S D E L N H T S I R T G V R L S G A A V R T F K H G D M V G L E K L I R E Q
ATAGTACTTTGTCAACCAAAAACAAATCGTCCATGGAAGAAAATTTTAATTTGCGCAGAAGGGTTGTTTTCCATGGAAGGTACTTTGTGTAACTTGCCAAAATTGGTTGAATTGAAGAAG
840 959
TATCATGAAACAGTTGGTTTTTGTTTAGCAGGTACCTTCTTTTAAAATTAAACGCGTCTTCCCAACAAAAGGTACCTTCCATGAAACACATTGAACGGTTTTAACCAACTTAACTTCTTC
I V L C Q P K T N R P W K K I L I C A E G L F S M E G T L C N L P K L V E L K K

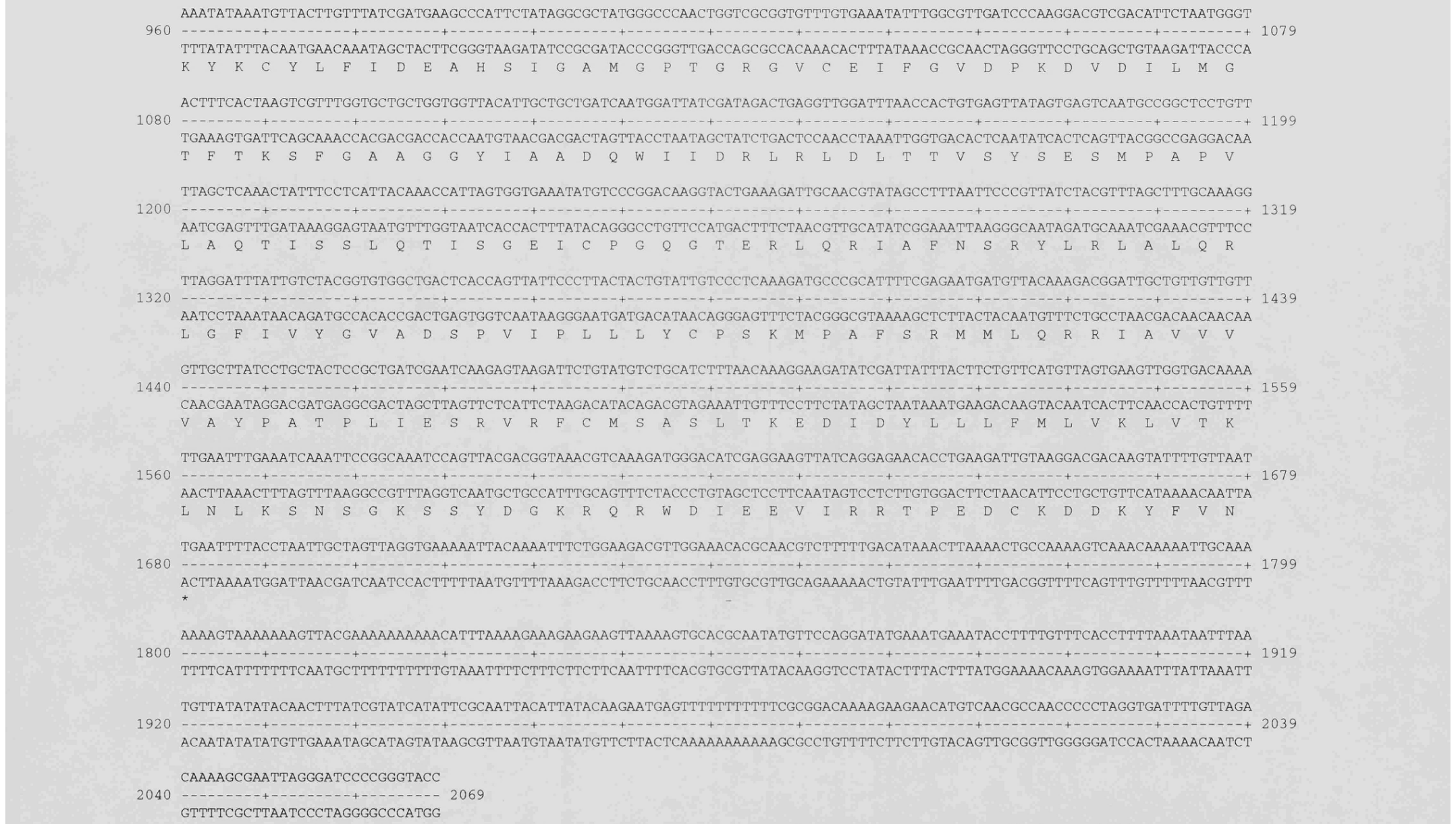


Figure 5.5: The complete sequence of the *NAL* gene

the yeast *S.cerevisiae* (Ballance, 1986). Additionaly, Dobson *et al.*, (1982) described a high efficiency promoter sequence for highly expressed yeast genes containing a block of residues rich in CT followed by CAAG at approximately 10 bases further along.

The complete *NAL* gene including the 5'- and 3'- non-coding region is described in figure 5.5. The adenine residue of the first ATG codon is designated as position +1. An adenine residue is present upstream of the initiation site at position -3, which is common in many yeast genes. Another purine residue is also present at position +4 as is the case with many yeast genes. Furthermore, a pyrimidine (T) residue can be found at position +6 which is thought to have an even more important role than that of purine at position +4 as previously mentioned.

A possible putative TATA box is found at position -93 (TATATTT). There is also an almost perfect CAAT box at -123. The hexanucleotide CACACA, as mentioned above, occurs at position -44. The function of this feature has yet to be defined. Despite those features commonly found in many yeast genes, the *NAL* gene lacks the feature described as a high efficiency promoter sequence which has been reported by Dobson *et al.*, (1982)

5.5.2. Open reading frame: codon usage in the *NAL* gene

The codon bias in the yeast *Saccharomyces cerevisiae* has been described by Bennetzen & Hall, (1982). They compared seven highly expressed genes in this yeast. Among their results, three points are of relevance here:

1) in amino acids having 3 or 4-fold coding degeneracy [XY(U/A/C/G)], the codons XYU and XYC are used with roughly equal probability, with the exception of glycine and proline;

Table 5.5.1: Codon usage of the *NAL* gene.

Underlined codons are codons that are rarely used by highly expressed gene but were used by the *NAL* gene.

Aa= Amino acid

Number= represent the number of amino acids encoded by corresponding codon

Fraction= procentage of the particular amino acids in the Nal protein

Table 5.5.1: Codon usage of the *NAL* gene.

Aa	Codon	Number	/1000	Fraction	Aa	Codon	Number	/1000	Fraction
Gly	GGG	5.00	7.69	0.21	Met	ATG	8.00	12.31	1.00
Gly	GGA	12.00	18.46	0.50	Ile	ATA	6.00	9.23	0.18
Gly	GGT	4.00	6.15	0.17	Ile	ATT	18.00	27.69	0.53
Gly	GGC	3.00	4.62	0.13	Ile	ATC	10.00	15.38	0.29
Glu	GAG	8.00	12.31	0.38	Thr	ACG	7.00	10.77	0.20
Glu	GAA	13.00	20.00	0.62	Thr	ACA	10.00	15.38	0.29
Asp	GAT	8.00	12.31	0.80	Thr	ACT	10.00	15.38	0.29
Asp	GAC	2.00	3.08	0.20	Thr	ACC	8.00	12.31	0.23
Val	GTG	3.00	4.62	0.10	Trp	TGG	16.00	24.62	1.00
Val	GTA	7.00	10.77	0.23	End	TGA	24.00	36.92	0.41
Val	GTT	15.00	23.08	0.50	Cys	TGT	21.00	32.31	0.58
Val	GTC	5.00	7.69	0.17	Cys	TGC	15.00	23.08	0.42
Ala	GCG	1.00	1.54	0.05	End	TAG	13.00	20.00	0.22
Ala	GCA	7.00	10.77	0.35	End	TAA	21.00	32.31	0.36
Ala	GCT	4.00	6.15	0.20	Tyr	TAT	25.00	38.46	0.66
Ala	GCC	8.00	12.31	0.40	Tyr	TAC	13.00	20.00	0.34
Arg	AGG	10.00	15.38	0.18	Leu	TTG	13.00	20.00	0.25
Arg	AGA	12.00	18.46	0.21	Leu	TTA	17.00	26.15	0.32
Ser	AGT	10.00	15.38	0.18	Phe	TTT	34.00	52.31	0.67
Ser	AGC	8.00	12.31	0.14	Phe	TTC	17.00	26.15	0.33
Lys	AAG	13.00	20.00	0.36	Ser	TCG	5.00	7.69	0.09
Lys	AAA	23.00	35.38	0.64	Ser	TCA	12.00	18.46	0.21
Asn	AAT	14.00	21.54	0.58	Ser	TCT	7.00	10.77	0.12
Asn	AAC	10.00	15.38	0.42	Ser	TCC	15.00	23.08	0.26
Arg	CGG	7.00	10.77	0.12	Gln	CAG	4.00	6.15	0.33
Arg	CGA	16.00	24.62	0.28	Gln	CAA	8.00	12.31	0.67
Arg	CGT	5.00	7.69	0.09	His	CAT	12.00	18.46	0.67
Arg	CGC	7.00	10.77	0.12	His	CAC	6.00	9.23	0.33
Leu	CTG	1.00	1.54	0.02	Pro	CCG	2.00	3.08	0.17
Leu	CTA	6.00	9.23	0.11	Pro	CCA	3.00	4.62	0.25
Leu	CTT	10.00	15.38	0.19	Pro	CCT	5.00	7.69	0.42
Leu	CTC	6.00	9.23	0.11	Pro	CCC	2.00	3.08	0.17

2) whereas XYC is used in 2-fold degenerate codons with a pyrimidine in the wobble position, XYU is not, with the exception of cysteine.

3) glycine and proline use respectively GGU and CCA as the predominant codon choices.

The "codonfrequency" programme of the molecular biology computer package is capable of comparing the codon used by a particular yeast gene to those codons used by twenty other highly expressed yeast genes (Devereaux *et al.*, 1984)

The table of codon usage of the *NAL* gene is shown in table 5.5.1. Certain unusual codons that are not predominantly, or very rarely, used in the yeast *S.cerevisiae* gene are utilised in the *NAL* gene. Among these are GAU encoding aspartic acid, CUC encoding leucine, CUG encoding glutamine CGA encoding arginine.

The use of other codons appears to be much like the other codon usage of the yeast genes in the 'codonfrequency' table.

5.5.3. The 3' non-coding region

Many eukaryotic mRNA have a poly(A) tail added on to their 3' ends. The mature mRNA is performed by the post-transcriptional processing of a longer precursor transcript. The capping of the 5' end, removal of non-coding intron sequences and the formation of a proper 3' end, such as the addition of a poly(A) tail, are crucial steps in mRNA maturation.

Mammalian poly(A) signals have been well defined. These contain two elements, the first of which is the AAUAAA sequence, located 10-30 nucleotides upstream of polyadenylation site (Fiztgerald & Shenk, 1981; Proudfoot, 1991). Mutagenesis experiments showed that this hexanucleotide is essential both for processing and poly(A) addition

(Wickens & Stephenson, 1984). The second element is a diffuse GU-rich sequence immediately after the 3'end of the mRNA. Both sequences are nearly always found at the end of mammalian mRNA. Most yeast (*S.cerevisiae*) DNA genes, however, lacks the AAUAAA processing signal. Zaret and Sherman (1982) found that a *cyc1* mutant defective in the normal *CYC1* 3'end formation lacked a 38 bp DNA region containing the tripartite sequence TAG...TA(T)GT...TTT. This sequence motif has been found near the poly(A) addition site of many yeast genes, and has been suggested by Zaret & Sherman (1982) to be a signal sequence involved in mRNA 3' end processing in yeast. Irniger *et al.,* (1991) found this signal to be weak in yeast *S.cerevisiae*. Site-directed mutagenesis of this sequence nevertheless reduced 3' end formation. They also found that polyadenylation sites contain the sequence element TTTTTAT. Besides this sequence motif, the polyadenylation sites have sequences which are highly AT rich and stretch out for longer than 10 bp at or near the mRNA.

The *NAL* gene lacks the TTTTTAT motif sequence. However, it does have the tripartite sequence TAG..TAGGT...TTT at a position starting from 11 bp downstream the first stop codon. A highly AT rich region is equally present, stretching for a few hundred basepairs, starting from about 60 bp downstream the first stop codon.

5.6. Comparing the *NAL* gene sequence to the DNA data bank

The *NAL* gene sequence was compared to the DNA data bank through the VAXcluster computer system. No identity to any gene in the DNA data bank was present. This shows that the *NAL* gene is novel in *S.cerevisiae*. These results also proved this gene to differ

Figure 6.6: Lineup of the Nal protein and its homology proteins

Kbl$Ecoli is KBL protein of *E.coli,* Hem1$Mous, Hem1$Rat, Hem1$Chic, Hem1$Huma and Hem1$Braja are δ-ALA synthetase of mouse, rat, chicken, human and *B.japonicum,* respectively. Nal is the Nal protein. Cons is a consensus sequence of proteins listed above.

Sequences underlined are amino acids which are conserved among those proteins.

The upper case symbols, in the consensus row, indicate that the particular amino acids are exactly conserved whereas the lower case indicate that those particular amino acid are the prefered amino acids among the protein listed.

Figure 6.6: Line up of the Nal protein and its homology proteins

```
          0                                                                                                  99
Kbl$Ecoli  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Hem1$Mous  .......... .......... .......... .......... .......... .......WFV LSAGQQDFGL RMVAAAMLLW SCPVLSQGPT GLLGKVAKTY
 Hem1$Rat  METVVRRCPF LSRVPQAFLQ KAGKSLLFYA QNCPKMMEVG AKPAPRTVST SAAQCQQVKE TPPANEKEKT AKAAVQQAPD ESQMAQTPDG TQLPPGHPSP
Hem1$Chic  .......MEA VVRRCPFLAR VSQAFLQKAG PSLLFYAQHC PKMMEAAPPA AARGLATSAS RGQQVEETPA AQPEAKKAKE VAQQNTDGSQ PPAGHPPAAA
Hem1$Huma  MDSVVGRCPF LSRVPQAFLQ KAGKSLLFYA QNCPKMMEVG AKQPSRIVHC SSTLPQDQET PPASEKDQTA KAKVQQTLMD PSRVQMAHSF RLDSVWTPLA
Hem1$Braj  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      Nal  .......... .......... .......... .......... .......... .......... .......... .......... .........M STPANYTRVP
     Cons  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          100                                                                                                199
Kbl$Ecoli  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Hem1$Mous  QFLFSIGRCP ILATQGPTCS QIHLKATKAG GELQDRKSKI VQRAAPEVQE DVKTFKTDLL STMDSTTRSH SFPSFQEPEQ TEGAVPHLIQ NNMTGSQAFG
 Hem1$Rat  STSQSSGSKC PFLAAQLARR AAASSARPVW SFRRTWQEMH AVRTEVAQSP VLPSLVNAKR DGEGPSPLLK NFQDIMRKQR PERVSHLLQD NLPKVVSTFQ
Hem1$Chic  VQSSATKCPF LAAQMNHKSS NVFCKASLEL QEDVKEMQVD RKGKEFAKIP TNSVVRNTEA EGEEQSGLLK KFKDIMLKQR PESVSHLLQD NLPKSVSTFQ
Hem1$Huma  ATSQGTASKC PFLAAQMIRE AAVSSAKPVL SFRRDVQEMN AVKKEGAETS AGPSVVSVKT DGGDPSGLLK NVQDIMQKQR PERVSHLLHD NLPKSVSTFH
Hem1$Braj  .......... .......... .......... .......... .......... .......... .......... .......... ..........
      Nal  LCEPEELPDD IQKENEYGTL DSPAHLYQVK SRHGKPLPEP VVDTPPYYIS FFVNISKLFD SDYIRSCSRL LRYDLPKKRH LDLLEHDGLA PWFSNFESFY
     Cons  ..s....... .......... .....a..v. s......... .v..e.a... .......... .g...s.llk .f.dim.kqr pe.vshll.d nlpk.vstf.

          200                                                                                                299
Kbl$Ecoli  ...MRGEFYQ QLTNDLETAR AEGLFKEERI ITSAQQADIT VADGSQVINF CANNYLGLAN HPD..LIAAA KAGMDSHGFG MASVRFICGT QDSHKELEQK
Hem1$Mous  YDQFFRDKIM EKKQDHTYRV FKTVNRWANA YPFAQHFSEA SMASKDVSVW CSNDYLGISR HPRV..LQAI EETLKNHGAG AGGTRNISGT SKFHVELEQE
 Hem1$Rat  YDHFFEKKID EKKNDHTYRV FKTVNRRAQI FPMADDYTDS LITNNQVSVW SSNDYLGMSR HPRV..CGAV IETVKQHGAG AGGTRNISGT SKFHVELEQE
Hem1$Chic  YDQFFEKKID EKKKDHTYRV FKTVNRKAQI FPMADDYSDS LITKKEVSVW CSNDYLGMSR HPRV..CGAV MDTLKQHGAG AGGTRNISGT SKFHVDLEKE
Hem1$Huma  YDRFFEKKSD EKNDDHTYRV FKTVNRRAHI FPMADDYSDS LITKKQVSVW CSNDYLGMSR HPRV..CGAV MDTLKQHGAG AGGTRNISGT SKFHVDLERE
Hem1$Braj  MDYSQFFNSA LDRLHTERRY RVFADLERMA GRFPHAIWHS PKGKRDVVIW CSNDYLGMGQ HPKV..VGAM VETATRVGTG AGGTRNIAGT HHPLVQLEAE
      Nal  VRRIKMRIDD CFSRPTTGVP GRFIRCIDRI SHNINEYFTY SGAVYPCMNL SSYNYLGFAQ SKGQCTDAAL ESVDKYSIQS GGPRAQI.GT TDLHIKAEKL
     Cons  yd.ffekkid ekkndhtyrv fktvnr.a.i fpmaddysds litkkqvsvw csndYLGmsr hprv..cgAv .etlkqhgag aggtrnIsGT skfhvelEqe
```

```
          300                                                                                                  399
Kbl$Ecoli LAAFLGMEDA ILYSSCFDAN GGLFETL..L GAEDAIISDA LNHASIIDGV RLCKAKRYRY ANNDMQELEA RLKEARERGA R.......HV LI.ATDGLFS
Hem1$Mous LAELHQKDSA LLFSSCFVAN DSTLFTLAKL LPGCEIYSDA GNHASMIQGI RNSGAAKFVF RHNDPGHLKK LLEKSDPKTP K......... .IVAFETVHS
 Hem1$Rat LADLHGKDAA LLFSSCFVAN DSTLFTLAKM MPGCEIYSDS GNHASMIQGI RNSRVPKYIF RHNDVNHLRE LLQRSDPSVP K......... .IVAFETVHS
Hem1$Chic LADLHGKDAA LLFSSCFVAN DSTLFTLAKM LPGCEIYSDS GNHASMIQGI RNSRVPKHIF RHNDVNHLRE LLKKSDPSTP K......... .IVAFETVHS
Hem1$Huma LADLHGKDAA LLFSSCFVAH DSTLFTLVKM MPGCEIYSDS GDHASMIQGI RNSRVPKYIF RHNDVSHLRE LLQRSDPSVP K......... .IVAFETVHS
Hem1$Braj LADLHGKEAA LLFTSGYVSN QTGIATIAKL IPNCLILSDE LNHNSMIEGI RQSGCERQVF RHNDLADLEA LLKAAGANRP K......... .LIACESLYS
      Nal VARFIGKEDA LVF.SMGYGT NANL.FNAFL DKKCLVISDE LNHTSIRTGV RLSGAAVRTF KHGDMVGLEK LIREQIVLCQ PKTNRPWKKI .LICAEGLFS
     Cons lAdlhgkdaA llfsScfvan dstlftlakl .pgceiySDs gnHaSmiqGi Rns..pkyif rhnDvnhL.e llk.sdps.p k......... .ivafetvhS

          400                                                                                                  499
Kbl$Ecoli MDGVIANLKG VCDLADKYDA LVMVDDSHAV GFVGENGRGS HEYC...... .....DVMGR VDIITGTLGK ALGGASGGYT AARKEVVEWL RQRSRPYLFS
Hem1$Mous MDGAICPLEE LCDVAHQYGA LTFVDEVHAV GLYGAWGAGI GERD...... ....GIM.HK LDIISGTLGK AFGCV.GGYI ASTWDLVDMV RSYAAGFIFT
 Hem1$Rat MDGAVCPLEE LCDVAHEFGA ITFVDEVHAV GLYGASGGGI GDRD...... ....GVM.PK MDIISGTLGK AFGCV.GGYI ASTSLLIDTV RSYAAGFIFT
Hem1$Chic MDGAVCPLEE LCDVAHEHGA ITFVDEVHAV GLYGARGGGI GDRD...... ....GVM.HK MDIISGTLGK AFACV.GGYI SSTSALIDTV RSYAAGFIFT
Hem1$Huma VDGAVLPLEE LCDVAHEFGA ITFVDEVHAW GLYGARGGGI GDRD...... ....GVM.PK MDIISGTLGK AFGCV.GGYI ASTRSLMDTV RSYAAGFIFT
Hem1$Braj MDGDVAPLAK ICDLAEKYNA MTYVDEVHAV GMYGPRGGGI AERD...... ....GVM.HR IDILEGTLAK AFGCL.GGYI AANGRIIDAV RSYAPGFIFT
      Nal MEGTLCNLPK LVELKKKYKC YLFIDEAHSI GAMGPTGRGV CEIF...... ....GVDPKD VDILMGTFTK SFGAA.GGYI AADQWIIDRL RLDLTTVSYS
     Cons mdGavcpLee lcdvah.yga itfvDevHav GlyGarGgGi gerd...... ....gvm.hk mDIisGTlgK afgcv.GGYi asts.lidtv Rsyaagfift

          500                                                                                                  599
Kbl$Ecoli NSLAPAIVAA SIKVLEMVEA GSELRDRLWA NARQFREQMS AAGFTLAGAD HAIIPVMLGD AVVAQKFARE LQKEGIYVTG FFYPVVPKGQ ARIRTQMSAA
Hem1$Mous TSLPPMMLSG ALESVRLLKG EEGQALRWAH QRNVKHMRQL LMDRGFPVIP CPSHIIPIRV GNAALNSKIC DLLLSKHSIY VQAINYPTVP RGEELLRLAP
 Hem1$Rat TSLPPMLLAG ALESVRILKS NEGRALRRQH QRNVKLMRQM LMDAGLPVIH CPSHIIPVRV ADAAKNTEIC DELMTRHNIY VQAINYPTVP RGEELLRIAP
Hem1$Chic TSLPPMLLAG ALESVRTLKS AEGQVLRRQH QRNVKLMRQM LMDAGLPVVH CPSHIIPIRV ADAAKNTEIC DKLMSQHSIY VQAINYPTVP RGEELLRIAP
Hem1$Huma TSLPPMLLAG ALESVRILKS AEGRVVRRQH QRNVKLMRQM LMDAGLPVVH CPSHIIPVRV ADAAKNTEVC DELMSRHNIY VQAINYPTVP RGEELLRIAP
Hem1$Braj TALPPAICSA ATAAIKHLKT SSWERERHQD RAARVKAILN AAGLPVMSSD THIVPLFIGD AEKCKQASDL LLEEHGIYIQ PINYPTVAKG SERLRITPSP
      Nal ESM.PAPVLA QTISSLQTIS GEICPGQGTE RLQRIAFNSR YLRLALQRLG FIVYGVADSP VIPLLLYCPS KMPAFSRMML QRRIAVVVVA YPATPLIESR
     Cons tslpPmllag alesvrilks .eg..lrrqh qrnvklmrqm lmdaglpv.h cpshiipirv adaaknteic d.lmsrh.iy vqainyptvp rgeellriap

          600
Kbl$Ecoli HTPEQITRAV EAFTRIGKQL GVIAERSYFG NMGPQYVTTY A
Hem1$Mous SPHHSPQMME NFVEKLLLAW TEVGLPLQDV SVAACNFCHR PVHFELMSEWE KAYFSGMSK MVSAQA
 Hem1$Rat TPHHTPQMMN YFLEKLLLTW KRVGLELKPH SSAECNFCRR PLHFEVMSERE RSYFSGMSK LLSVSA
Hem1$Chic TPHHTPQMMS YFLEKLLATW KDVGLELKPH SSAECNFCRR PLHFEVMSERE KSYFSGLSK LVSAKA
Hem1$Huma TPHHTPQMMN YFLENLLVTW KQVGLELKPH SSAECNFCRR PLHFEVMSER
Hem1$Braj YHDDGLIDQL AEALLQVWDR LGLPLKQKSL AAE
      Nal VRFCMSASLT KEDIDYLLLF MLVKLVTKLN LKSNSGKSSY DGKRQRWDIEE VIRRTPEDC KDDKYFVN
     Cons tphhtpqmmn yflekllltw k.vgLelkph ssaecnfcrr plhfevmserE.syfsgmsk lvsa.a.N
```

from the *PDR* or *STE6* genes, which have been implicated in multiple or pleiotropic drug resistance in the yeast *S.cerevisiae*.

5.7. Conclusion

The wild type *NAL*-containing clone has been sequenced using the double-stranded DNA sequencing method. The size of the *NAL* gene is about 1.8 kb, with an open reading frame containing 563 amino acids. The sequence of this gene displays many of the features common to eukaryotic genes, such as the CAAT, the TATA box, and the sequence enviroment for translational initiation. No identity to the sequence was found in the EMBL Database, showing that the *NAL* gene is novel. This also proved the *NAL* gene to be neither the *PDR* nor the *STE6* gene.

Chapter VI : Analysis of the Nal protein

6.1. Introduction

The molecular biology package program enables the translation of DNA sequences to polypeptide sequences. The predicted proteins can then be compared to the protein data bank to find any similarity or homology to existing proteins. Additionaly, certain physical properties can be predicted.

Further analysis of the Nal protein involves a determining of its location the cell. This objective can be achieved by raising antibodies against the protein to be used in immunofluorescence microscopy experiments.

This protein can be over-produced in *E.coli* or *yeast,* purified for raising antibodies or use in studying its biochemical properties. The *NAL* gene was subcloned in a pET3a vector (figure 6.1). The pET3a vector contains an *amp* gene for selection and a unique *Bam*HI site in the *tet* gene as a fusion cloning site.

6.2. Analysis of the predicted Nal protein

The *NAL* gene was translated into a polypeptide sequence using the molecular biology package program. The Nal protein consists of 563 amino acid residues with a molecular mass of approximately 63.5 kDa. This protein has a predicted isoelectric point at pH 8.46.

The predicted protein was compared to the protein data bank. The result showed the Nal protein to have a high homology both to β-amino-γ-ketobutyrate coenzyme A ligase (AKB ligase) produced by the *kbl* gene and to δ-aminolevulinic acid synthase (δ-ALA synthetase) produced by the *HEMA* gene from various organisms (figure 6.6).

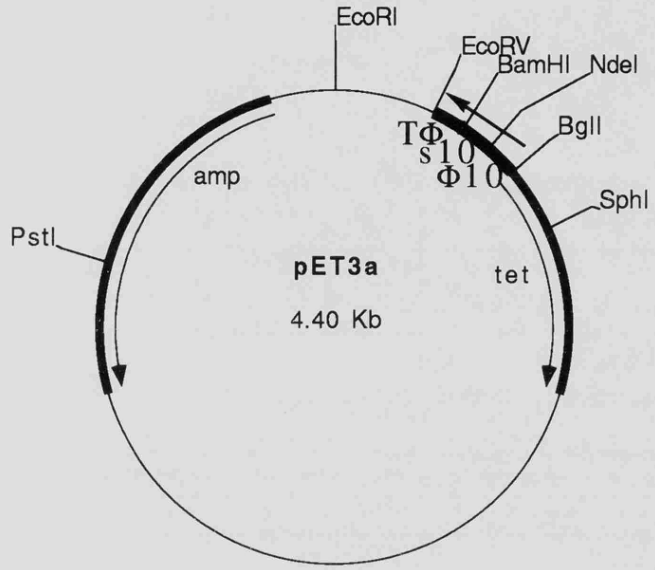


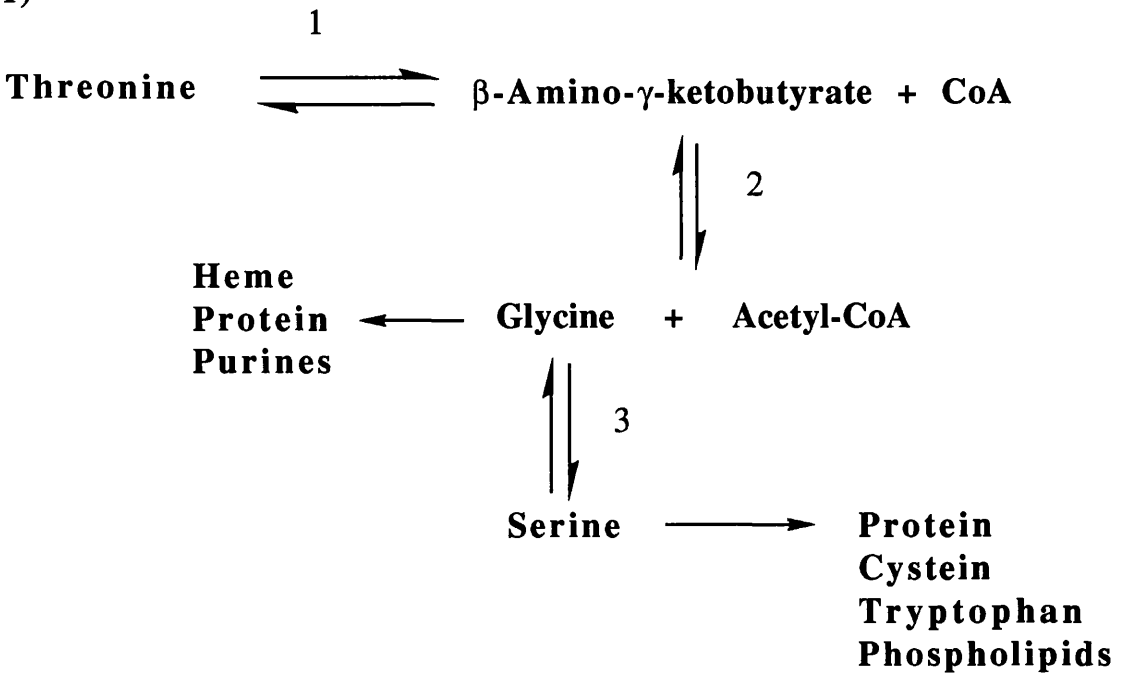
Figure 6.1: pET3a vector

The T7 promoter (Φ10), the gene *10* translation start site (s10) and transcription terminator (TΦ) were inserted in BamHI site of pBR322. The positions of the unique *Nde*I, *Bam*HI cloning sites of the vector. Transcription is controlled by the strong Φ*10* promoter for T7 RNA polyme rase

Figure 6.4: A) Threonine biosynthesis and degradation
This reaction is considered as the main pathway for threonine utilization (Tut cycle). Threonine is synthesized from glycine + acetyl-CoA and catalyzed by β-amino-γ-ketobutyrate CoA ligase producing β-amino-γ-ketobutyrate. This is then converted to threonine catalysed by the threonine dehydrogenase enzyme. The degradation of threonine is the reverse reaction above. The resulting glycine is synthesized to serine, catalysed by serine hydroxymethyl transferase enzyme.

B) δ-Aminolevulinic acid biosynthesis
δ-Aminolevulinic acid synthesis is the first step of heme biosynthesis. δ-Aminolevulinic acid synthetase (δ-ALA) catalyses the reaction of succinyl-CoA and glycine yielding δ-aminolevulinic acid, which is converted to heme through subsequent steps in the pathway.

A)

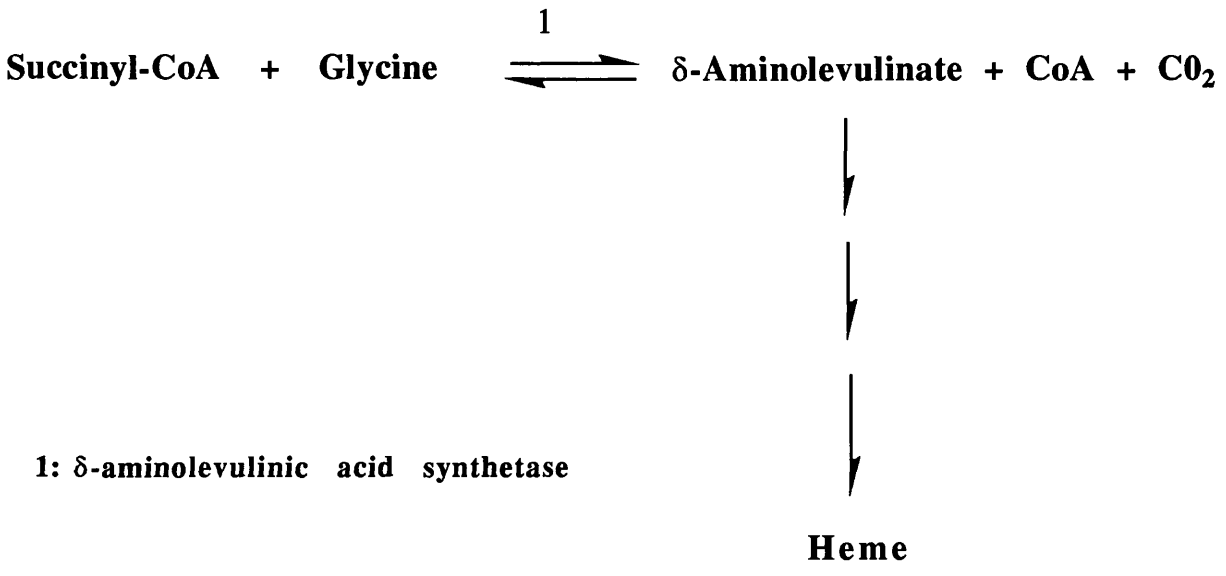


1: threonine dehydrogenase

2: β-amino-γ-ketobutyrate CoA ligase

3: serine hydroxymethyltransferase

B)



1: δ-aminolevulinic acid synthetase

Figure: 6.4

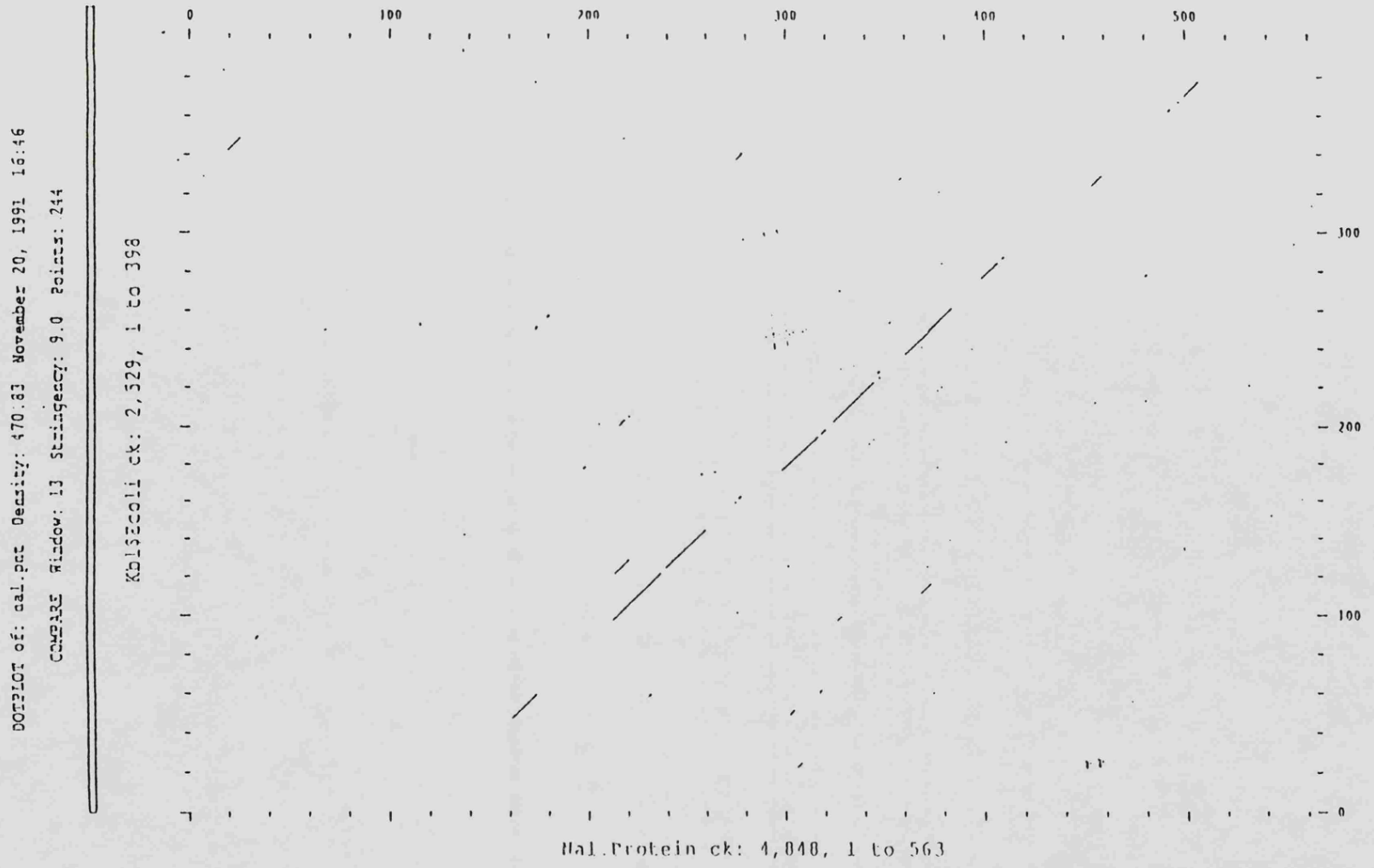


Figure 6.5.1: Comparison of the Nal protein and AKB ligase of *E.coli* using the dotplot program in the Genetics Computer Group computer suite. The straight diagonal lines show the homology between the two proteins

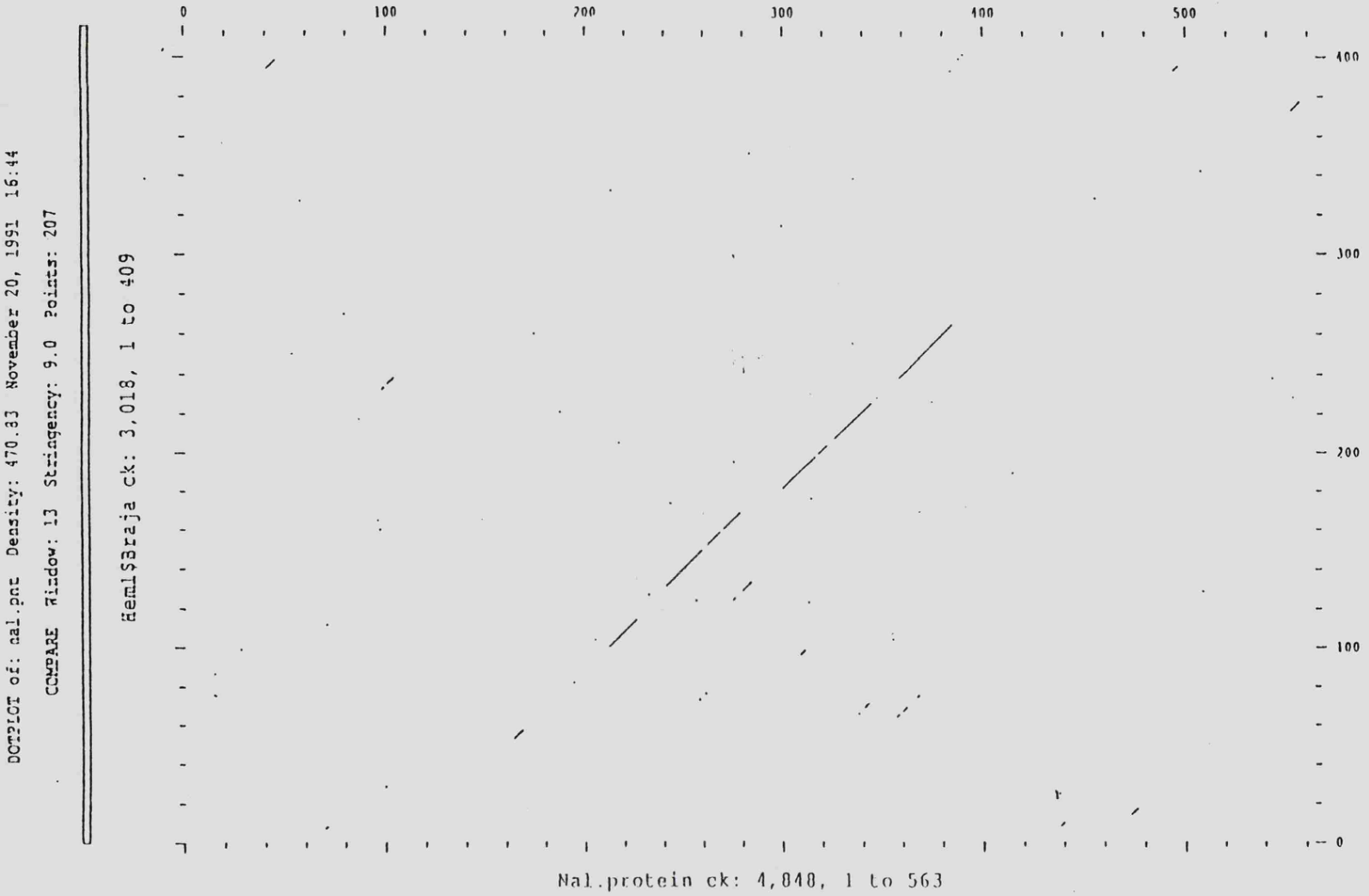


Figure 6.5.2: Comparison of the Nal protein and δ-ALA synthetase of *B.japonicum* using the dotplot program in the Genetics Computer Group computer suite. The straight diagonal lines show the homology between the two proteins

The *kbl* gene is found in both prokaryotes such as *E.coli* (Mukherjee, *et.al.*, 1987) and eukaryotes. The AKB ligase catalyses the reversible cleavage/condensation reaction between β-amino-γ-ketobutyrate and glycine + acetyl-CoA (figure 6.4). This reaction is the second step in the threonine dehydrogenase-initiated pathway, where threonine is converted to glycine and subsequently to serine via Tut cycle (Ravnikar and Somerville, 1987a, 1987b) (figure 6.4). This pathway has been shown to be the major route for metabolism and degradation of threonine in both eukaryotes (Dale, 1978) and prokaryotes (Komatsubara, *et.al.*, 1978; Boylan and Dekker, 1981). In eukaryotes, this enzyme is found in mitochondria. The only *kbl* gene sequence available in the data bank is that of *E.coli*, causing the Nal protein to recognise only that enzyme.

The δ-ALA synthetase is produced by the *HEMA* gene. This gene is also found in both prokaryotes and eukaryotes. In eukaryotes, the δ-ALA synthetase is found in mitochondria. This enzyme catalyses the synthesis of δ-aminolevulinate from glycine and succinyl-CoA (figure 6.4), a reaction which constitutes the first step of heme biosynthesis.

Comparison of the Nal protein and AKB ligase using a dotplot program in the GCG computer suite showed a straight diagonal line (figure 6.5.1). This dotplot graph confirmed the above finding, namely that some similarity to AKB ligase is one characteristic of the Nal protein. The same analysis was applied to the δ-ALA synthetase. The dotplot graph showed the Nal protein to be highly homologous to the δ-ALA synthetase, but not homologous to the extent of AKB ligase (figure 6.5.2). Application of the same method comparing the AKB ligase and δ-ALA synthetase shows the homology between the two enzymes to be very high (figure 6.5.3). This homology is most likely due to the similarity of their substrates. Both enzymes use glycine and

CoA as substrates and pyridoxal-phosphate as a cofactor. The high homology of the Nal protein to both enzymes suggests that it might share the same substrate.

The comparison of the Nal protein to the homologous proteins mentioned above, using the lineup program revealed three conserved regions (figure 6.6) the features of which include:

a) **YLG** residues (position 168 in the Nal protein) present in all the proteins compared;

b) **GGYIA** residues (position 374 in the Nal protein) present in all the δ-ALA enzymes compared, but **GGYTA** residues in AKB enzyme:

c) **SDELNH** residues at position 246 in the Nal protein exactly matched only those of δ-ALA enzyme of *B.japonicum*. These residues are also present in all the δ-ALA and AKB enzymes compared with a tolerance one to three mismatches.

Discovery of these features might well provide additional evidence supporting the assumption that those enzymes share the same or similar substrates.

6.3. Subcloning the *NAL* gene in the pET3a vector.

As there was no any suitable restriction site within the open reading frame to subclone the whole *NAL* gene, only part of it has been subcloned into the pET3a vector. The first step was to digest the pNUC182B, the orientation of which is as described in figure 4.8, with the restriction endonuclease *Xba*I. After running the fragments on an agarose gel, the *Xba*I-*Xba*I fragment of the *NAL* gene (1.2 kb) was isolated from the agarose gel by the "death wish" method. These fragments were then ligated to *Xba*I-digested pUC19 vector. The ligation mixture was transformed into DH5α *E.coli* competent cells

Figure 6.7: Subcloning of the *Xba*I-*Xba*I fragment of the *NAL* gene in pUC19

The pNUC192B was digested with the restriction endonuclease *Xba*I. The 1.6 kb fragment was isolated and ligated with the *Xba*I-digested pUC19. The ligation mixture was transformed into *E.coli* DH5α. Cells were plated on nutrient agar containing X-Gal 25μgml^{-1}, IPTG 25μgml^{-1} and ampicillin 100 μgml^{-1} and incubated at 37°C overnight. The white colonies were picked up and examined for recombinant plasmids. The correct recombinant plasmid is designated pNUC192X

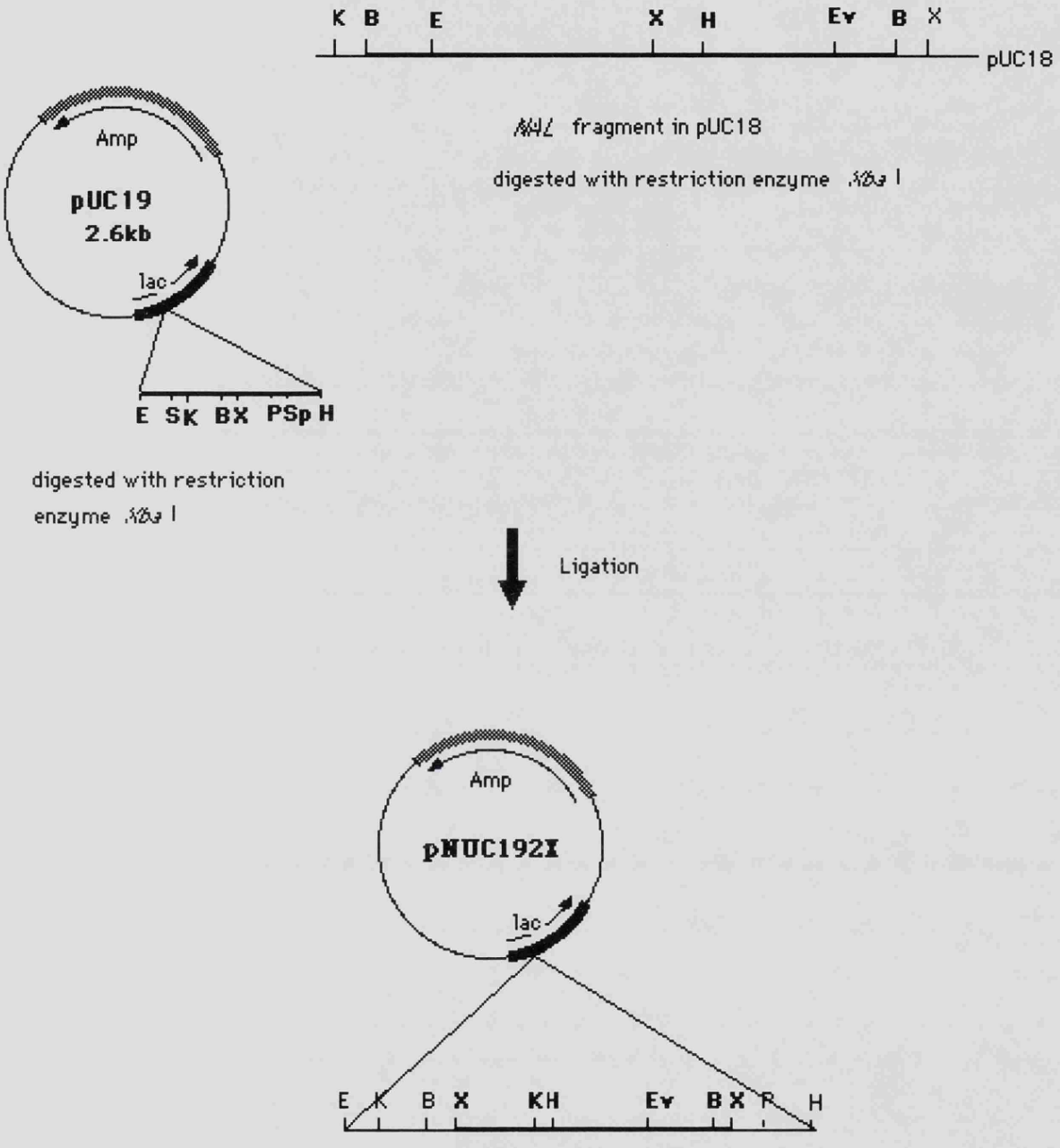

K B E X H Ev B X
pUC18
fragment in pUC18
digested with restriction enzyme
Amp
pUC19
2.6kb
lac
E SK BX PSp H
digested with restriction
enzyme
Ligation
Amp
pNUC192X
lac
E K B X KH Ev B X P H

Figure: 6.7

Figure 6.8: Subcloning the *Bam*HI-*Xba*I of the *NAL* gene into an expression vector.

pNUC192X was digested with the restriction endonuclease *Bam*HI. The 1.6 kb fragment was isolated and ligated with dephosphorylated *Bam*HI-digested pET3a vector. The ligation mixture was transformed into *E.coli* BL21. The transformants were analyzed for recombinant plasmids. The correct recombinant plasmid designated pET3a::NAL2 was purify for over-producing for fusion protein.

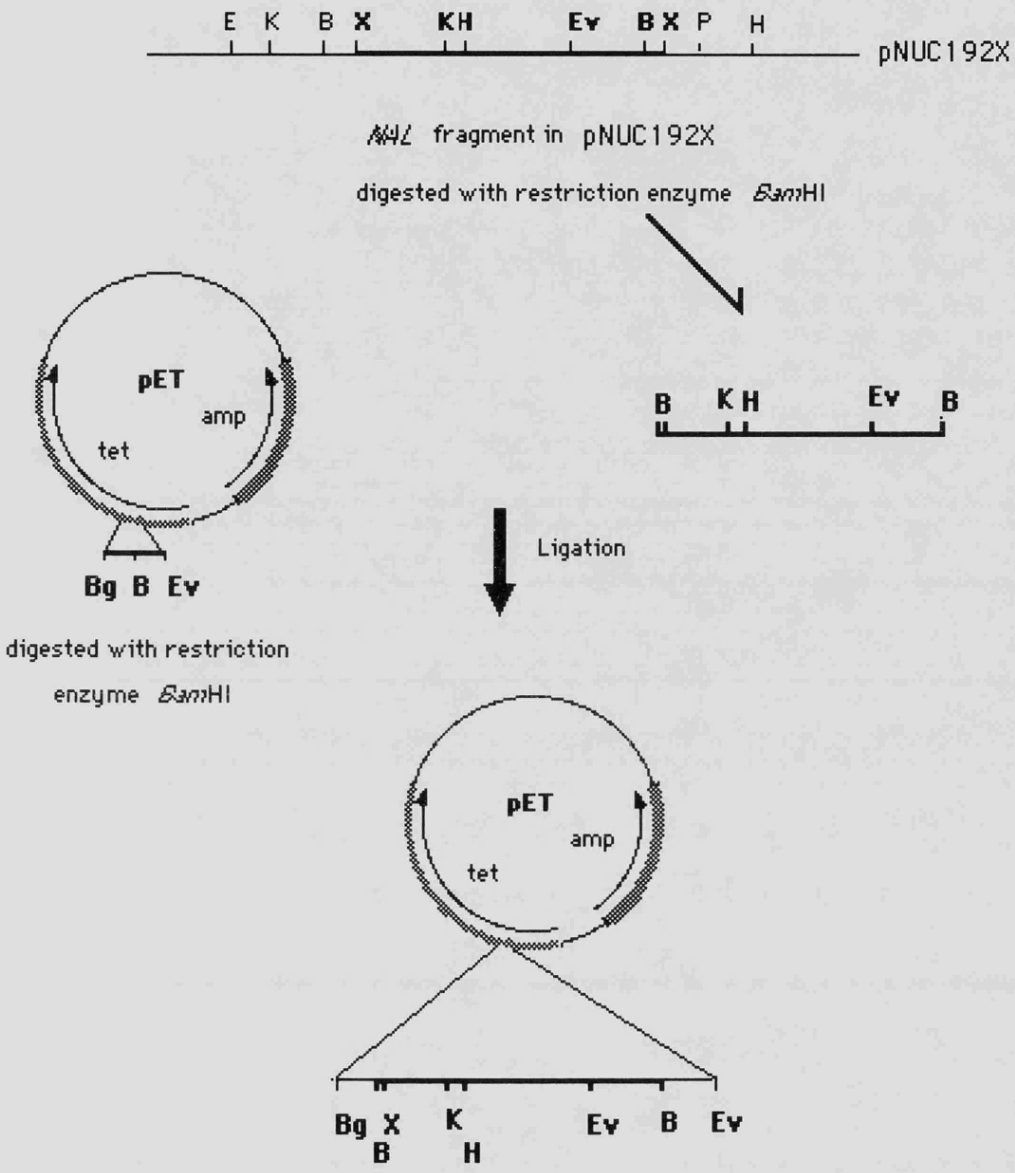

E K B X KH Ev B X P H
pNUC192X
NAL fragment in pNUC192X
digested with restriction enzyme BamHI
B K H Ev B
pET
amp
tet
Bg B Ev
digested with restriction
enzyme BamHI
Ligation
pET
amp
tet
Bg X K Ev B Ev
B H

Figure: 6.8

followed by plating on nutrient agar containing IPTG, X-Gal and ampicillin. The recombinant plasmid with the orientation as described in figure 6.7 was chosen and designated pNUC192X.

Secondly, the pNUC192X was digested with the restriction endonuclease *Bam*HI. The digests were run on an agarose gel and the 1.2kb *Bam*HI-*Bam*HI fragment was isolated from the agarose gel. It was further ligated to the *Bam*HI-digested PET3a1. The ligation mixture was transformed into competent cells of *E.coli* BL21, plated on ampicillin-containing nutrient agar. A dozen transformants were randomly picked and their recombinant plasmids analysed. The required recombinant plasmid, pET3a1::*NAL*, with the orientation as described in figure 6.8, was selected for amplification and purification.

6.4. Over-producing part of the Nal protein

The transformant containing the proper recombinant construct as mentioned above was incubated in ampicillin-containing nutrient broth at 37°C. When the OD_{600} reached approximately 0.1, the inducer IPTG was added at a final concentration of 50 $\mu g ml^{-1}$ to induce the synthesis of the fusion protein of X-Nal. The incubation continued for 3-4 hours after which inclusion bodies could be observed using a light microscope.

Inclusion bodies were isolated as described in chapter II. The protein in the inclusion bodies was dissolved by boiling in sample buffer for 5 minutes. The mixture was run on an acrylamide gel to determine the size of the fusion protein, which was approximately 49.5kD (figure 6.9). It was later proved that the transformant containing the recombinant construct, pET3a1::*NAL*, produced less protein when it was stored for longer than one week at 4°C. Because of

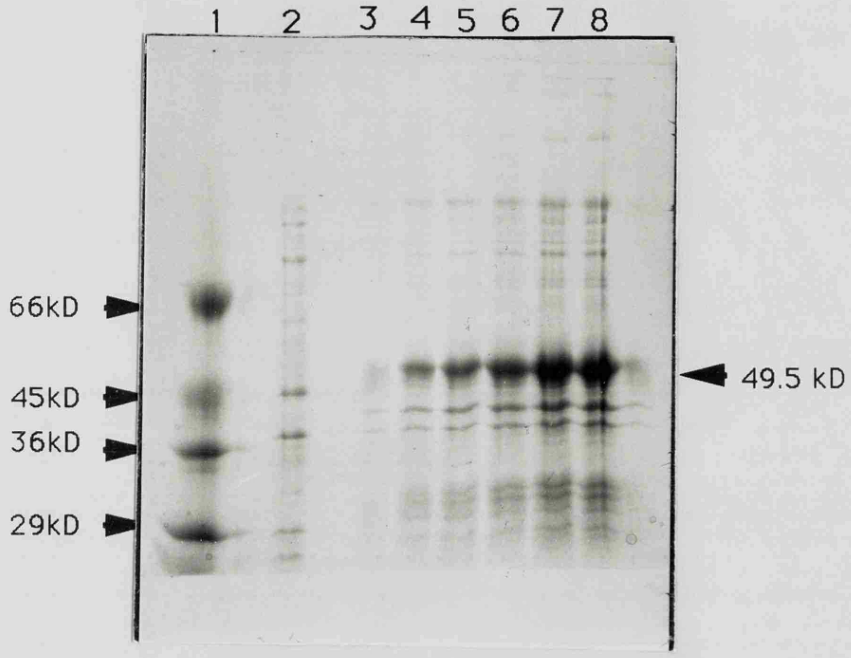


Figure 6.9: SDS-PAGE analysis of the Nal protein fusion
Lane 1 is a molecular mass marker, lane 2 is proteins extracted from *E.coli* containing pET3a vector lane 3-8 are proteins extracted from inclusion bodies containing the fusion protein, at a volume of 0.5, 1, 2, 3, 4, 5 µl, respectively. The molecular mass of the Nal protein is approximately 49.5kD.

this, and in the interest of obtaining a high yield of the Nal protein, the transformant had to be freshly prepared.

6.5. Isolation of the Nal protein fragment to raise polyclonal antibodies

The protein isolated from the inclusion bodies was run on a 15% acrylamide gel. The gel was stained with Coomasie Blue for 10 minutes and destained with destaining solution. The appropriate bands were cut out of the gel and further destained until they were free from the Coomasie Blue. The protein-containing gel pieces was was in 30% ethanol at 4°C overnight. The following day, the gel was washed 3 times with sterile TBS buffer.

The gel pieces containing the protein were sonicated in sterile TBS buffer until there were no visible particles in the suspension. An equal volume of Freund's Complete Adjuvant was added to the suspension so that the total volume would be 1 ml. An egg-laying hen was injected with the emulsion containing its protein fragment. The procedure was repeated on the 12th and 24th day. The eggs were collected daily for 8 weeks.

So as to raise polyclonal antibodies in a rabbit, a slightly different procedure was applied. The protein on an acrylamide was electro-blotted onto nitrocellulose, as described in chapter II. The protein-containing nitrocellulose was ground in the presence of liquid nitrogen. Its particles were resuspended in sterile TBS buffer and injected into the rabbit. The second injection was given 6 weeks after the first and the bleeding tests were performed the following week. The injection was continued with interval of 4 weeks until the titer of antibodies reached the peak.

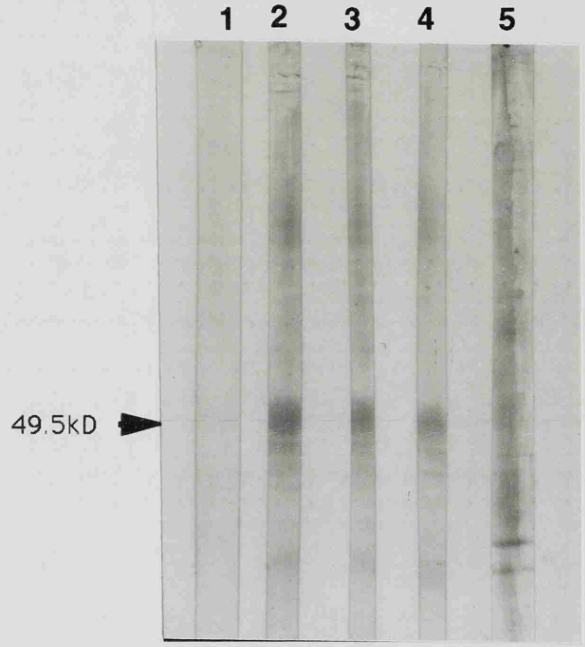


Figure 6.10: Western blot analysis of the Nal protein fusion from inclusion bodies of *E.coli.*

The extract proteins were separated by SDS-PAGE and blotted onto a nitrocellulose filter. Lane 1 is a negative control (no primary antibodies), lane 2-4 - extract proteins incubated with chicken anti-Nal antibodies at dilutions of 500 X, 1000 X and 2000 X, respectively. Lane 5 is incubated with pre-immune serum. The second antibodies was goat anti chicken coupled to peroxidase. The chicken anti-Nal antibodies recognize the Nal protein fusion with molecular mass of approximately 49.5 kDa.

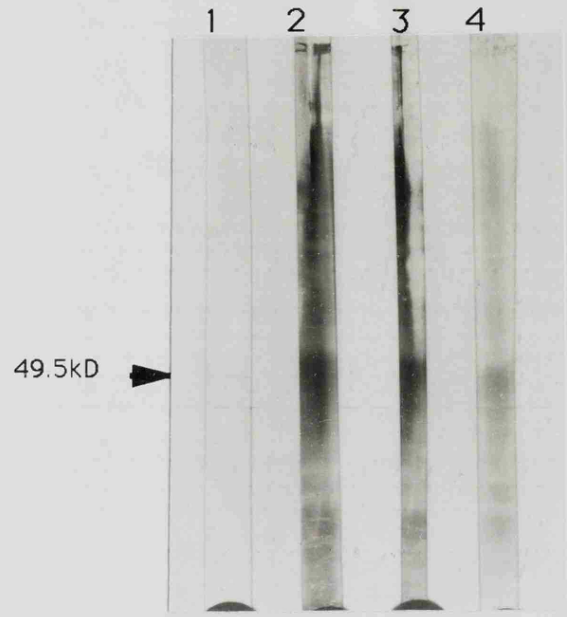


Figure 6.11: Western blot analysis of the Nal protein.
The protein was extracted from inclusion bodies of *E.coli*, separated by SDS-PAGE and electroblotted onto nitrocellulose. Lane 1 is a negative control (no primary antibodies). Lane 2-4 are incubated with affinity purified anti-Nal antibodies at 50 X, 100 X and 1000 X dilution, respectively.

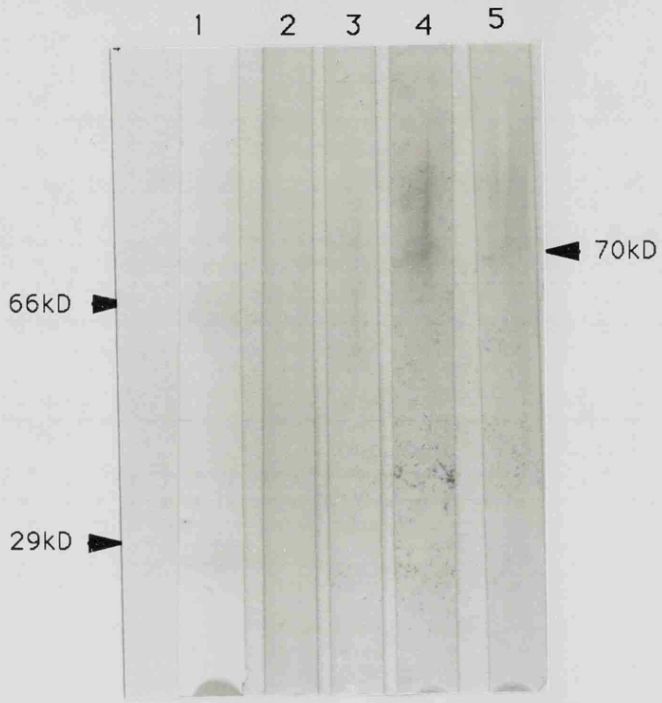


Figure 6.12: Western blot analysis of the Nal protein in yeast extract

Lane 1 is a molecular mass marker, lane 2 is a negative control (no primary antibodies), lane 3 is yeast extract incubated with pre-immune antibodies, lane 4 and 5 are yeast extracts incubated with affinity purified chicken antibodies at 20 X and 50 X dilution, respectively. The antibodies recognize a protein with a molecular mass of approximately 70 kDa.

6.6. Western blot experiments

In order to determine whether antibodies against the Nal protein fragment have been produced by the chicken and rabbit, Western blot experiments were preformed. Antibodies were extracted from chicken yolk and rabbit blood as described in chapter II. The protocol of the Western blot has also been described in chapter II.

Results of the blots are presented in figure 6.10. It is shown that antibodies at dilution of 1:2000 can still detect the Nal protein made in *E.coli*. The anti-Nal antibodies were purified using the affinity purification method described in chapter II and tested against the Nal protein (figure 6.11). These affinity-purified chicken and rabbit antibodies were further used to detect the Nal protein in yeast extracts. The result shown in figure 6.12, suggests that the anti-Nal antibodies recognise a protein extract with a molecular mass of approximately 70 kDa in yeast.

6.7. Indirect immunoflourescence microscopy

Wild-type yeast cells were fixed as described in chapter II. The chicken anti-Nal antibodies and a positive control: mouse anti yeast tubulin antibodies were added to the fixed cells and incubated at 4°C overnight. After washing the first antibodies, the second antibodies, goat anti-chicken IgG-FITC and rabbit anti-mouse IgG-TIRFC were added and incubated at 37°C for 1 hour in dark. These second antibodies were washed away and the cells were resuspended in TBS.

A drop of the cell suspension in TBS was spread and left to dry on a glass slide. The cells were observed under UV light using a Zeiss photomicroscope. The photographs are presented in figure 6.13. The location the nuclear and mitochondrial DNA were indentified as large and small dots, respectively (figure 6.13A), whereas the location of

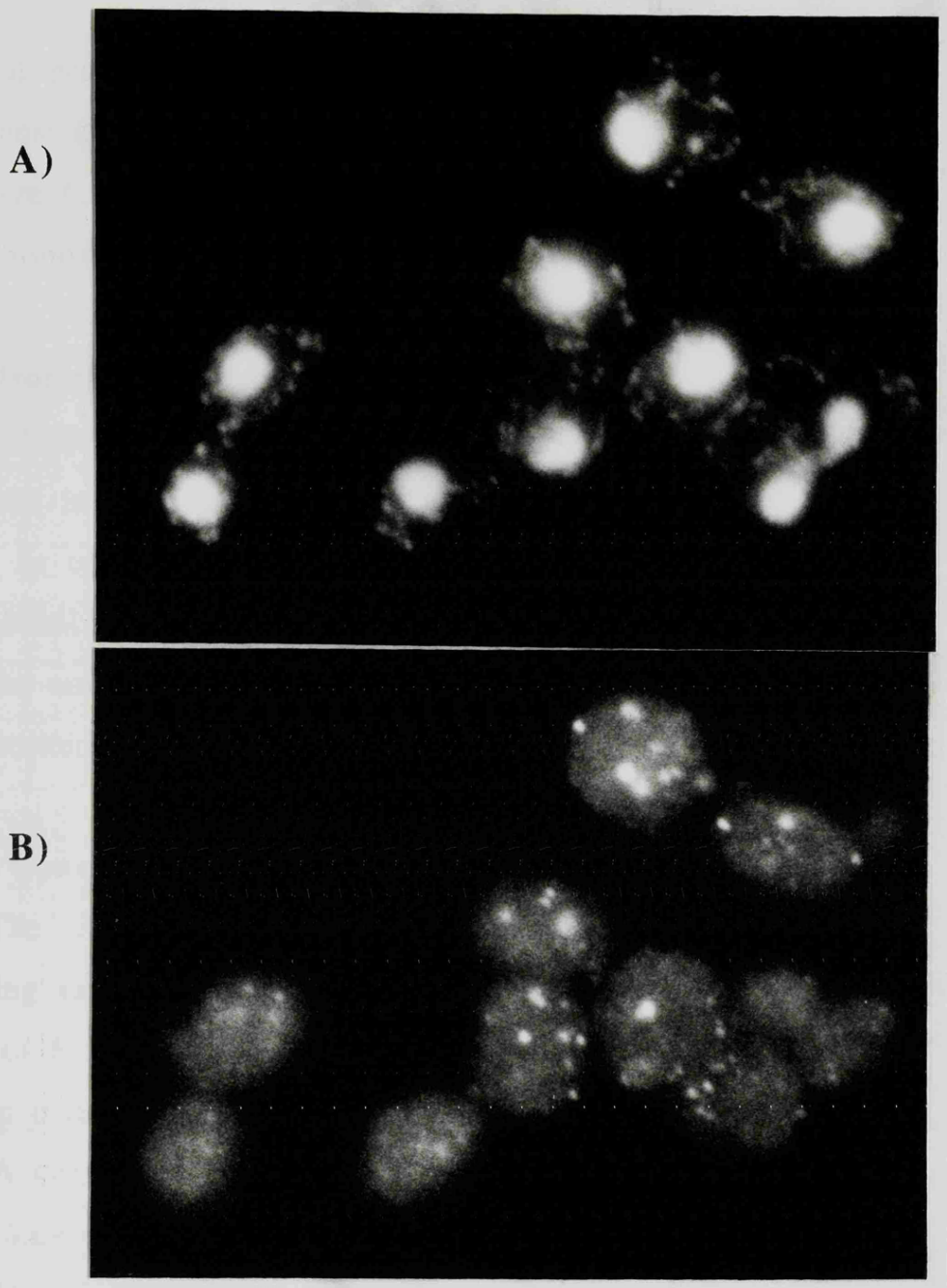


Figure 6.13: A) Fluorescence microscopy of DAPI-stained cells of 842D the big dots are nucleus, and the small dots are mitochondria. B) The same cells as above stainned with chicken Nal-anti bodies as primer antibodies and goat anti-chicken-FITC antibodies as secondary antibodies. The small dots correspond to the Nal protein which seems to be at the same location as mitochondria (A). (3000 X).

the Nal protein was identified as small dots (figure 6.13B). It seemed that some of the dots in figure 6.13A were at the same location as dots in figure 6.13B. This suggests that the location of the Nal protein is in mitochondria.

6.8. Isolation of the *nal(ts)* rho^0 mutant.

nal(ts) rho^0 mutants have been prepared through selecting viable colonies in the presence of ethidium bromide. These mutants were raised in order to determine whether the Nal protein has any effect in the absence of mitochondrial DNA. The result showed that these mutants are still temperature sensitive. This suggests that the absence of mitochondrial DNA does not abolish the effect of the Nal protein.

6.9. Conclusion

The predicted Nal protein has been translated from the *NAL* gene resulting in a polypeptide of 563 amino acid residues with a molecular mass of 63.5 kDa. Comparison of this protein to existing proteins in the protein data bank reveals some homology to AKB enzyme of *E.coli* and δ-ALA enzymes from human, chicken, rat, mouse, *B.japonicum* and the yeast *S.cerevisiae.*

The Nal protein has also been over-produced in *E.coli* using the fusion method, producing a fusion protein with a molecular mass of 49.5 kDa. Polyclonal antibodies against the protein have been raised in a chicken and a rabbit. The affinity-purified anti-Nal antibodies recognise a protein in yeast extract with a molecular mass of approximately 70 kDa, which is in agreement with the predicted molecular mass calculated from the sequenced *NAL* gene.

The indirect immunofluorescence microscopy indicates that the location of the protein is likely to be in the mitochondria. If this

protein is a mitochondrial protein then it should have a targeting signal in the amino-terminal. However, since no conserved sequenced to identify a targeting signal, it is difficult to evaluate the existence of such signal in the Nal protein. Hartl *et al.* (1989) suggested that mitochondrial targeting signals in the amino-terminal of prepeptides are typically hydrophilic and positively charged. The Nal protein has a hydrophilic and negatively charged region at the predicted N-terminal. Although this region is not positively charged, it does not rule out the possibility that this region is the targeting signal. In order to determine this possibility, the Nal protein should be purified, its N-terminal sequenced and compared to the DNA sequence.

Chapter VII: Discussion

In this study, I have isolated a yeast mutant (*nal*) which confers simultaneously temperature sensitivity and nalidixic acid-resistance in a drug-sensitive (*mds*) background. Genetic analysis showed that the mutation is nuclear, it is not a reversion of the Mds phenotype nor a "translational" suppressor. Morphological analysis, using light microscopy, demonstrated that at the non-permissive temperature, the mutant cell cycle is arrested at or around the S (DNA replication) phase, though the arrest is not immediate. The mutant is hyper-sensitive to drugs and chemicals that affect the plasma membrane, suggesting that the mutation might alter membrane permeability. The wild-type gene was cloned by complementation of the Ts phenotype and sequenced. The reading frame corresponding to the *NAL* gene was unambiguously identified by subcloning fragments complementing the mutation. The Nal protein, the predicted product of the *NAL* gene, has a molecular mass of 63.5 kDa.

Considering the data, the main questions which should be addressed in this work are: 1) the identity of the Nal protein, and 2) the nature of the nalidixic acid-resistant mutation, since resistance to drugs can potentially be achieved by many different mechanisms (see introduction).

The main genetic target of 4-quinolones in prokaryotes is the subunit A of DNA gyrase, the bacterial type II topoisomerase (Gellert, 1976b); other resistant mutants identify proteins which are involved in the permeability of the plasma membrane (Michea-Hamzehpour, 1991). It is clear that the *nal(ts)* mutation is not a *top2* mutation, and therefore cannot be the eukaryotic type II topoisomerase.

The predicted sequence of the Nal protein shows high homology to the β-amino-γ-ketobutyrate CoA ligase (AKB ligase) of *E.coli.* This homology suggests that the two proteins could share similar substrates, which are glycine and acetyl-CoA. It is possible that the Nal protein is the yeast counterpart of the AKB ligase, and is thus involved in the synthesis and degradation of threonine. This process has been shown to be the major route of threonine utilisation in both prokaryotes and eukaryotes (Dale, 1978). In addition, threonine residues are thought to play an important role in the synthesis of glycoproteins of the cytoplasmic membrane. In *Saccharomyces cerevisiae,* O-linked oligosaccharides are bound to peptides via a linkage to the hydroxyl oxygen of serine and threonine (Ballou and Raschke, 1974; Darnell *et al.,* 1990). Glycosylation defects could account for cell wall permeability and consequently the drug-resistance of the *nal(ts)* mutant.

The second protein to which the *NAL* gene product exhibits high homology is the δ-aminolevulinic acid (δ-ALA) synthetase from various organisms. This enzyme catalyzes the reaction of glycine and succinyl-CoA yielding δ-aminolevulinic acid. This reaction is the first step of heme biosynthesis, essential for cytochrome activity. Cytochromes are involved in energy transduction which takes place in the mitochondria. In the *nal(ts)* mutant, at the non-permissive temperature, both DNA and RNA synthesis stop almost simultaneously but not immediately. This may suggest a defect in a general process such as energy metabolism. However, rho^{o} mutants, defective in the respiratory chain, are still viable. So a cytochrome defect can not be the cause of the temperature sensitivity.

The Nal protein is most likely localized in the mitochondria, as shown by the immunofluorescence experiments. The two partially

homologous enzymes, the δ-ALA synthetase and the AKB ligase from bovine liver are mitochondrial. So if the Nal protein is mitochondrial, how does it affect: a) drug resistance and b) the *cdc* phenotype of the mutant? It has been shown that the yeast *pdr* and *pma* genes produce mitochondrial proteins that affect membrane permeability (Rank *et al.*, 1975; Ulaszewski *et al.*, 1983) although the exact mechanism of their activity remains unclear. Perhaps the Nal protein is a mitochondrial protein which is involved in the biosynthesis of a membrane component. Any defect in this protein could consequently lead to alterations in the membrane structure. Alternatively, the Nal protein might be involved in the detoxification of nalidixic acid through its chemical modification e.g. acetylation or through sequestration into mitochondria. If the protein interacts with other mitochondrial proteins, a defect in this protein, at the non-permissive temperature, could make it poisonous and eventually lead to the cell death.

Ravnikar and Somerville (1987a), showed that the major alternative pathway for serine biosynthesis is by threonine utilization via glycine (figure 6.4a). Serine is essential in the biosynthesis of phosphatidylserine, which is a major component of the plasma membrane. A defective in its biosynthesis may lead to alterations in the structure of the plasma membrane. So if the Nal protein is involved in threonine metabolism it could affect the structure of the membrane.

There is now evidence that interference with the structure of the cytoplasmic membrane can lead to abnormal DNA synthesis (Leno and Laskey, 1991). When a *fas1* mutant of *S.cerevisiae* is incubated in a medium lacking fatty acid supplement, its cell cycle is arrested at the S phase similar to the *nal(ts)* mutant at the non-permissive

temperature (G.Shiels, personal communication). This *fas1* mutant is defective in fatty acid synthetase, an enzyme involved in the synthesis of lipids which are necessary for membrane and particularly phospholipid biosynthesis. Moreover, *in vitro* studies have demonstrated that phospholipids interact with DNA polymerase α of *S.cerevisiae* (Capitani *et al.,* 1984) and DNA polymerase I and III of *E.coli* (Novello *et al.,* 1975; Wickner and Kornberg, 1974) and are necessary for the correct control of DNA replication (Yoshida *et al.,* 1989).

In future work concerning the target of nalidixic acid, it would be nice to demonstrate whether the Nal protein binds to nalidixic acid directly. It is interesting in this regard that the *mds1* mutant is sensitive to nalidixic acid but not to other 4-quinolones. Nalidixic acid is more hydrophobic than other 4-quinolones (Hirai, *et al.,* 1986a). It is therefore possible that the permeability barrier in the *mds1* mutant allows nalidixic acid, but not other 4-quinolones less hydrophobic, to enter the cell. Consequently, I was unable to isolate mutants conferring resistance to other 4-quinolones. In order to find out whether the *nal(ts)* mutation is the direct target of 4-quinolones the Nal protein should be purified and used to determine whether it binds to the drugs. If this protein is the target for 4-quinolones then it should bind all 4-quinolones.

Another important experiment which should enable us to understand the role of the Nal protein in the drug resistance phenotype is the disruption of the *NAL* gene, an experiment which has not yet been successful. It should also be interesting to isolate the *nal* (mutant) gene. This gene could then be used to determine whether the mutation is dominant with regard to the nalidixic acid resistance.

Appendix A: The complete sequence of the NAL-cloned

```
   1  GATCCAGCCG CAATAGCAAC CATTTCACAA TTTTTAGCCA AATATGATAG
  51  TATAGAAGTC AGTGGCGGAT GTCCAATCGT CATTGGTTTG AGGTATCAAG
 101  ATACCATTCC AGCATGGTGT ACTCATATAT GTTGCGTTGA TGAGAAAAAC
 151  GGTATATTGT TTGAAGGCCC AATCGAAAAA CTTCAAAGTA AAATGGATGA
 201  AACCAGATCA AGGGCACTAA AAGAACTAGA GCAGCTCAAA AAAGCGTCTA
 251  ATTCCAAAGA GGACATTTCC ATTAACGATT TGATTTGTAT ACATCCTATG
 301  TATGGCAAGA AAGGGCACGA AATATCAAAA TGCCTCATCT TATAGAATTA
 351  GACGGGTTGA GCGTTTCATA CAAAGCGAAG CTGTTTTGGA AAATCTGCAC
 401  TGGAAAGTTC AGCCGGGTTC GAAATGGCAT ATAAGAGGTG ACAATGGGTC
 451  AGGTAAGTCG ACCTTATTAT CTTTGCTTAC GGCGGAACAT CCCCAATCGT
 501  GGAATTCGAG GGTGATAGAT AATGGCGTCC CACGAAGAAC AGGTAAAACA
 551  AACTACTTTG ACCTAAACAG CAAAATAGGT ATGTCATCAC CCGAATTACA
 601  CGCAATTTTT TTGAAGAATG CTGGAGGAAG GCTAAACATT CGGGAAAGTG
 651  TTGCAACTGG CTATCATGAG GCGTCCTCCA ACAATTACCT ACCCATATGG
 701  AAGCGCTTGG ACAAAAATAG CCAAGAAATA GTGAATATGT ATCTGAAATA
 751  TTTTGGCCTG GACAAAGACG CCGATAGTGT TTTATTTGAA CAGTTGTCCG
 801  TAAGTGACCA GAAACTAGTC CTTTTTGTCA GGTCTTTAAT TAAGATGCCG
 851  CAAATATTGA TTCTCGATGA GGCATTTTCT GGAATGGAGG TAGAACCTAT
 901  GATGCGTTGT CATGAATTTT TAGAGGAGTG GCCTGGAACA GTCCTTGTAG
 951  TGGCACACGT TTGCCGAAGA GACACCAAAA TGTGCCCATT ACTTAAGGCT
1001  CATATCTCCT GGAGAGTATG AAATAGGCGA TATGGAAAAT TAAAGTTTTC
1051  TGTTGTCGTG GCAGCAAGAG ACAGAACCTC GATAATTTGA CATACGTATA
1101  TAATAGTACA TGTACATAAA AAGCACGCAA ATATCGTATA TCTGTTATAC
1151  TACAAAACAA TTACTTCTAT ATCATAGCCA GTTAGCGGGA ACGACTTCAG
1201  CTAAATGGAC TATCCATGCT TTAGGCAGAG GCGAAGGCGG TGATTGGGTG
1251  TAACATCATC TCCTTTTCTC TACGACAAAT TCCAAAAAAA AAATTTATGC
1301  TATGTTAATA CCTGCACAAT TCAACCGTGC TGAAACGTAA AATTAAGGTG
1351  ATTATACGGA TAGTATACGA TATTATCAAT CTCATAAGAA AAATCTCTTT
1401  TGAATTTAAC GGAGGGATTA TTCATTAGAA AGCGTTCTTA CCATTCACTA
1451  GGAGCGAATC CGTGGAAGGT GTTTTAACGT TGCCACGAAA AACAGCTCTA
1501  CATCGAAATA AAAGACAACA ATCAGTGCCC GTAAGTTTCA TTACTATTTT
1551  CTATTATTAT CTGCAACTTT TTATTAGTTA GGTTTTTTTT GTTTGTTTGT
1601  TTGTTTTCAA TTGATTAATT TACAAGACAA AGAACCTTAT ATTTCGTGTT
1651  TTTCATTCTA AAGGAAAAAA AGCATAAAGA AGATTCCACA CACTTTATTG
1701  TGATAGTTTT CAAAGTAAAA AGTAATAGAT TATGAGTACT CCTGCAAACT
1751  ATACCCGTGT GCCCCTGTGC GAACCAGAGG AGCTGCCAGA CGACATACAA
1801  AAAGAAAATG AATATGGTAC ACTAGATTCT CCGGCGCATT TGTATCAAGT
1851  CAAGTCACGT CATGGGAAGC CACTACCTGA GCCCGTTGTC GACACCCCTC
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1901 CTTATTACAT TTCTTTCTTT GTTAACATAT CTAAATTATT TGATTCTGAT
1951 TATATTAGGT CATGTTCACG ACTTCTTAGG TATGACCTTC CAAAAAAACG
2001 ACATCTGGAT CTTTTAGAGC ATGATGGGTT AGCACCTTGG TTTTCAAATT
2051 TCGAGAGTTT TTATGTCAGG AGAATTAAAA TGAGAATTGA TGATTGCTTT
2101 TCTAGACCAA CTACTGGTGT TCCTGGTAGA TTTATTCGTT GTATTGATAG
2151 AATTTCTCAT AATATAAATG AGTATTTTAC CTACTCAGGC GCAGTGTATC
2201 CATGCATGAA CTTATCATCA TATAACTATT TAGGCTTCGC ACAAAGTAAG
2251 GGTCAATGTA CCGATGCCGC CTTGGAATCT GTCGATAAAT ATTCTATTCA
2301 ATCTGGTGGT CCAAGAGCTC AAATCGGTAC CACAGATTTG CACATTAAAG
2351 CAGAGAAATT AGTTGCTAGA TTTATCGGTA AGGAGGATGC CCTCGTTTTT
2401 TCGATGGGTT ATGGTACAAA TGCAAACTTG TTCAACGCTT TCCTCGATAA
2451 AAAGTGTTTA GTTATCTCTG ACGAATTGAA CCACACCTCT ATTAGAACAG
2501 GTGTTAGGCT TTCTGGTGCT GCTGTGCGAA CTTTCAAGCA TGGTGATATG
2551 GTGGGTTTAG AAAAGCTTAT CAGAGAACAG ATAGTACTTT GTCAACCAAA
2601 AACAAATCGT CCATGGAAGA AAATTTTAAT TTGCGCAGAA GGGTTGTTTT
2651 CCATGGAAGG TACTTTGTGT AACTTGCCAA AATTGGTTGA ATTGAAGAAG
2701 AAATATAAAT GTTACTTGTT TATCGATGAA GCCCATTCTA TAGGCGCTAT
2751 GGGCCCAACT GGTCGCGGTG TTTGTGAAAT ATTTGGCGTT GATCCCAAGG
2801 ACGTCGACAT TCTAATGGGT ACTTTCACTA AGTCGTTTGG TGCTGCTGGT
2851 GGTTACATTG CTGCTGATCA ATGGATTATC GATAGACTGA GGTTGGATTT
2901 AACCACTGTG AGTTATAGTG AGTCAATGCC GGCTCCTGTT TTAGCTCAAA
2951 CTATTTCCTC ATTACAAACC ATTAGTGGTG AAATATGTCC CGGACAAGGT
3001 ACTGAAAGAT TGCAACGTAT AGCCTTTAAT TCCCGTTATC TACGTTTAGC
3051 TTTGCAAAGG TTAGGATTTA TTGTCTACGG TGTGGCTGAC TCACCAGTTA
3101 TTCCCTTACT ACTGTATTGT CCCTCAAAGA TGCCCGCATT TTCGAGAATG
3151 ATGTTACAAA GACGGATTGC TGTTGTTGTT GTTGCTTATC CTGCTACTCC
3201 GCTGATCGAA TCAAGAGTAA GATTCTGTAT GTCTGCATCT TTAACAAAGG
3251 AAGATATCGA TTATTTACTT CTGTTCATGT TAGTGAAGTT GGTGACAAAA
3301 TTGAATTTGA AATCAAATTC CGGCAAATCC AGTTACGACG GTAAACGTCA
3351 AAGATGGGAC ATCGAGGAAG TTATCAGGAG AACACCTGAA GATTGTAAGG
3401 ACGACAAGTA TTTTGTTAAT TGAATTTTAC CTAATTGCTA GTTAGGTGAA
3451 AAATTACAAA ATTTCTGGAA GACGTTGGAA ACACGCAACG TCTTTTTGAC
3501 ATAAACTTAA AACTGCCAAA AGTCAAACAA AAATTGCAAA AAAAGTAAAA
3551 AAAGTTACGA AAAAAAAAAC ATTTAAAAGA AAGAAGAAGT TAAAAGTGCA
3601 CGCAATATGT TCCAGGATAT GAAATGAAAT ACCTTTTGTT TCACCTTTTA
3651 AATAATTTAA TGTTATATAT ACAACTTTAT CGTATCATAT TCGCAATTAC
3701 ATTATACAAG AATGAGTTTT TTTTTTTCGC GGACAAAAGA AGAACATGTC
3751 AACGCCAACC CCCTAGGTGA TTTTGTTAGA CAAAAGCGAA TTAGGGATCC
3801 CCGGGTACC

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