STUDIES OF CYTOPLASMICALLY INHERITED GENES FOR COMPONENTS OF THE MITOCHONDRIAL ATPase COMPLEX:

Analysis of the Oli-2 region of the mitochondrial genome

of *Saccharomyces cerevisiae*

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"When all other contingencies fail, whatever remains,
however improbable, must be truth."

"Method of Sherlock Holmes"

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ABBREVIATIONS

A	Adenosine nucleotide
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BSA	Bovine serum albumin
BAP	Bacterial alkaline phosphatase
BCIG (X-gal)	5-bromo-4-chloro-3-indolyl-β-D-galactoside
bp	Base pair
C	Cytidine nucleotide
CIP	Calf intestinal phosphatase
CCCP	Carbonyl cyanide m-chloro phenyl hydrazone
CT DNA	Calf thymus DNA
DAPI	4', 6- diamidino-2-phenyl indole dihydrochloride
DCCD	N, N'-Dicyclohexyl-carbodiimide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DNase	Deoxyribonuclease
dNTPs	deoxy nucleotides
ddNTP	dideoxy nucleotides
dATP	deoxy Adenosine triphosphate
dGTP	deoxy Guanidine triphosphate
dCTP	deoxy Cytidine triphosphate
dTTP	deoxy Thymidine triphosphate
ddATP	dideoxy Adenosine triphosphate
ddGTP	dideoxy Guanidine triphosphate
ddCTP	dideoxy Cytidine triphosphate
ddTTP	dideoxy Thymidine triphosphate
DTT	Dithiothreitol
D-loop	Dihydro uridine loop
EDTA	Ethylene diamine-tetra-acetic acid
EGTA	Ethyleneglycol-bis-(β-amino-ethyl ether)N, N'-tetra acetic acid.
EtBr	Ethidium bromide
G	Guanidine nucleotide
HAP	Hydroxy apatite
IPTG	Isopropyl -β-D-thiogalactopyronoside
Kbp	Kilo base pair
LMP agarose	Low melting point agarose
mt-DNA	Mitochondrial DNA
mt-genome	Mitochondrial genome
mt-RNA	Mitochondrial RNA
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
OSCP	Oligomycin sensitive conferring protein
O.S.ATPase	Oligomycin sensitive ATPase
OLI(Oli)	Oligomycin
ORF	Open reading frame
Oss	Ossamycin
O.D.	Optical density
PEG	Polyethylene glycol

PNK buffer	Polynucleotide kinase buffer
p.s.i.	Pound per square inch
RF	Replicative form
Rh6G	Rhodamine 6G
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rDNA	Ribosomal DNA
rpm	Revolution per minute
RNase	Ribonuclease
ρ	Rho factor, loss of which involves deletion in mt-DNA leading to the 'petite' phenotype (ρ or ρ^0)
S	Sedverg's constant
SDS	Sodium dodecyl sulphate
SMP	Sub mitochondrial particles
ssDNA	Single stranded DNA
SSC	Salinated sodium citrate; see Appendix for the composition
1799	α, α-bis (hexafluoro acetonyl) acetone
T	Thymidine nucleotide
TBE	Tris-borate EDTA buffer; see Appendix for composition
TEMED	N, N, N', N', tetramethyl ethylene diamine
TET	Triethyl tin
URF	Unknown reading frame
UV	Ultra violet
VEN	Venturicidin
Ve2383	(2-[(dimethylamino) methyl] diethyl tin halide

SUMMARY

This thesis describes studies of the structure and organisation of the Oli-2 region of the mitochondrial genome from wild type, drug resistant ($Oli2^R$, $Oss1^R$) and *mit* strains of the yeast *Saccharomyces cerevisiae*. In addition attempts have been made to identify the locus of cytoplasmic, nonmitochondrial markers (VEN^R, TET^R, $Rh6G^R$), to drugs which affect mitochondrial energy metabolism.

Firstly, a fine structure genetic map of the Oli-2 region of the mitochondrial genome has been generated by petite deletion mapping. Two *mit* mutations (pho8 and pho9) have been mapped upstream and one *mit* mutation (mit-175) has been mapped downstream of the Oli2 locus (Chapter-2). A problem was identified regarding the effect of a nuclear gene (*kar-1*) on the copy number and transmission of petite (ρ) mitochondrial genomes (Chapter-3). To circumvent this problem the relevent portions of the mitochondrial DNA (mt-DNA) from various mutants of the Oli-2 region have been cloned in a multicopy plasmid, pAT 153 of *E. coli*, for their amplification and propagation (Chapter-4).

The cloned DNAs have been sequenced using the single stranded phage, M 13 as a sequencing vector. A sequence of about 4000 bp, starting from the Carboxyl terminal end of cytochrome oxidase subunit-1 (Oxi-3) to 1500 bp downstream of Oli2 has been sequenced. Three main reading frames have been identified in this stretch of the DNA segment. The mutations ($Oli2^R$, $Oss1^R$) leading to the resistivity towards the drugs oligomycin and ossamycin have been located in the reading frame for subunit-6. The *mit*⁻ mutation (pho9) has been found not to lie on the structural gene for subunit-8 which is located upstream of the gene for subunit-6. It is assumed that the mutation is possibly located in the intergenic regulatory region of the genes. A putative reading frame has been identified downstream of subunit-6 reading frame, which could be the possible site for the genetic locus, mit-175 (Chapter-5).

Models for the secondary and tertiary structures of subunit-6, subunit-8, and subunit-9 have been proposed on a theoretical basis using hydrophobicity plots and a modified Chou and Fasman method (Chou & Fasman, 1978). Suggestions as to the mechanism of inhibition of oxidative phosphorylation by oligomycin and ossamycin have been made on the basis of these models. The present studies also indicate that subunit-8 has structural analogies to subunit-b of *E. coli* ATP synthetase (Chapter-6).

An attempt has also been made to identify a cytoplasmic candidate for nonmitochondrial, cytoplasmic genetic markers (VEN^R, TET^R, $Rh6G^R$) with special emphasis on studies of the 3 μ plasmid (Chapter-7). It has been demonstrated that the 2 μ plasmid, or dsRNAs found in the cytoplasm are not the bearer of these genetic markers. In spite of the fact that the 3 μ DNA species under investigation has an insertion, no evidence has been obtained that the 3 μ plasmid contain the above markers. The existence of a high molecular weight plasmid in the cytoplasm of *S. cerevisiae* has also been demonstrated.

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CHAPTER - 1

GENERAL INTRODUCTION

1. Mitochondria: power plant of living cells

Mitochondria are called the power plants of living cells because most of the energy in a living cell with the exception of the prokaryotes is provided by mitochondria. Energy is produced in the form of ATP by a process of aerobic metabolism called oxidative phosphorylation. The participation of mitochondria in aerobic metabolism in fact starts with the oxidative de-carboxylation of pyruvate (a glycolytic product of sugars) to acetyl-CoA which are further oxidised to CO_2 by a cycle called the tricarboxylic acid (TCA) cycle. During this process, NAD^+ molecules are reduced to NADH.The NADH molecules are reoxidised to NAD^+ and, in the process electrons are transferred through a respiratory chain and generate a proton gradient across the membrane. At the end of the process, a multiprotein enzyme called ATP synthase, pumps these protons through its intermembrane complex channel to its catalytic site where the protons (H^+) facilitate the reaction of ADP molecules with inorganic phosphate (P_i) to produce ATP (Mitchell, 1961, 1966, 1979a, 1979b).

The central intermediate in mitochondrial metabolism is acetyl-CoA which can not only be derived from pyruvate, but also from the oxidation of fatty acids and aminoacids. The enzymes required are all located in the matrix of mitochondria. The relationship among the major oxidative pathways of mitochondria and their ultimate connection to the terminal ATP generating proton pump is outlined in Fig. 1.1.

2. Mitochondria as semi-autonomous organelles

The biochemical reactions outlined in the earlier section underline the fact that it requires a considerable number of protein products: some are soluble catalytic enzymes while others are membrane integrated structural proteins which may also have catalytic functions. However, most of these proteins (about 95%) are transported from the cytoplasm where

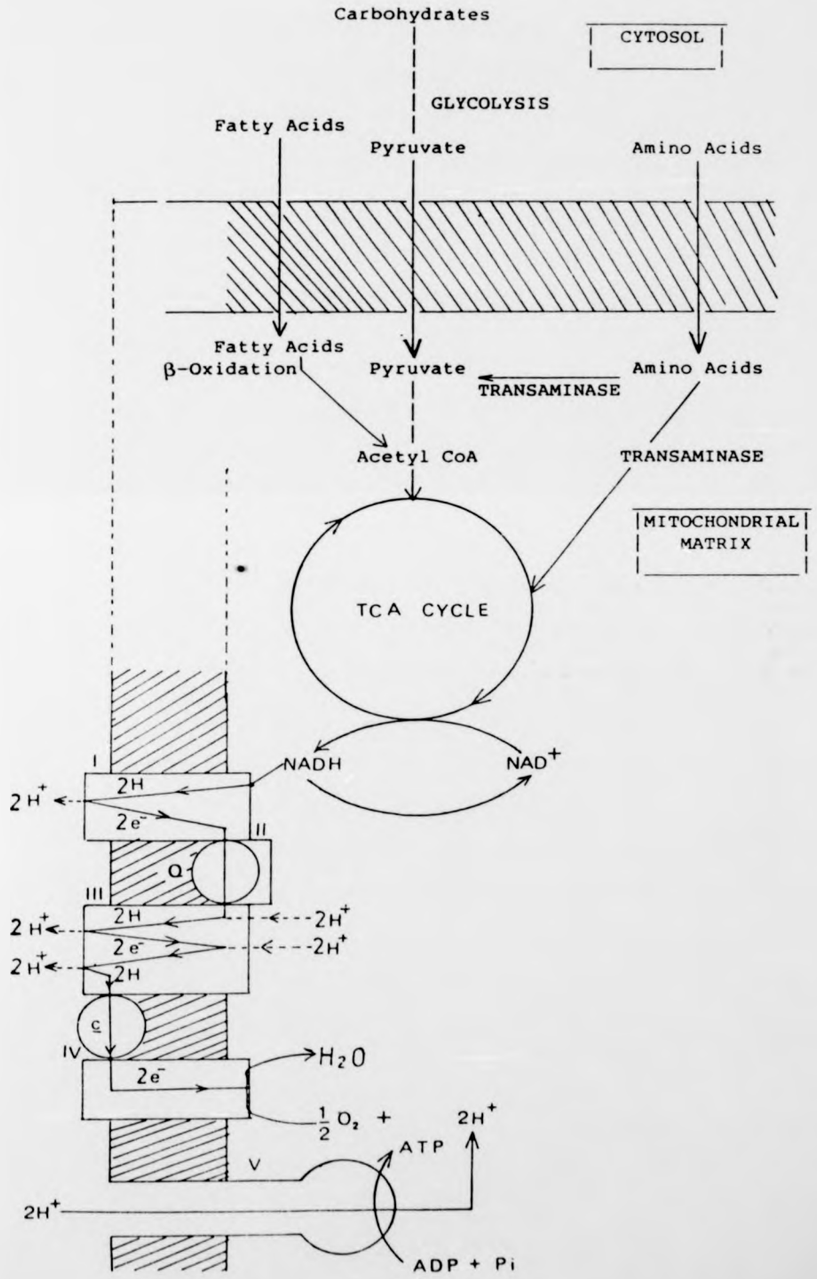


Figure 1.1. The relationship among the major oxidative pathways of mitochondria and their ultimate connection to the terminal ATP generating proton pump (the ATP synthase or the O.S.ATPase or the Complex-V). For details See Nicholls (1982). I, II, III, IV, and V are various respiratory complexes found in the mitochondrial inner membrane (See Hatefi et al, 1962; Racker, 1979). I, NADH - UQ oxidoreductase; II, Succinate dehydrogenase (succinate UQ reductase); III, UQH_2 - Cytochrome c reductase (cytochrome bc_1 complex; IV, Cytochrome c oxidase; V, ATP synthase or the O.S.ATPase.

they are synthesised under the direction of nuclear coded DNA. A small (about 5%) but significant amount are coded by mitochondrial DNA (mt-DNA), which controls the whole machinery of respiration, electron transport and oxidative phosphorylation inside mitochondria. The mt-DNA does not only provide the information for these polypeptides of the inner membrane but also possesses its own machinery (rRNAs, tRNAs) to synthesize them. This autonomy controlled by the transport of some other factors which are nuclear coded cytoplasmic proteins has made the organelles semi-autonomous. The extent of autonomy can be best understood by the study of the mitochondrial genome itself.

3. The mitochondrial genome and its diversity

The mitochondrial genome or mt-DNA is clearly distinguishable from the nuclear genome by its structure, size, shape and base composition. The genome is highly A+T rich and the buoyant density is lighter than that of nuclear DNA. It does not form a nucleo-histone complex (or nucleosome) and with the exception of ciliates forms circular, superhelical structures. However, study of the mt-DNA from various organisms studied sc far, have little in common. Table 1.1 summarises some of the features from various phylogenetic groups. For further detail, the reader can consult the reviews: Gillham, 1978; Gray, 1982; Gray & Doolittle, 1980; Wallace, 1982; Evans,1983; and Dujon, 1983. Briefly, metazoan mt-DNAs are smaller and circular with a size of 15-16 kilobase pair (Kbp). Fungal mt-DNAs are circular with variable sizes (18 Kbp - 78 Kbp) (exceptions: *Hansenula mrakii*, Wesolowski & Fukuhara, 1981; *Candida rhagii*, Kovak *et al*, 1984); plant mt-DNAs are heterogenous and may, or may not be circular and size varies from 95 Kbp to about 200 Kbp. Mt-DNA of *Saccharomyces cerevisiae*, which is under investigation in the present study, is a circle of about 25 μ in contour length corresponding to a molecular weight of about 75 Kbp (Hollenberg *et al*,1970). The following section describes the mt-genome of the yeast *S.cerevisiae*.

TABLE 1.1. Size and Shape of Mitochondrial DNAs

Organisms from Various Taxanomic Categories	Shape	Length (µm)	Molecular weight (daltons) x 10^6	Molecular weight (Kbp)	References
PROTOZOA					
Acanthamoeba castellanii	Circular	12.7	26	38	Bohnert and Herrmann (1974)
Leishmania terantolae	Circular	9.9(maxi)	20	60	Borst & Hoeijma-kers (1979); Challberg and Englund (1980)
		0.28(mini)	0.56	1.7	
Paramecium aurelia	Linear	13.8	30-35	~ 42	Goddard and Cummings (1975)
Plasmodium lophura	Circular	10.3	21.5	32	Kilejian (1975)
Tetrahymena pyriformis	Linear	~ 15	~ 30	~ 45	Goldbach et al (1977)
Trypanosoma mega	Circular	9.0(maxi)	16.1	27	
		0.74(mini)	1.49	2.2	Borst & Hoeij-makers (1979)
SLIME MOLDS					
Physarum polycephalum	Circular	19.1	41	57.3	Bohnert (1977)
FUNGI					
Yeasts					
Brettanomyces anomalus	Circular	18.2	~ 38	~ 54	Clark-Walker & McArthur (1978)
Brettanomyces custersii		~ 34	~ 71	10.2	Clark-Walker et al. (1981b)
Candida parapsilosis	Circular	11.1	23.1	33	O'Connor et al (1975)
Candida rhagii	Linear	10	15	30	Kovac et al. (1984)
Hansenula mrakii	Linear		36.4		Wesolowski and Fukuhara (1981)
Hansenula wingei	Circular	8.2	17.3	~ 25	O'Connor et al. (1975)
Kloeckera africana	Circular	8.3	~ 17.5	~ 25	Clark-Walker & McArthur (1978)
Kluyveromyces lactis	Circular	11.4	24.0	~ 34	O'Connor et al. (1975)
Saccharomyces cerevisiae	Circular	21-25	46-52	70-76	Gillham (1978)
Saccharomyopsis lipolytica	Circular	~ 14	~ 29	~ 42	Wesolowski et al. (1981)
Saprolegina sp.	Circular	14	28	42	Borst & Flavell (1976)
Schizosaccharomyces pombe	Circular	6.0	12.5	18	O'Connor et al (1975)
Torulopsis glabrata	Circular	6.0	12.8	18	O'Connor et al (1975)

TABLE 1.1 Contd.

Organisms from Various Taxanomic Categories	Shape	Length (μm)	Molecular weight (daltons) x 10^6	Molecular weight (Kbp)	References
Filamentous fungi					
Aspergillus nidulans	Circular	10-10.6	20-21	~ 32	Lopez-Perez & Turner (1975)
Neurospora crassa	Circular	18-22	41	~ 54	Bernardi et al. (1975)
Podospora anserina	Circular	31	61-63	95	Cummings et al. (1979)
METAZOA					
Nematoda					
Ascaris lumbricoides	Circular	4.8	~ 10.1	~ 15	Borst and Flavell(1976)
Crustacea					
Aretemia salina	Circular	5.1	~ 10.7	~ 16	Borst and Flavell(1976)
Insecta					
Drosophila melanogaster	Circular	6.2	12-35	18	Fauron and Wolstenholme (1976)
Echinodermata					
Lytechinus pictus	Circular	4.7	~ 9.9	~ 15	Borst and Flavell(1976)
Pisces					
Carrassius carrassius	Circular	5.4	~ 11.3	~ 16.5	Borst and Flavell(1976)
Amphibia					
Xenopus laevis	Circular	5.8	~ 12.2	~ 18	Borst and Flavell(1976)
Reptilia					
Terrapene ornata	Circular	5.3	~ 11.1	~ 16.5	Borst and Flavell(1976)
Aves					
Gallus domesticus	Circular	5.4	~ 11.3	~ 16.5	Glaus et al (1980)
Mammalia					
Bos sp.	Circular	~ 5.2	~ 10.9	~ 16.3	Anderson et al(1982)
Homo sapiens	Circular	~ 5.2	~ 10.9	~ 16.3	Brown et al. (1979)
ALGAE					
Chlamydomonas reinhardtii	Circular	4.5	9.8	~ 15	Mahler and Perlman(1979)
EMBRYOPHYTA					
Monocotyledoneae					
Triticum aestivium	Circular	1-30	140-200		Bonen & Gray (1980); Quetier and Vedel (1977, 1980)

TABLE 1.1 Contd.

Organisms from Various Taxanomic Categories	Shape	Length (μm)	Molecular weight (daltons) x 10^6	Molecular weight (Kbp)	References
Zea mays	Circular	0.5-30	~ 300	570	Ward et al. (1981); Lonsdale et al (1984)
Dicotyledoneae					
Brassica oleracea	Circular	20-30		217	Chetrit et al (1984)
Glycine max	Circular	5.9-29.9	150-240		Synenski et al (1978); Levings & Pring (1979)
Oenothera berteriana	Circular	~ 31	~ 66		Brennicke (1980)
	Linear	~ 22	~ 45		
Parthenocissus tricuspidata	Linear & Circles	5-30	60-165		Quetier & Vedel (1977, 1980)
Pisum sativum	Circular	5-30	~ 231		Kolodner and Tewari (1972); Ward et al (1981)
Solunum tuberosum	Linear		~ 100		Vedal and Quetier(1974)
Vicia faba	Circular		~ 70		Kolodner and Tewari (1972)

4. The mitochondrial genome of *Saccharomyces cerevisiae*

Over the past fifteen years, the mitochondrial genome of the baker's yeast *S. cerevisiae*, has attracted a great deal of attention from many molecular biologists and molecular geneticists, for intensive studies. This is partly due to the well known genetics of this yeast species and partly due to the ease with which various types of mutations in the mt-DNA could be isolated (Gillham,1974). Being a facultative anaerobic organism, it can live on non-fermentative media (glycerol, ethanol or TCA cycle intermediates), while its mt-DNA is functional. In the case of mitochondrial mutations it can survive on fermentative media such as glucose. Therefore, it would be useful to consider briefly the various types of mutations isolated in *S. cerevisiae* and the methods which have been employed to map these mutations genetically and physically on the mt-genome. It is, however, worth mentioning that the various mutations and their localisation on mt-DNA by genetic and physical mapping has demonstrated that within the various strains of *S. cerevisiae*, the size of these genomes may vary from a length of 70 Kbp (Short form) to about 76 Kbp (Long form) (Prunell *et al*, 1977; Sanders *et al*, 1977; Morimoto & Rabinowitz, 1979).

I. Mitochondrial mutations:

All types of mitochondrial mutations isolated from *S. cerevisiae* can be broadly divided into five main types: petite mutations, drug resistant mutations, *mit*$^-$ mutations, *syn* mutations and *mim* mutations. In the following paragraphs there is a brief description of each type of mutation obtained from *S. cerevisiae*. For details the reader is referred to reviews by Borst & Grivell (1978); Dujon (1983).

(a) Petite mutations

Petite mutations, first described by Ephrussi *et al* (1949) were the first cytoplasmic mutations to be shown associated with mt-DNA or the so-called rho-factor (ρ). They result from the large deletions of grande mt-genome and lead to a non revertible respiratory deficiency (Locker *et al*, 1974, Faye *et al*, 1973). A petite might lose all of its mt-DNA which is termed as ρ° or, may have a part of the grande genome (hence called ρ) which is

amplified to the extent of wild type genome (Fukuhara & Wesolowski,1977). These ρ petites are useful to biochemists and molecular biologists for three reasons. First, amplification of residual mt-DNA sequences provide a useful source of a smaller segment of grande mt-DNA in large quantities for various types of molecular analysis, hybridization analysis, DNA sequencing analysis etc. Secondly, by having a defined portion of the genome which can be transmitted to further generations without major errors, they provide a system to understand the molecular mechanism of replication, recombination, gene regulation on isolated segments and their contribution to the total function of the mt-genome. Third, petite mutants due to their ability to mate with wild type cells ($\rho^- \times \rho^+$) and to transfer the genetic markers contained in their mt-DNA to the wild type ρ^+ DNA, allow the characterisation of the genetic character of the mt-DNA segment to be retained (Carignani *et al*, 1979). ρ° strains, on the other hand, help to locate any newly isolated genetic marker on mt-DNA and to transfer one mt-genome of one particular nuclear background to another (by the process of cytoduction) while studying the nuclear effect on specific mitochondrial markers (Lancashire & Mattoon, 1979a).

(b) Antibiotic or inhibitor-resistant mutations

Various antibiotics or inhibitors which impair the mitochondrial function have been used to study the steps of complex biochemical reactions, such as electron transport and oxidative phosphorylation etc. (See Tzagoloff,1982). Mutations offering resistance against these antibiotics or inhibitors provide a useful tool to look deeper into the molecular mechanism of those reactions. Such mutations are broadly of two categories: those which block oxidative phosphorylation or electron transport and those which inhibit mitochondrial protein synthesis. Based on these mutants chloramphenicol(C) (Bunn *et al*,1970), erythromycin (E), Spiramycin (Spi) (Linnane *et al*,1968), antimycin-A (A) (Schweyen *et al*, 1978), Paromomycin (P) (Wolf *et al*, 1973, Linnane and Nagley, 1978), Oligomycin (O) (Avner *et al*, 1973, Avner & Griffiths, 1973a, 1973b), Ossamycin (Oss) (Houghton *et al*, 1974; Lancashire & Mattoon, 1979b), venturicidin(VEN), triethyltin(TET) (Lancashire & Griffiths, 1975a, 1975b), etc. loci have been identified. Table 1.2 describes a list of various drugs

TABLE 1.2. Antibiotic inhibitors of mitochondrial functions and the associated genetic loci on yeast mitochondrial genome (After Tzagoloff, 1982)

Antibiotic	Genetic locus	Site of inhibition
Chloramphenicol	cap	Large ribosomal subunit
Erythromycin	ery	Large ribosomal subunit
Paromomycin	par	Small ribosomal subunit
Oligomycin	Oli1, Oli3	ATPase
	Oli2, Oli4	ATPase
Venturicidin	Ven	ATPase
Ossamycin	Oss1, Oss2	ATPase
Antimycin	ana1, ana2	CoenzymeQH_2-cytochrome c reductase
Funiculosin	fun	CoenzymeQH_2-cytochrome c reductase
Mucidin	muc	CoenzymeQH_2-cytochrome c reductase
Diuron	diu	CoenzymeQH_2-cytochrome c reductase

and antibiotics with their sites of inhibition and associated genetic loci on the mt-DNA.

(c) *mit*$^-$ mutations

Tzagoloff *et al* (1975a, 1976) were the first to isolate and characterise a useful category of mutations designated as *mit*$^-$. Those mutations which affect a specific region of the mitochondrial genome and impair cellular respiration so that the mutations cannot grow on non-fermentative carbon sources (e.g. glycerol or ethanol) but retain mitochondrial protein synthesis unlike ρ^- cells. The *mit*$^-$ mutations are often revertible and respiration can be restored after crossing to a ρ^+ genome possessing normal sequences of the affected area.

(d) *Syn*$^-$ mutations

Syn$^-$ mutations are specifically defective in mitochondrial protein synthesis and normal alleles of these mutants are essential for the maintenance of the wild type (ρ^+) state. They have been localised either on the rRNAs (Davenish *et al*, 1978; Bolotin-Fukuhara, 1979), tRNAs (Dujon *et al*, 1977) or on the tRNA biosythetic locus (Martin & Underbrink-Lyon, 1979)

(e) *Mim* mutations

A type of mitochondrial point mutations which suppress the *mit*$^-$ phenotype, are termed *mim* mutations. These mutations themselves do not confer a mutant phonotype. At least two loci for mim mutations have been assigned.

Mim-1 is located within the large rRNA gene and restores the wild type phenotype of some mit-mutations, probably by decreasing the fidelity of translation (Dujardin *et al*, 1980). *Mim-2* on the contrary, is a locus within intron 4 of the Oxi-3 gene which can suppress the mutation of intron-4 of the Cob-box gene, probably by activating an otherwise dormant maturase (Dujardin *et al*, 1982). An ochre suppressor has also been discovered, which maps near the small rRNA gene (Fox & Staempfli, 1982).

II. Mapping of mitochondrial genes

Mapping of mitochondrial genes has been acheived both genetically (genetic mapping) and

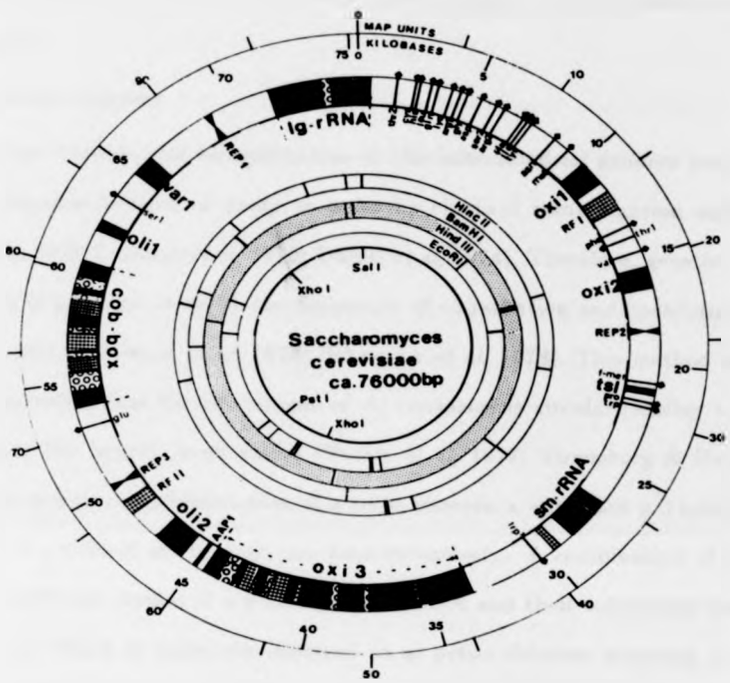


Figure 1.2. Physical and genetic map of the mitochondrial genome of the yeast *Saccharomyces cerevisiae*. The map has been taken from Dujon (1983). Restriction map (inner circles) shown here is from the "long strain" (KL14-4A). For comparison with the short strain, see Morimoto & Rabinowitz (1979). The genes have been placed on the genetic map (outer circle) on scale with respect to restriction sites. The references for various genes have been given in the text. ■ exons, ▩ open reading frames, ▩ introns.

physically at DNA level (physical mapping). A combination of both has led to the construction of the circular 76 Kbp map of mt-genome from *S. cerevisiae* which is shown in Fig. 1.2.

(a) Genetic mapping

The high frequency of recombination in the mitochondrial genome poses the problem of unambiguous location of genes in order by classical recombination analysis (Thomas & Wilkie, 1968; Linnane *et al*, 1972; Dujon *et al*, 1974). Therefore, genetic mapping of mitochondrial genes are done by the frequency of co-retention and co-deletion studies (Molloy *et al*, 1975; Schweyen *et al*, 1976; Schweyen *et al*, 1978). This method allows the genetic demonstration that the mt-genome of *S. cerevisiae* is circular (Molloy *et al*, 1975). Other methods like zygotic gene rescue (Butow *et al*, 1977; Strausberg & Butow, 1977) which involve genetic complementation of a cross between a *mit*$^-$ (but ρ^+) tester strain and a ρ strain of a defined mt-genome, are used extensively. A combination of both co-retention and co-deletion studies of a petite genomic DNA and their subsequent cross to a (ρ^+) tester strain which is sometimes referred to as petite deletion mapping, is a very powerful tool for generating a fine structure map of a piece of mt-DNA segment (Carignani *et al*, 1979).

(a) Physical mapping

Physical mapping is achieved by any one or a combination of the following methods:

i) Restriction digest analysis

The specificity of restriction enzymes (Roberts,1981) enables generation of an ordered structure of fragments. Using a combination of restriction enzymes, a restriction map of mt-DNA from two strains of *S.cerevisiae* (Short form and Long form) has been obtained (Morimoto *et al*, 1977; Morimoto & Rabinowitz, 1979a,1979b)

(ii) Hybridization analysis

Using mt-DNA from a defined petite as a probe the relation between the genetic markers

carried by various petites could be analysed by Southern hybridization analysis (Morimoto *et al.* 1976; Sanders *et al*, 1976) This could be related back to the wild type mt-genome to construct a detailed map (Borst *et al*, 1979).

Labelled mt-RNAs could also be employed to find out the location of a specific gene. tRNAs & rRNAs were located on mt-DNA by such methods (Morimoto *et al*, 1978; Sanders *et al*, 1975).

(iii) *In vitro* transcription and translation analyses

Transcription and translation of mt-DNA segments *in vitro* has been employed to identify and locate a particular gene product on the mt-genome (Morimoto *et al*, 1978; Grivell & Moorman, 1977).

(iv) EM studies

Heteroduplex analyses including partial denaturation mapping and RNA-DNA heteroduplexes under electron microscope have been employed to localise the structure-function relationship of a particular gene on the wild type mt-genome (Bos *et al*, 1978; Faye *et al*, 1979).

5. Mitochondrial genes

I. In *Saccharomyces cerevisiae*

Most genes and their products identified in *S. cerevisiae* have been shown in Fig. 1.2 and Table 1.3 on the basis of genetic analysis and DNA sequence analysis from various strains of yeasts ("long form" or "short form"). This section, however, provides a synopsis of all these results. For details see Borst & Grivell,1978; Morimoto & Rabinowitz,1979a,1979b; Dujon,1983; Evans,1983).

(a) Genes for ribosomal RNAs (components for protein synthesising machinery)

In *S. cerevisiae*, the two rRNA genes, large rRNA & small rRNA genes are separated by a length of about 25Kbp DNA and they are not co-transcribed, a phenomenon which is quite common in nuclear rDNA clusters (Sanders *et al*, 1975; Borst, 1977). Controlling ele-

Table 1.3 Gene and Gene Products of Yeast Mitochondria

Mitochondrial Complexes	Gene Products	Associated Genetic Loci	Approximate Map Locations
Cytochrome c oxidase (Complex IV)	Subunit-I	Oxi-3	53-56
	Subunit-II	Oxi-1	13
	Subunit-III	Oxi-2	21
Cytochrome bc_1 Complex (Complex III)	Apocytochrome b	cob-1 & cob-2, box 1-10 *ana*^{r}, *fun*^{r}, *diu*^{r}, *muc*^{r}	72-82
O.S.ATPase Complex (Complex V)	Subunit-6	Oli2, oli4, Oss1, pho1	60-65
	Subunit-8	pho8 (?)	58
	Subunit-9	Oli1, Oli3, pho2	82-84
	Subunit-3 (?)	?	
Ribosome Complex	Mitoribosome associated polymorphic protein	var-1, var-2, var-3	87
	21S rRNA	rib-1, rib-2, rib-3 *cap*^{r}, *spira*^{r}, *ery*^{r}	96-100
	15S rRNA	*par*^{r}	~35-38
25 tRNAs	tRNAs	Several *syn*$^-$ mutants	
tRNA biosynthetic locus	Catalytic RNA (?)	*syn*$^-$	29

ments regulating equal production of the two RNAs, however, are unknown.

(i) 21S rRNA gene

This gene is transcribed to produce 21S rRNA (analogous to the prokaryote 23S & eukaryotic 26S rRNAs) needed for the biogenesis of the large ribosomal subunit (50S) of the mito ribosome. DNA sequence analysis of this gene has been completed (Dujon, 1980). Polarity of recombination, associated with the genetic locus omega (ω), in this region was shown to be due to an insertion of 1.1 Kbp DNA in ω$^-$ strains (Dujon, 1980; Jacque *et al*, 1977). DNA sequence analysis has confirmed the location of an intron (of precise length 1.143 Kbp) and identified another smaller insertion of 66 bp, located 156 bp upstream of the former. The latter insertion however, is not spliced unlike the longer intron from the functional 21S rRNA and does not impair the normal function of the ribosome. Part of the small insertion (5'-TTCGGGGTTCCGG-3') is identical to other (G + C) rich clusters found elsewhere in the genome (Prunell & Bernardi,1977) which led Dujon to suggest its probable transposon activity. The discovery of an open reading frame (of 235 aminoacids) within the long intron by DNA sequencing analysis has raised the question of the function of this hypothetical protein, either in ω polarity or as a reminiscent of a "fossil" gene. The 3' end of the rRNA gene is highly homologous with 23S rRNA from *E. coli*, and chloroplasts, which suggests that the sequence of this region is functionally important. In fact, two chloramphenicol resistant mutations have been localised in this region (Dujon,1980).

(ii) 15S rRNA gene

This gene codes for 15S rRNA (analogous to prokaryotic 16S rRNA and eukaryotic 18S rRNA) which assemble with ribosomal proteins to form small (37S) mito-ribosomal subunits. The gene has been sequenced (Sor & Fukuhara, 1980) and is estimated to be 1659 bp long (the rRNA sequence is not known) based on comparison with *E. coli* 16S rRNA. Association of paromomycin resistivity with this gene, which had been a controversy for several years, has however, been confirmed by sequencing analysis of *par*^{r} gene by Li *et al*

(1982). No Shine & Dalgarno sequence (Shine & Dalgarno,1975), which is involved in mRNA binding to the ribosome for translational initiation, has been found. However, a putative Shine & Dalgarno like sequence (3'-AAATTCTATA-5') has been proposed by Tzagoloff (1982) on the basis of a complementary sequence found upstream of the initiation codon of several mit-genes (See also Li *et al*, 1982).

(b) Genes for tRNAs (components for protein sythesising machinery)

25 tRNA genes have been localised on mt-DNA of which DNA sequences from 23 are known (See Dujon,1981). Hybridization analysis of labelled tRNAs and DNA sequence analysis has established that the majority of them (19) are located between 21 S rRNA and the *Oxi-2* locus (encoding subunit 3 of cytochrome oxidase). Four tRNAs ($tRNA^{met}$, $tRNA^{pro}$, $tRNA^{trp}$-1 and 2) are scattered within 20-50 map units of the genome (see Fig. 1.2), while one each of $tRNA^{glu}$ and $tRNA^{ser}$ occur in the remaining portion of the genome. The tRNAs in the cluster are separated by varying lengths of DNA from 3 bp to 85 bp (Bos *et al*, 1979; Bonitz & Tzagoloff, 1980). The genes are mostly 70-80 bp long and lack coding sequences for the 3'-CCA (aminoacid binding site) terminus which is presumably added post-transcriptionally. The mt-tRNAs have a low (~ 18-35%) (G+C) content, but show typical "clover-leaf" structures (Holley *et al*, 1965) with -TΨC-, D, anticodon loops and acceptor stem. An anomaly is found in $tRNA^{asp}$ (Bonitz & Tzagoloff, 1980). The existence of only 25 tRNAs available for the recognition of all possible codons in mRNA undermines Crick's wobble hypothesis (Crick,1966) which requires a minimum of 32 tRNAs.

(c) tRNA biosynthesis locus

At 29 map units of the mt-DNA of *S. cerevisiae*, a locus has been described to be responsible for processing tRNAs (Underbrink & Lyon et al, 1983). DNA sequencing analysis suggests that this region codes for a 9S (A+U) rich RNA which is necessary for the catalytic activity of a RNA processing enzyme similar to the RNase P observed in *E. coli* (Miller *et al*,1983).

(d) Genes for cytochrome oxidase (components of the respiratory chain)

Three large subunits of cytochrome-c oxidase (subunit-I, subunit-II and subunit-III) are coded by the mitochondrial genome. They are not arranged in an operon, a feature found in the prokaryotic genome.

i) Subunit I of cytochrome oxidase

This subunit is encoded by a complex genetic locus Oxi-3 which produces a polypeptide of molecular weight of 55,933 daltons. The complete DNA sequence has been determined by Bonitz *et al* (1980) in "short form" (D273-10B) and by Hensgens *et al* (1983) in "long form" (KL14-4A and 777-3A) of yeast strains. The DNA sequence analysis indicates that the yeast gene consists of seven to eight exons separated by 6 to 7 introns (aI_1 to aI_7) in short form while in long form, nine to ten exons are separated by eight to nine introns (aI_1 to aI_9). The uncertainty arises due to the lack of protein sequencing data for this subunit from yeast. Potential exon - intron boundaries and coding sequences have been determined by comparison with human CO1 (cytochrome oxidase I) gene. The first four introns, (five in the case of "long form"), however, contain long open reading frames (ORFs) in phase with their upstream exons and are potentially able to specify polypeptides of 30 to 80 Kilodaltons (Bonitz *et al*, 1980a; Hensgens *et al*, 1983). The expression of subunit-I has been shown to be controlled by "maturase" coded by Cob-box intron (bI_4). Surprisingly, no *Oxi-3* mutations have been identified to occur in the introns, a remarkable difference from the Cob-box or apocytochrome b gene (see next). The recent discovery of *mim*-3-1 mutations and *nam*-3-1 mutations (Dujon *et al*, 1983) have demonstrated that intron aI_1 which has a strong sequence homology with the fourth intron of the cytochrome b gene (bI_4) can mutate to specify an active maturase. Recently, Groudinsky *et al* (1983) have provided some evidence that the first intron (aI_1) might code for a maturase.

The molecular weight of this subunit derived from DNA sequence analysis is different from the observed mol. wt. (44 000 daltons) in SDS gels (Rubin & Tzagoloff, 1973). Post-

translational modification could be a reason for this anomaly.

ii) Subunit II of cytochrome oxidase

This subunit of cytochrome oxidase is encoded by the *Oxi-1* genetic locus. The DNA sequence analysis of this gene has been completed by Coruzzi & Tzagoloff (1979) and Fox (1979). The gene is 756 nucleotides long (specifying 251 aminoacid containing polypeptides) with a 27% (G + C) content. The deduced aminoacid sequence from nucleotide sequence analysis is consistent with the aminoacid composition and molecular weight of yeast cytochrome oxidase subunit II (Poyton & Schatz, 1975; Poyton *et al*, 1978).

iii) Subunit III of cytochrome oxidase

This polypeptide is encoded by the genetic locus *Oxi-2*. The DNA sequence has been determined by Thalenfeld and Tzagoloff (1980). No intron has been observed within a coding frame of 807 nucleotides (269 aminoacids). The deduced aminoacid composition is consistent with the aminoacid composition data obtained directly by analysing the protein (Thalenfeld & Tzagoloff, 1980). The molecular weight (30, 340 daltons) deduced from DNA sequence analysis is 20% higher than the previous estimates by SDS-gel electrophoresis (Poyton & Schatz, 1975).

(e) Gene for apocytochrome b (component of respiratory chain)

This is another example of a spliced gene in yeast mitochondria, coded by a complex locus called "Cob-Box". The complete DNA sequence from a "short form" strain (D273-10B) has been determined by Nobrega & Tzagoloff (1980) and that from a "long form" strain by de la Salle *et al* (1982) and partially by Lazowaska *et al* (1980). Mutants resistant to antimycin A (*ana*), diuron (*diu*), funiculosin (*fun*) and mucidin (*muc*) map to this region (Mahler *et al*, 1978). Extensive biochemical genetic and molecular studies have produced many interesting results as well as publications, with reference to the splicing mechanism of this gene (Church *et al*, 1979; Lazowska *et al*, 1980; Nobrega & Tzagoloff, 1980; Halbreich *et al*, 1980; Dujardin *et al*, 1980a, 1980b; de la Salle *et al*, 1982).

Genetic studies revealed that mutations in the Cob-box region fall into two groups: (A) box-1, box-2, box-4, box-5, box-6, box-8, and box-9 which were assumed to contain exons and (B) box-3, box-7 and box-10 which contain the non coding introns (Slonimski *et al*,1978). Moreover, box-3, box-7 and box-10 mutants were pleiotropic and blocked expression of the *Oxi-3* gene ("box" effect). When more detailed physical structures of the Cob region were obtained, it was found that the gene structure was strain dependent. In its "long form", the gene contains six exons (B_1 - B_6) and five introns (I_1 - I_5) while in its "short form" it contains three exons and two introns. The short form of the gene is related to the long form and can be derived by removal of I_1 - I_3 and fusion of B_1 - B_4 (Fig. 1.3a). In addition, the long form of the gene contains at least two long open reading frames which are continuous with the proceeding exon. These are I_1 - B_2 - I_2 (span over box-3) and B_4 - I_4 (span over box-7, Fig. 1.3b).

A mechanism for the maturation of the primary transcript of the Cob-box region has been proposed by Lazowska *et al* (1980) which is depicted in Fig. 1.3c. The first splicing reaction fuses B_2 to B_1 with the removal of I_1 as a circular 10S rRNA molecule (Church *et al*, 1979; Van Ommen *et al*, 1980). This step is regulated by nuclear coded enzyme(s) and produces a mRNA for synthesis of a protein called box-3 RNA maturase. The translation of the protein follows open reading frame from B_1, B_2 into box-3 of I_2 (Lazowska *et al*, 1980). This could be visualised by the presence of the first 143 aminoacids of cytochrome b at its N-terminal end, connected to 280 aminoacids of I_2. The box-3 maturase is necessary for the second processing step which specifically removes I_2 and catalyses the fusion of B_3 to B_2 B_1. The next steps of the processing are still not well characterised, but eventually lead to fusion of B_1 B_2 B_3 and B_4 B_5 B_6 by removing I_3 I_4 I_5. Box-7 or I_4 has also an open reading frame and probably codes for another trans acting factor (TAF) which is homologous to aI_4 of cytochrome oxidase subunit-I (see above). The pleiotropic effect of box-7 has a direct effect on *Oxi* -3 expression while the effect of box-3 seemed to be a consequence of its effect on box-7 (Dhawale *et al*,1981). Although no protein homologous to I_2 or I_4 intron coded maturases are seen in the gel, the antibodies against a synthetic

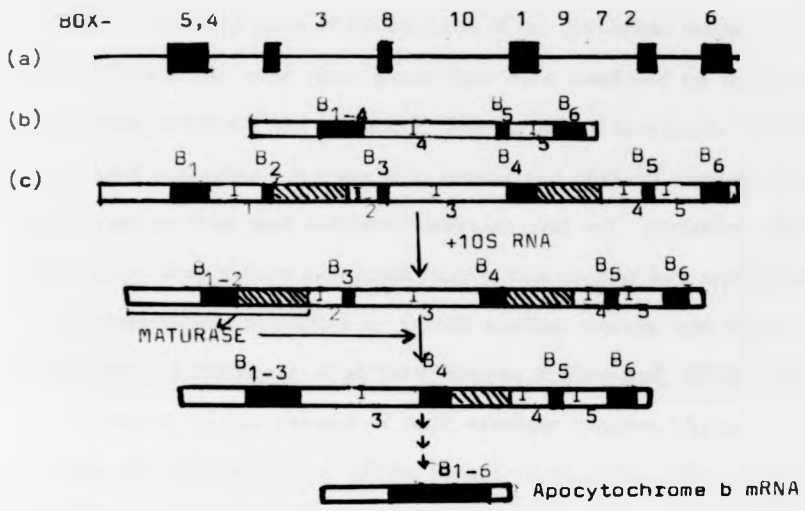


Figure 1.3 Organisation and expression of the COB gene in yeast mitochondria (after Church *et al*, 1979).

(a) Location of the *box* mutation clusters in relation to the exon (shaded boxes) and introns (thin line) components of the gene.

(b) Short form of the gene with three exons (shaded) and two introns (unshaded).

(c) Proposed scheme for the synthesis of mature apocytochrome b mRNA from primary transcript of long form of the gene (with six exons (shaded) and five introns (unshaded)). Open reading frames within introns are cross-hatched.

oligo-peptide (deduced from the ORF of the intron) has identified a protein emphasising the fact that the reading frame is expressed at the protein level (Banroques *et al*, 1984; Guiso *et al*, 1984).

(f) Genes for the O.S. ATPase (Complex V)

Mt-DNA codes for some of the subunits of the membrane sector (F_o) of ATP synthase or O.S.ATPase. At least three genes have been identified on the mt- genome: genes for subunit-6, subunit-8 and subunit-9. The purpose of this thesis is to identify and sequence subunit-6 and subunit-8 genes with genetic and physical mapping involving both normal and various drug and antibiotic resistant and *mit* mutation related to these genes. Therefore, they will be mentioned later in this chapter in a separate section on oxidative phosphorylation. Subunit-9 or DCCD binding protein and its gene has already been characterised (Hensgens *et al*, 1979; Macino & Tzagoloff, 1979). Various mutations have been located in this subunit of ATP synthase complex (Avner & Griffiths, 1970; Lancashire & Griffiths, 1975a, 1975b; Sebald *et al*, 1979). This proteolipid is the smallest subunit (Mol. wt. 7.5Kilodaltons) in this complex. Conventionally the subunit is called proteolipid, but no lipid molecule has been shown to remain conjugated with it covalently. The stability of this subunit in organic solvent (phenol/chloroform) has led to the use of this term. The gene has been sequenced independently by Hensgens *et al* (1979) and Macino & Tzagoloff (1979). The reading frame (228 nucleotides long) has been correlated with the aminoacid sequence from direct sequencing of the protein (Sebald & Wachter, 1978).

(g) Var-1 gene

This has been related to the Var-1 locus which has been extensively studied by Butow and his colleagues (Douglas & Butow, 1976; Butow *et al*, 1977, 1980). The gene encodes a protein associated with the small subunit of the mitoribosome (Groot *et al*, 1979). As the name implies, the size of the Var-1 protein is variable (40-44 kilo dalton) without any alteration of function. The variation in mol. wt. of the protein has been correlated with

variation in the size of the genes (Hudspeth *et al*, 1984). After the initial controversy regarding identification of this gene due to high A+T content (see Tzagoloff *et al*, 1980), the existence of Var-1 gene has been settled and the gene has been sequenced by Hudspeth *et al* (1982). An insertional phenomenon has been correlated with the variation of the size of the protein as well as to the polarity phenomenon, similar to the ω locus of 21S rRNA gene (Strausberg & Butow,1981). Recently, Hudspeth *et al* have sequenced 6 species of Var-1 gene and have demonstrated how in-frame insertion of this locus brings about the molecular weight size variability of this protein (Hudspeth *et al*, 1984).

(h) Unassigned Reading Frames (URFs)

DNA sequencing studies of parts of mt-DNA of yeast have yielded some 10 unknown reading frames:

(i) One has been found in the long optional intron of the 21S rRNA gene (Dujon, 1980)

(ii) Three have been found in the introns of the gene for apocytochrome b (Cob -box) (Nobraga & Tzagoloff, 1980; Lazowska *et al*, 1980, 1981).

(iii) Four lie in the introns of *Oxi*-3 or the cytochrome c oxidase subunit-1 gene (Bonitz *et al*, 1980).

(iv) One is situated near the *Oxi*-3 gene (Coruzzi *et al*, 1981)

(v) One lies between the Oli-2 and the Cob-box gene.

The URFs found within the introns have been speculated to be "maturases" which are involved in the splicing mechanism of the associated RNAs. One near the Oxi-1 gene has also been advocated to have maturase like function, as a result of the homology with the intron coded reading frame. However, none of them has been shown to be homologous with the URFs observed in mammals or other organisms (see next section). One recent publication (Zimmerman, 1983) has revealed that a group of plant RNA viruses encodes some proteins which have a substantial homology (15 - 23%) with the yeast mitochondrial introns. The significance of this is not known.

II. Mitochondrial genes in other organisms

The mitochondrial genes in *S. cerevisiae* described in the earlier section are common to all mitochondrially coded genes from other organisms, except for the following deviations:

(i) Genes for subunit-9 of O.S. ATPase or ATP synthase are specified by the nuclear genome in other organisms (Human, Bovine, Mouse, Rat, *Neurospora*, *Aspergillus*, *Drosophila*). In *Neurospora* however, besides its functional nuclear gene, one silent gene (mal) has been discovered on the mitochondrial genome (Van den Boogaart *et al*, 1982).

(ii) In mammals, only 22 tRNAs genes exist (Anderson *et al*, 1981, 1982; Bibb *et al*, 1981) as opposed to 25 in yeast.

(iii) In plant mitochondria, mt-DNA seems to encode more proteins than others. It has already been shown that the 5S rRNA gene which is absent in mitochondrial ribosome from other organisms, does exist on plant mt-DNA and is transcribed (Leaver & Harmey,1976). The α subunit of the F_1 sector of the O.S. ATPase has also been shown to be coded by the mt-genome in maize (Leaver *et al*, 1983).

(iv) At least 8 URFs have been found to occur in mammalian mt-DNA which have been sequenced from human (Anderson *et al*, 1981), mouse (Bibb *et al*, 1981), rat (Grosskopf & Feldman, 1981; Gortz & Feldman, 1982; Cantatore *et al*, 1982), bovine (Anderson *et al*, 1982). 11 URFs have been identified in *Aspergillus* (Grisi *et al*, 1982; Netzkar *et al*, 1982; Scazzacchio *et al*, 1983). Some of the mammalian URFs have been shown to be expressed (Mariottini *et al*, 1983; Oliver *et al*, 1983) but the functions of these still remain unknown. Four *Aspergillus* URFs have been correlated with four of the mammals on the basis of homology (Scazzacchio *et al*, 1983). URF X of Aspergillus and URF A6L of mammals have been shown to be homologous to the subunit-8 of the O.S. ATPase complex of yeast.

(v) Intron coded URFs found in *S. cerevisiae* have not been observed in other organisms.

6. Organisation of the mitochondrial genome of *Saccharomyces cerevisiae*

Mt-DNA of *S.cerevisiae* has one of the lowest G+C contents (18%) of any functional

DNA. Bernardi and his co-workers have studied the genomic organisation of *S. cerevisiae* in detail and has established four distinct sequence elements in mt-DNA (Bernardi *et al*, 1972; Prunell & Bernardi, 1977; Prunell *et al*, 1977; Fonty *et al*, 1978):

(i) (A+T) rich stretches (GC<5%) with an average mass of 9 x 10^5 daltons; these make up about 50% of the DNA and are called "spacers" by Bernardi.

(ii) Intermediate GC-rich stretches (GC varying from 22-65%) about the same size as the AT rich stretches and called "genes" by Bernardi; these also constitute about 50% of the DNA.

(iii) Site clusters, which are short elements containing several recognition sites for restriction endonucleases *Hae* III (GGCC) and *Hpa* II (CCGG).

(iv) GC rich clusters which are also short but characterised by the absence of any of the tetranucleotides GGCC, CCGG and GCGC.

Bernardi *et al* proposed that these sequence elements are arranged in units as follows: -(G+C) rich cluster - site cluster - genes - spacer-. A possible function of this organization as proposed by Bernardi *et al*, is that repeated sequences within the GC clusters and AT spacers provide regions of homology for illegitimate, site specific recombination, giving rise to the observed polymorphism of the mitochondrial genome as a result of duplication, inversion, translocation and deletion. In a more extreme case, DNA sequence analysis has revealed that the rearrangements of the genome that occur during the formation of the petite mutation follow the excision of a small fragment of the genome and this excision occurs preferentially at a repeated site within the GC clusters or AT spacers (Faugeron-Fonty *et al*, 1980; Baldacci *et al*, 1980; Gaillard *et al*, 1980). This function, however, has been questioned by Borst *et al* (1977) and they have proposed that AT rich segments are involved in the processing of precursor RNAs and illegitimate recombination and consequent rearrangements of genome are the side effects. Another intriguing possibility has been suggested by the observations on the control of gene expression by regions distant from the coding sequences, particularly the possibility that supercoiling and superhelix

density may be important in the regulation of transcription (Smith,1981). The AT-spacers may, therefore, influence mitochondrial gene expression by an effect on DNA structure, rather than by a direct effect on the processes themselves.

7. Mitochondrial transcription

Transcription of mt-DNA of *S. cerevisiae* appears to be different from that of mammals. In mammals, the whole mt-DNA is transcribed completely and symmetrically (Murphy *et al*, 1975), even though most of the genes are localised on the H strand with a few on the L strand. Evidence for the existence of a single promotor near the H strand replication origin (Cantatore & Attardi, 1980) has been cited (Amalric *et al*, 1978). A "tRNA punctuation" model for processing mRNA has been advocated (Mokaka *et al*, 1981), due to the occurrence of tRNAs at the 5' and 3' end of most of the protein coding reading frames. However, the occurrence of a 30 to 60 fold higher level of rRNA transcripts has led to questioning of such a simple control mechanism (Attardi *et al*, 1983). In *S. cerevisiae*, the existence of multiple promotors and splicing machinery to process "mosaic" genes and co-transcription of certain genes have made the whole process much more complex (See Grivell,1983).

(i) Mitochondrial RNA polymerase

Levens *et al* (1981), purified a DNA-dependent RNA polymerase from yeast mitochondria which uses mt-DNA as a preferred natural template and poly d(AT) as synthetic template. This is similar to the properties of *Xenopus laevis* mitochondrial RNA polymerase (Wu & David, 1972), with respect to its salt sensitivity and α-amanitin and rifamicin insensitivity but different from equivalent nuclear and bacterial enzymes.

(ii) Promotors and initiation of transcription

Some 19 different RNAs are capable of capping reactions with guanylyl transferase and $[\alpha-^{32}P]$-GTP, suggesting that they represent separate distinct transcription initiation sites with distinct promotors (Edwards *et al*, 1983). 13 of them have been mapped in the sequenced region of the *S. cerevisiae* mt-genome, either upstream of known genes or in the

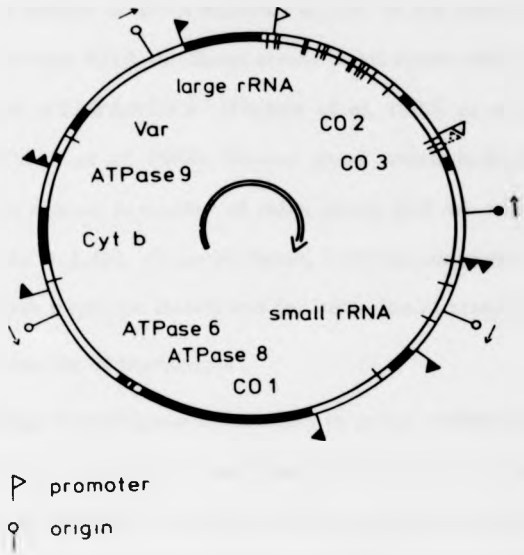
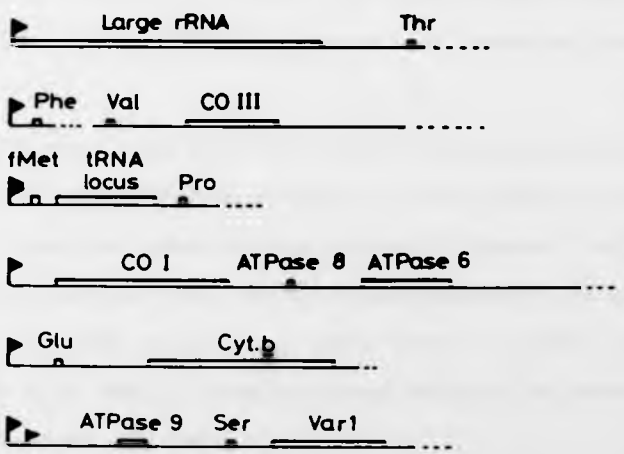


Figure 1.4 Transcriptional map of the mitochondrial genome of *Saccharomyces cerevisiae* (afterTabak *et al*, 1983) (a) Physical of yeast mt-DNA showing sites for initiation of transcription and origins of DNA replication. Filled in flags are proven sites for initiation and open flags are positions containing the initiation sequence (see text). Circles indicate replication origins with the arrow showing the direction of replication.



(b) Possible multigene transcription units in yeast mt-DNA. Stippled lines indicate uncertainty in the exact limits of primary transcripts, which are not drawn to scale.

putative origins of DNA replication (Ori or rep sequences, see later section). It has been observed that RNA synthesis starts in all cases within a short nonanucleotide conserved sequence: ATATAAGTA^{-} (Osinga *et al*, 1982) or a close variant of it (Edwards *et al*, 1983; Tabak *et al*, 1983). Several genes which lack this motif are transcribed together with the nearest promotor of other genes and are assumed to be processed subsequently (Fig. 1.4a & 1.4b). Some evidence, however, suggests that these "nonanucleotide boxes" themselves might be insufficient for initiation of transcription (Tabak *et al*, 1983).

(iii) Processing of transcripts

Processing of multigenic transcripts in yeast, unlike tRNA signalling processing of mammalian and *S. pombe* transcripts is thought to be mediated via some sort of signal sequences occuring within the intergenic region of mt-DNA. The cleavage signal of a hepta nucleotide (AAUAUAA) in between the Var-1 gene and ATPase subunit-9 + $tRNA^{arg}$ gene does not seem to be universal (Zassenhaus & Butow, 1983). Thalenfeld *et al*, (1983) have proposed a sequence of "ATTCTTA" which is involved as a processing signal between subunit-9 and $tRNA^{arg}$ of the same transcript. The role of the G+C rich palindrome as a processing signal which occurs in the intergenic portion of the mitochondrial genome has also been suggested (Tzagoloff *et al*, 1980). The tRNA clustered region which is co-transcribed, is processed via a "tRNA biosynthetic locus" dependent factor (mentioned earlier).

Processing of "split genes" appears to be a complex mechanism involving intron coded maturases (Lazowska *et al*, 1980; Weiss-Brummer *et al*, 1982; Dujardin *et al*, 1982; Anziano *et al*, 1982); numerous nuclear encoded products (Dieckmann *et al*, 1982; Faye & Simon, 1983; Dieckmann *et al*, 1984); critical sequences away from the spliced junctions (Weiss-Brummer *et al*, 1982; Anzaino *et al*, 1982; Netter *et al*, 1982; Schmelzer *et al*, 1982; de La Salle *et al*, 1982) and probable internal secondary structure of the introns (Davies *et al*, 1982; Michel *et al*, 1982).

Mitochondrial introns are broadly of two types:

(a) Group I introns have a common potential secondary structure, essential for splicing, and reading frames, when present, contain a region of sequence homology bounded by distinctive decapeptide blocks (Waring *et al*, 1982; Hensgens *et al*, 1983).

(b) Group II introns have a different but distinctive potential secondary structure (Michel & Dujon, 1983); they have a sequence homology at 3' ends (Schmelzer *et al*, 1982) and produce covalently closed circular RNAs after excision (Annberg *et al*, 1980; Halbreich *et al*, 1980; Hensgens *et al*, 1983). Reading frames might also occur in them (Bonitz *et al*, 1980).

As Group I introns are conserved in the nuclear rDNA of *Tetrahymena* and *Physarum*, Davies *et al* (1983) suggest that there might be an evolutionary relationship of a splicing mechanism, irrespective of whether they code for "maturases".

8. Mitochondrial translation

Detailed reviews on this aspect can be found in Gray (1982) and Evans (1983) Three points must be mentioned here:

(i) Mitochondrial rRNAs show a low level of post transcriptional modification, a feature common amongst prokaryotes rather than eukaryotes. How this is related with ribosome structure and translation is yet to be discovered.

(ii) Like prokaryotes, protein translation always starts with f-met which remains uncleaved in the matured protein (Bianchetti *et al*, 1977).

(iii) The "Non-standard" genetic codes used in mitochondria are different from the "universal code" as well as from each other. Table 1.4 shows some of the characteristic features of the mitochondrial code from different organisms. For details see Grivell (1983).

Table 1.4 "Non standard" genetic code in Mitochondria

Codon	Universal code	Yeast	Mammal	Insect	Plant
UGA	STOP	Tryptophan	Tryptophan	Tryptophan	STOP
AGA	Arginine	Arginine	STOP	Serine	
AUA	Isoleucine	Methionine	Methionine	Methionine	Isoleucine
CUN	Leucine	Threonine	Isoleucine	Isoleucine	Isoleucine

Why the mt-DNA codon recognition rule is so different among different organisms is not clear. The dissimilarity of the codon rule establishes the fact that if they have a common origin they have been fixed at different times in different organisms during the course of evolution (see Gray, 1982).

9. Replication of the mt-genome

Replication of yeast mt-DNA is not as well understood as it is in the mammalian mit-genome. In mammalian mt-DNA replication begins at a unique origin (Crews *et al*, 1979; Gilum & Clayton, 1979) with the synthesis of about 680 nucleotides of H strand (7S DNA). This forms a displacement loop (D-loop) by base pairing to the parental L strand. Replication continues by the extension of some of the 7S DNA molecule, thus extending the D-loop. In yeast mitochondria, similar D-loop structures have been identified (Locker *et al*, 1974). Mapping and hybridization studies have established that the yeast mt-genome contains at least seven putative origins of replication ("ori") sequences unevenly distributed over the genome (de Zamaroczy *et al*, 1981). All "ori" sequences have a common 265 bp structure with two short G+C rich clusters (A & B) flanked by a 23 bp A+T rich palindrome, at one end, separated from another G+C rich cluster (C) at the other end by an extensive A+T rich sequence. In "hyper-suppressive" petites, Blanc & Dujon (1980) have demonstrated that their mt-genomes contain amplified "ori" sequences which could be the reason for their hyper-suppressivity. ("ori" sequences are represented as "rep" sequences by Blanc & Dujon, 1982). The lack of "ori" sequences in some petites

indicates that mt-DNA polymerase can recognise and replicate virtually any mt-DNA sequence, but does so most efficiently in the presence of "ori" sequences.

Evidence exists that "ori" sequences can behave like ARS (autonomous replication sequences) in the cytoplasm of yeast (Fangman *et al*, 1984). In a particular clone "rep-2" of *S. cerevisiae*, it has been suggested that ARS activity of "ori" sequences could be due to two types of sequences:

(i) a palindrome in the GC cluster of rep-2

(ii) a consensus 11bp ARS like sequence $\frac{A}{T}AA\frac{C}{T}ATAAA\ \frac{T}{A}$

It has also been noted that "ori" sequences of mitochondria of *S.cerevisiae* work like ARS in their own but undergo rearrangement in *S. pombe* and do not exhibit ARS activity. On the contrary, "ori" sequences from *S. pombe* exhibit ARS activity in the cytoplasm of both species.

A mt-DNA polymerase from yeast which is very sensitive to Mn^{2+} and different from that of the nuclear polymerase has also been reported (Wintersberger & Blutson, 1976).

10. Mitochondrial recombinantion

Very little is known of the mitochondrial recombination mechanism in *S. cerevisiae*. For details see Birky, 1978; D. Wilkie, 1983; Fonty *et al*, 1978. From genetic analysis it is known that the rate of recombination is very high (Birky, 1978). Recombination studies in our laboratory have demonstrated that intra-codon recombination could be as high as 0.01 to 0.1% (Connerton *et al*, 1984b). E. Sena (1984) has reported, from EM studies, that 2.2% of the total mt-DNA molecules observed on the EM grids possess a "X" structure. Higher percentages of A+T sequences in mit-DNA probably provide some sort of homologous blocks for recombination.

11. Genetic interaction between nuclei and mitochondria

Mitochondria, privileged by their own genomic DNA, albeit limited in coding capability,

regulate some of their own functions. Nuclear DNA still plays the major role in encoding most of the proteins needed for the maintenance of their genome as well as other functions. Therefore, it is expected that there would be a continuous interaction along the nuclear-mitochondrial axis.

(a) From nucleus to mitochondria

About 95% of mitochondrial proteins are encoded by the nuclear DNA. It is therefore obvious that the nucleus of the cell has most control over mitochondrial function. Various mutations have been identified in the nucleus which modulate the mitochondrial functions. They are generally of three types: *pet* mutations (Michaelis *et al*, 1982; Sherman, 1963), nuclear drug resistant mutations (see Evans, 1983), and NAM (nuclear accomodation of mitochondria), a type of nuclear suppressor mutation that acts on specific *mit* loci (Dujardin *et al*, 1980). Some temperature sensitive mutants which block the transport of proteins to the mitochondria are also worth mentioning (Burke *et al*, 1976; Yaffe, 1983; Yaffe & Schatz, 1984). Molecular analyses of these mutants would be instrumental in understanding the basic control of the nucleus in mitochondrial function.

(b) From mitochondria to nucleus

There is no direct evidence that mitochondria can control nuclear function. Some circumstantial evidence however, suggests that mitochondria are somehow involved in functions outside their own membrane territory. It has been observed that respiratory deficient petite mutations (ρ^-) exhibit changes in the cell surface characterisitics, for example concavanavalin A agglutination, permeability to certain sugars and other metabolites, flocculation characteristics and partitioning behaviour in a biphasic polymer system (see Wilkie & Evans, 1982). It has been suggested that these effects might not be due to lesions in mt-DNA, but due to certain mitochondrial functions that modulate the function of nuclear genes expressed in the cell surface. A possible candidate for such a function could be Ca^{2+} dependent protein or Ca^{2+} itself whose amount and distribution in the cytoplasm is controlled by the mitochondrial membrane system (Wilkie, 1983). Energy,

or ATP molecules, could also be a key element in the mitochondrial control of nuclear functions. However, mt-DNA itself might have some regulatory mechanism of nuclear functions. It is known that ρ strains of yeast could be well maintained in contrast to ρ° strains which grow and divide, albeit slowly, and tend to die in the stock culture. The fact that ρ and ρ° cells are both respiratory deficient and depend on energy produced by fermentation raises the question of what contribution the ρ DNA make for relative stability of ρ over ρ° (see Wilkie, 1983).

There are, in addition, several studies which suggest that the function of mt-DNA and mitochondria is much wider than previously thought:

(i) Some genes of Class I antigen of the histocompatibility complex are controlled by a maternally transmitted factor, *mtf* (Lindahl *et al*, 1983).

(ii) Changes in mitochondrial protein components occur following transformation of the mouse 3T3 cells by the viral SV40 DNA (Zuckerman *et al*, 1984).

(iii) Many carcinogenic drugs specifically interfere with the mitochondrial transcriptional process (Wilkie, 1983).

(c) Mobility of mt-DNA

Although both direct and indirect evidence suggest that the nuclear mitochondrial interactions play a major role in the living processes of cells across their DNA/RNA impermeable membrane, yet another mechanism might play a major role in such interaction. That is, "promiscuity" of organelle DNA (Ellis, 1982). Discoveries that homologous chloroplast DNA can integrate stably into plant mt-DNA (Stern & Lonsdale, 1982) and mt-DNA can translocate to nuclear DNA in yeast (Farrelly & Butow, 1983), sea urchin (Jacob *et al* , 1983), locust (Gellissen *et al*, 1983), Podospora (Wright & Cummings,1983) indicate the fact that the so-called DNA/RNA impermeable membrane might not be impermeable in the evolutionary scale and this has probably played a major role in the evolution of organelle genomes as well as in speciation.

Evidence from maize (Scherdl *et al*, 1984) suggests that the mitochondrial genome undergoes recombination with mitochondrial plasmids which happen to be linear, and can produce linear mitochondrial chromosomes. Hence, plasmid mediated transfer of characters within different genomes (eg. nuclear, chloroplast or mitochondrial) could be a possible mechanism for the promiscuous nature of DNA in living organisms.

12. Oxidative phosphorylation

Oxidative phosphorylation, the single most important function of mitochondria, is the process whereby the energy released from the oxidation reaction of the electron transfer chain is used for the synthesis of ATP molecules. The enzyme complex which catalyses this reaction is called ATP synthase, which is also found in the *E. coli* plasma membrane and the chloroplast membrane. The mitochondrial ATP synthase however, shows specific sensitivity towards the antibiotic oligomycin and is hence referred to as the oligomycin sensitive ATPase or O.S. ATPase. With substantial effort and time invested (40 years), biochemists are still not in a position to describe oxidative phosphorylation substantively. However, a broad outline of the mechanism is now known (See Nicholls, 1982). To understand the process in detail, attempts have been made recently along with a classical biochemical approach using inhibitors, uncouplers or ionophores, to use genetical and molecular biological methods. The latter two branches have opened a new horizon of understanding of the biogenetic process of the enzyme concerned and the mechanism of biochemical reaction and its inhibition by various inhibitors.

The present thesis is directed towards these new approaches with the same purpose, to understand the mechanism of oxidative phosphorylation and its impairment by various inhibitors. Therefore, in this section I shall describe briefly the structural aspects of the enzyme which have been discovered biochemically and some basic genetics studies which have been carried out during the last decade,with special reference to the yeast *Saccharomyces cerevisiae*.

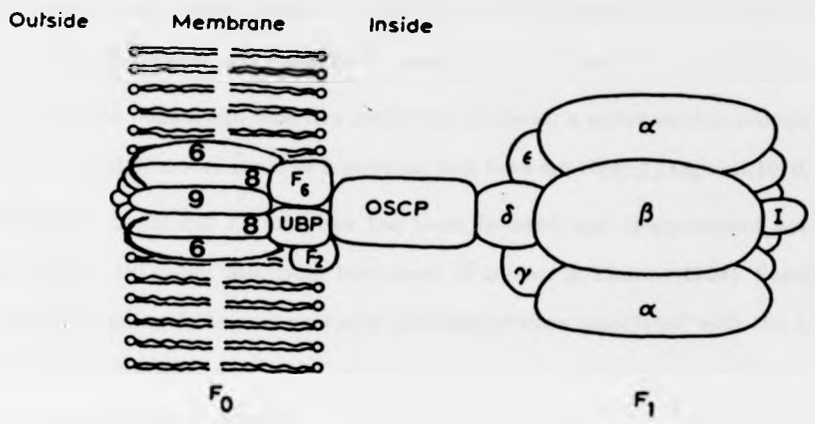


Figure 1.5 The possible structure of the O.S.ATPase or ATP synthase. The scheme is hypothetical. α, β, γ, δ, and ε are various subunit components of the F_1 segment. 6, 8, 9 are three recognised subunits of the F_0 sector. The Oligomycin-sensitivity conferring protein (OSCP), the uncoupler binding protein (UBF), Factor-6 (F_6), Factor-B (F_2) have been tentatively placed in the diagram. I is the ATPase inhibitor protein which probably remains attached to the β subunit.

I. The structure of the O.S. ATPase complex

The functional component of mitochondrial O.S. ATPase has been resolved into three segments (Fig. 1.5)

(1) F_1, an oligomycin-insensitive and water soluble ATPase

(2) F_o or membrane sector which remains buried in the lipid bilayer and is the assumed site of action for oligomycin, ossamycin, venturicidin, Tri-ethyl tin compounds, etc.

(3) O.S.C.P (or oligomycin sensitive conferring protein), a water soluble protein that binds F_1 and F_o, and probably serve as a physical link between them (Tzagoloff,1970).

Additionally, a natural F_1 inhibitor has been isolated and characterised not only from yeast (Ebner, 1974) but also from beef-heart (Pullman & Monroy,1963). Some properties of various coupling factors of oxidative phosphorylation associated with the O.S.ATPase are given in table 1.5a.

a) F_1 sector of the O.S. ATPase

F_1 ATPase has a complex structure which consists of five subunits: α, β, γ, δ and ε (see Table 1.5b) with a stoichiometry of 3α : 3β : 1γ : 1δ : 1ε (Todd *et al*, 1980). It can be isolated by mechanical disruption or by chloroform extraction. Isolated F_1 has only ATP hydrolysis activity (i.e. ATPase activity) but no oligomycin sensitivity. β-subunit has been proposed to have the catalytic site (Ho & Wang, 1983; See Tzagoloff, 1982). Recently the DNA sequence of α and β genes from mutants have been determined from *E. coli* (Noumi *et al*, 1984) and yeast (Saltzgaber-Muller *et al*, 1983). Biogenetic studies using yeast mutants have proved that all the subunits in F_1 are coded by the nucleus and synthesized on the cytoribosomes. In yeast, an extra protein band of mol. wt. 33 000 is found whose function is not known (See Table 1.5b and Tzagoloff,1982).

b) O.S.C.P. and F_1 inhibitor protein

O.S.C.P. or oligomycin sensitivity conferring protein has been isolated from yeast (Tzagoloff, 1970). It has a molecular weight of 18 000 daltons and is synthesized on the cytoribosome. Unlike beef heart, OSCP from yeast remains loosely bound to F_1 and does not

Table 1.5a Properties of Coupling Factors of Oxidative Phosphorylation

Factor	Alternate name	Mol.wt	Intrinsic activity	Functions *in vitro*
F_1		360,000	ATPase	Hydrolysis of ATP, Stimulation of: P/O, ATP - P_i exchange, NAD^+ reduction, Transhydrogenation.
F_0		200,000	None	Proton translocation
F_6	F_c	8,000	None	Stimulation of: P/O, ATP - P_i exchange, NAD^+ reduction, Transhydrogenation.
OSCP		18,000	None	Stimulation of: P/O, ATP - P_i exchange, NAD^+ reduction, Transhydrogenation.
Factor B	F_2	12,000	None	Stimulation of: P/O, ATP - P_i exchange, NAD_+ reduction, Transhydrogenation. Stimulation of ATP-P_i exchange in O.S.ATPase

TABLE 1.5b Components and properties of the O.S.ATPase complex of Yeast

Subunit	Molecular weight	Function	Component
α	58,000	?	F_1
β	55,000	Nucleotide binding site and catalytic site	F_1
	33,000	?	F_1
γ	29,000	?	F_1
δ	14,000	Binding site for OSCP	F_1
ϵ	8,000	?	F_1
5	28,000	?	F_0
6	22,000	?	F_0
7	18,000	Binding of F_1 to F_0	OSCP
8	12,000	?	F_0
9	7,500	Binding site for DCCD and Oligomycin	F_0

form a complex with F_1 (Tzagoloff,1970).

F_1 inhibitor (mol. wt. 10 000 daltons) polypeptide (Ebner, 1974) is also synthesised on the cytoribosomes but is not coupled to the synthesis of F_1.

c) F_o sector of the O.S. ATPase

F_o is the intriguing part of the whole O.S. ATPase complex in yeast. It is still not known definitely, how many subunits or polypeptides constitute this membrane sector of the protein. Early biogenetic studies using radiolabelled aminoacids in the presence of cycloheximide which inhibits the cytoplasmic protein synthesis, demonstrated that four mitochondrially synthesized polypeptides in the O.S. ATPase from yeast could be precipitated using antiserum against F_1 (Tzagoloff & Meagher, 1972). The radioactive peaks of the labelled products were correlated with the O.S. ATPase subunits of 29 000, 22 000, 12 000 and 7 500 daltons. Later experiments suggested that the 22 000 and 7 500 daltons (DCCD binding protein) polypeptide species are the real components of the F_o sector and are named as subunit-6 and subunit-9 respectively. The 29 000 dalton species was shown to be a contaminant from the cytochrome oxidase subunit and the existence of the 12 000 daltons subunit (later described as 10 000 daltons) with O.S. ATPase was questioned (Orian *et al*, 1981; Orian & Marzuki, 1981). It will be demonstrated in this thesis and has been shown by other groups (Macreadie *et al*, 1983; Velours *et al*, 1984) during the course of my research, that the 10 000 dalton species (subunit-8) is a real component of the O.S. ATPase and encoded by a gene (named Aap1) between Oxi-3 and Oli-2 genetic markers on mt-DNA. Therefore, as of now, only three components of F_o sector can be positively identified. Recently Alkoutayani *et al* (1983) have reported a 30 000 dalton hydrophobic protein which is synthesized on mitoribosomes and immunologically different from the 32 000 dalton cytochrome bc_1 complex protein. Whether or not this protein is a component of the O.S.ATPase complex is not known.

d) Other coupling factors associated with the O.S. ATPase

Three more coupling factors have been claimed to be associated with the O.S. ATPase of

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d) Other coupling factors associated with the O.S. ATPase

Three more coupling factors have been claimed to be associated with the O.S. ATPase of

mitochondria. Coupling factor F_1X (Van de stadt *et al*, 1974) has been related with F_1 (See Tzagoloff,1982), while F_6 (Kanner *et al*,1976) and Factor B (Sanadi, 1982) have been presumed to remain associated with the membrane sector. All these factors have been reported from mammalian mitochondria. Among them, F_6 (MW 8 000) and Factor B (MW 15 500) deserve special attention. The F_6 has been purified and shown to be involved in the binding of F_1 to F_o as well as in the restoration of oxidative phosphorylation and ATP dependent reactions in depleted particles (Kanner *et al*, 1976). Factor B (cadmium binding protein) has also been purified and has been shown to be inhibited by sulphydryl (SH) reagents in the stimulation of oxidative phosphorylation (Kaplay *et al*, 1984; Sanadi *et al*, 1984; Lakshmikantham *et al*, 1984) It has been suggested that the protein may exert its effect by decreasing the permeability of the membrane to protons.

II. Genetics of oxidative phosphorylation

The genetic studies of oxidative phosphorylation started with the development of different compounds that specifically inhibit mitochondrial respiration, electron transport, energy coupling and consequently block the growth of yeast cells on non-fermentable media. In Dr. Griffith's laboratory, extensive use has been made of mitochondrial drug-resistant mutants (See Griffiths *et al*, 1972) based on the following criteria:

(i) Drugs which affect mitochondrial energy conservation reactions (inhibitors, uncouplers, ionophores) and have specific inhibitor sites with specific protein subunits of the oxidative phosphorylation complex located in the inner mitochondrial membrane, have been used to generate resistant mutants.

(ii) Drug resistant mutants thus generated have been shown to exhibit the modified sensitivity to the drug at the whole cell, mitochondrial, sub-mitochondrial and specified enzyme levels.

(iii) The inheritance of these drug resistant markers and their location on specific map point of mitochondrial genome indicate that the specific mitochondrial gene product of oxidative phosphorylation has been modified.

Table 1.6 Mitochondrial drugs used in the genetic studies of oxidative phosphorylation and their possible location of action (after Griffiths, 1976)

Drug	Biochemical locus of action	Resistant mutants isolated and locus
Antimycin A	Electron transport	Yes; nuclear
CCCP	Uncoupling agent	Yes; cytoplasmic
TTFB	Uncoupling agent	Yes; cytoplasmic
1799	Uncoupling agent	Yes; cytoplasmic
Oligomycin	O.S.ATPase	Yes; nuclear & cytoplasmic
Ossamycin	O.S.ATPase	Yes; cytoplasmic
Venturicidin	O.S.ATPase	Yes; nuclear, mitochondrial & cytoplasmic
Trialkyltin	O.S.ATPase	Yes; nuclear & cytoplasmic
Aurovertin	F_1 ATPase	Yes; nuclear
Dio 9	F_1 ATPase	Yes, nuclear
Rhodamine 6G	ADP translocase(?)	Yes; cytoplasmic
Bongkrekic acid	ADP translocase	Yes; nuclear & cytoplasmic
Valinomycin	K^+ transport	Yes; mitochondrial

CCCP, Carbonylcyanide m-chlorophenylhydrazone; TTFB, 4, 5, 6, 7-tetrachloro-2-trifluoromethylbenzimidazole; 1799, α, α - bis (hexafluoroacetonyl) acetone

A list of the drugs used in the study (Griffiths,1976) which satisfy the general criteria outlined above is shown in the table 1.6.

The table reveals that the locus of the drug resistant marker may lie in the nucleus, in the mitochondria and also on some other unidentified genome (cytoplasmic or mitochondrial plasmid?). The emphasis in such a study has been given on biochemical genetic studies of oligomycin resistant, venturicidin resistant and tri-ethyltin-resistant mutants for a number of good reasons. The most important one is that all of them have definite binding sites on the O.S. ATPase complex (Griffiths & Houghton,1974). The major conclusions drawn from these studies (Avner & Griffiths, 1973a, 1973b; Lancashire & Griffiths, 1975a, 1975b; Griffiths *et al*, 1975 Lancashire & Mattoon, 1979b) are:-

1. Oligomycin-resistant mutants can be divided into two general classes (class I and class II) on the basis of cross resistance to other mitochondrial drugs. Class I mutants show cross resistance to aurovertin, Dio-9, venturicidin, tri-ethyltin, uncouplers and other mitochondrial drugs. In contrast, class II mutants are specifically resistant to oligomycin and structurally related antibiotics and show no cross resistance to venturicidin, tri-ethyltin, DCCD or uncoupling agents (Table 1.7A). All class II mutants exhibit typical cytoplasmic inheritance and resistance determinants have been located at two distinct loci, OLI (Oli1) and OLII (Oli2) of mt-DNA by genetic mapping analyses. Biochemical studies are consistent with the modification of two of the mitochondrially synthesised components of the O.S. ATPase complex.

2. Venturicidin resistant mutants also fall into two general classes (Table 1.7B). Among them class I is cross-resistant to a wide variety of other drugs including some inhibitors of protein synthesis in mitochondria. A membrane drug-permeability, or other non-specific effect seems to be involved. Class II (V.O) mutants show cross resistance to oligomycin and the resistance allele is located on mt-DNA at a locus which is closely linked to OLI, called OLIII (Oli3). Class II (V.T.) mutants are cytoplasmic mutants which show cross resistance to triethyltin, and are similar to cytoplasmic triethyltin mutants (Table 1.7C). Genetic analysis indicates that these resistance alleles are unlinked to other mitochondrial

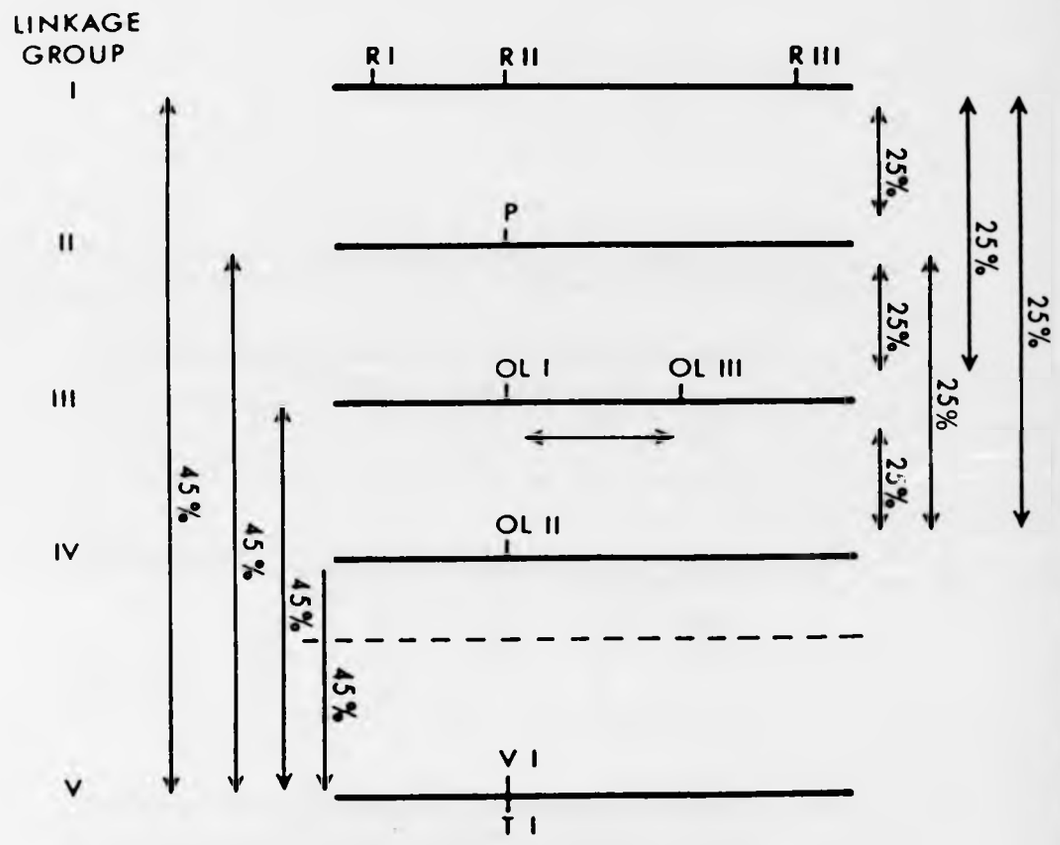


Figure 1.6 Linkage map of various oligomycin-, triethyltin-, and venturicidin resistant mutations in yeast (After Griffiths *et al*, 1976a) OL I (Oli1), OL II (Oli2) and OL III (Oli3) are three oligomycin resistant genetic loci of which OLIII (Oli3) shows cross resistant to venturicidin. The non mitochondrial cytoplasmic markers (VEN^R, TET^R) have been placed on the locus V I/T I, outside the limit of the mitochondrial genome (dotted line). The values shown are total recombination frequencies observed in homosexual ($\omega^- \times \omega^-$ and $\omega^+ \times \omega^+$ genetic crosses (Lancasire & Griffiths, 1974). See Griffiths *et al* for details.

Table 1.7 Cross resistivity of various classes of mutants of oxidative phosphorylation (after Avner & Griffiths, 1973a, 1973b; Lancashire & Griffiths 1975a, 1975b; Lancashire & Mattoon, 1979b).

Table 1.7a. Cross resistance of Oligomycin resistant mutants					
Class	Resistance level X parental strain				
	OLIGO	VEN	TET	1799	(MD)
I	> 50	>50	10-25	>20	3-5
II	>50	1	1	1	1

Table 1.7b Cross resistance of venturicidin resistant mutants					
Class	Resistance level X parental strain				
	OLIGO	VEN	TET	1799	(MD)
I	>50	>50	10-20	>20	3-5
II (V,O)	100	100	1	1	1
II (V,T)	1	50	20	10	1

Table 1.7c Cross resistance of triethyltin resistant mutants					
Class	Resistance level X parental strain				
	OLIGO	VEN	TET	1799	(MD)
I	>50	>50	>20	>20	5-10
IIa	1	>50	>20	1	1
IIb	1	>50	>20	10	1

loci and are located on a different molecule of DNA.

3. Interaction and co-operative effects between different binding sites on the mitochondrial ATPase have provided strong evidence for separate but interacting reaction sites for oligomycin, triethyltin and venturicidin. This in turn indicates that these mutants are due to modification of drug receptor sites on the membrane bound subunits of the O.S. ATPase complex.

The whole linkage group in this region as shown by Lancashire & Griffiths (1975a, 1975b) is shown in Fig. 1.6. Recombination rates within the groups shown in the Figure, seem unexceptional. Between the first four groups the recombination rate is approximately constant at about 25%. This behaviour is not understood. Of equal interest is the high (about 45%) and approximately constant recombination rate found to occur between the fifth group and the other four. The exceptionally high rate indicates that the four linked groups are not linked to the group V markers (i.e., totally random assortment). As the group I-IV markers have been proved to be located on the mitochondrial genome, it is expected that group V markers are on a second molecule of cytoplasmic DNA. It has been shown that VEN^R, TET^R alleles (Group V markers) are retained in a ρ° petite in which mt- DNA is absent (Griffiths *et al*, 1975). The authors concluded that the Group V markers are probably situated on a second mt-DNA molecule and may form part of the mitochondrial genome. They also tentatively suggested that 2 μ plasmid DNA, termed omicron-DNA (o-DNA) (Clark-Walker, 1973) might be the possible candidate. However, it will be seen in Chapter 7 that this 2 μ does not carry these markers.

13. The aim of the present project

On the basis of observed autonomy of the mitochondrial genome of yeast discussed in the earlier sections, and its major contribution to the formation of the F_o sector of the O.S. ATPase evidenced by genetic analyses, it was apparent that molecular study of the specific sector of the mitochondrial genome from various drug-resistant mutants would be instrumental in elucidating some of the mechanisms of oxidative phosphorylation.

Therefore, the aims of the present thesis were:

(i) To generate a fine structure map of the Oli-2 region, together with discrete petites, each having separate genetic markers. This is very important because the segment spans a length of about 4.4 Kbp region of yeast mt-DNA between Oli-2 and Cob-box with multiple drug resistant and *mit* loci.

(ii) Sequencing analyses of the mt-DNA from each individual petite clones.

(iii) To establish from the DNA sequence analyses how many genes are coded by this region and to determine what sort of alteration in the DNA sequences as well as predicted aminoacid sequences leads to drug resistance and the *mit* phenotype.

(iv) To correlate specific genetic locus (or loci) with a specific protein subunit of the O.S. ATPase.

(v) To predict, if possible, the probable membrane conformation of various subunits and their probable interaction with various antibiotics (oligomycin & ossamycin) during oxidative phosphorylation.

(vi) Comparison of the primary aminoacid sequences of the yeast mitochondrially coded subunits of O.S. ATPase with those of other various organisms, for which data are available, and to establish the conservation of the subunits, if any, during the course of evolution.

(vii) Examination of various autonomously replicating elements in the cytoplasm of *S. cerevisiae* to identify a candidate which could be a carrier of some yet unidentified genetic loci (e.g., VEN^R, TET^R, $Rh6G^R$, etc.).

CHAPTER-2

Isolation of discriminating petites from the Oli-2 Region by further Fine Structure Mapping of the Oli-2 Region of the Mitochondrial Genome of *Saccharomyces Cerevisiae.*

Introduction

To elucidate the role of the part of the mitochondrial genome containing the Oli-2 locus between Oxi-3 (the locus responsible for synthesising subunit 1 of cytochrome oxidase) and Cob-box (complete locus coding for apocytochrome-b), it was felt to be important to generate a fine structure map of this region using the available *mit*$^-$ mutants and drug resistant mutants directed towards this particular stretch of the mitochondrial genome. The procedure was to use the powerful genetic tool of petite deletion mapping. This involves the generation of several discrete petites of this part of the mitochondrial genome, and to cross them with different *mit*$^-$ and drug resistant mutants. The resultant diploids would then be tested for complementation analysis of the respective petites (restoration of respiratory function or suppression of the *drug*^{R} phenotype. The resulting co-retention and co-deletion studies of the petites, together with complementation results helped to order different genetic markers (*mit*$^-$ or *drug*^{R}) on the mitochondrial genome and thereby yielding a fine structure map (Carignani *et al*, 1979).

Other procedures such as recombination analysis from the cross of different mutants helps to generate linkage maps. This is of limited value in generating a fine structure map, at least as far as the mitochondrial genome is concerned, due to high recombination values and map expansion effects observed in mitochondrial genetic crosses (See Birky, 1978). Nonetheless the approach helped to produce a gross linkage map, providing the basis for generating a fine structure map.

A further method employed together with petite deletion mapping was the physical map-

ping of the petite genome and its comparison with the parental genome by restriction analysis and DNA/DNA hybridisation (Morimoto *et al*, 1976; 1977; Borst *et al*, 1979). This is a very powerful technique indeed. But this method, as will be described in the next chapter was found to be unsuccessful due to the interference of a nuclear mutation *kar-1* on the mitochondrial DNA copy number and/or mt-DNA replication, thereby yielding an insufficient quantity of mitochondrial DNA for molecular analyses. Nevertheless some methods were employed to circumvent this difficulty which will also be described in the next chapter. This chapter however, describes a fine structure map of the Oli-2 region by petite deletion mapping.

Materials and Methods

A) Materials

1. Yeast Strains

The genotypes and origin of the grande $drug^R$ and *mit* tester strains of *Saccharomyces cerevisiae* used in this study are listed in Table 2.1. The discriminative ρ^- clones presented here are all derived from the starting grande CD40 ($Oli2^R$, $Oss1^R$) by ethidium bromide mutagenesis.

2. Media and Chemicals

NO, N3, WO, YPD, YP10 are essentially as described by Lancashire (1974) and their components are given in the Appendix. For solid media 2.3% agar was added to the above. Antibiotic media (N30l and N30S)were prepared by adding methanolic solutions of Oligomycin (1.0 μg/ml) and Ossamycin (2.0 μg/ml) respectively to N3 medium after autoclaving as described previously (Lancashire and Griffiths 1975b). Oligomycin was purchased from Sigma and ossamycin was a gift from Dr. H. Schmitz, Bristol Myers Co.

Table 2.1 Strains of *Saccharomyces cerevisiae*

Strain	Genotype		Origin
	Nuclear	Mitochondrial	
D273-10B/A1	α met	ρ^+	Dr. Tzagoloff
D27	α met	ρ^+	Subclone of D273-10B/A1
JC8/AA1	a leu kar-1	ρ^0	Lancashire & Mattoon (1979b)
D27/76	α met	ρ^+ *Oli*2^R-76	Lancashire & Mattoon (1979a)
CD15	a leu kar-1	ρ^+ *Oss*1^R-92	Lancashire & Mattoon (1979a)
DB1	a/α leu/+ kar-1/+ +/met	ρ^+ *Oss*1^R-92 *Oli*2^R-76	JC8/AA1 x D27/76
DB1-1C	α met	ρ^+ *Oss*1^R-92 *Oli*2^R-76	Meiosis of DB1
CD40	a leu kar-1	ρ^+ *Oss*1^R-92 *Oli*2^R-76	Cytoductant from cross JC8/AA1 x DB-1C
CD24	α his kar-1	ρ^+ *mit* pho9	Darlison & Lancashire (1980)
CD41	α his kar-1	ρ^+ *mit* pho8	Darlison & Lancashire (1980)
mit-175	α ura	ρ^+ *mit*	Bolotin-Fukuhara *et al* (1977)
M28-81	α met	ρ^+ *mit* pho1	Foury & Tzagoloff (1976)
M6-239/L	α lys2	ρ^+ *mit* pho1	Darlison & Lancashire (1980)
M17-231	α met	ρ^+ *mit* cob-1	Tzagoloff *et al* (1976)
M17-162	α met	ρ^+ *mit* cob-2	Tzagoloff *et al* (1976)
M9-3	α proto	ρ^+ *mit* oxi-2	Slonimski & Tzagoloff (1976)
M9-3/L	α lys2	ρ^+ *mit* oxi-2	Selection of lysine auxotrophy
M9-94	α proto	ρ^+ *mit* oxi-1	Slonimski & Tzagoloff (1976)
M9-94L	α lys2	ρ^+ *mit* oxi-1	Selection for lysine auxotrophy
M5-16	α proto	ρ^+ *mit* oxi-3	Slonimski & Tzagoloff (1976)
M5-16/L	α lys2	ρ^+ *mit* oxi-3	Selection for lysine auxotrophy
DS-14	α met	ρ^- *Oli*2^R-48 *Oli*4'	Macino & Tzagoloff (1980)
CDS-14	a leu kar-1	ρ^- *Oli*2^R-48 *Oli*4'	Cytoductant from cross of JC8/AA1 x DS-14

B) General Methods

1. Sterilization

Sterilization was normally achieved by autoclaving at 15 p.s.i. pressure for 15 minutes. Pipettes were sterilized either by auto claving in the same way, or by heating to 160^0 C overnight.

2. Subcloning and Subculturing

Newly isolated strains were subcloned twice on NO or N3 solid media before storage on agar slopes (prepared in Bijou bottles) at 4^0 C. Strains were routinely subcultured every four to six months.

3. Starter Culture

Starter cultures were normally grown for 24 to 48 hours in 50 ml NO liquid media in 250 ml conical flasks on a rotary shaker at 29^0 C.

4. Lawns for Mating

Cells, grown as described above, were harvested by low speed (1500 x g) centrifugation, and washed twice in sterile distilled water. Approximately 2 x 10^7 cells were then plated onto the appropriate media.

5. Temperature

Unless otherwise stated, all incubations were performed at 29$\pm$1^0 C

C) Special Methods

1. Ethidium Bromide Mutagenesis

The grande CD40 was grown to late log phase in liquid YPD media. Approximately 5 X 10^5 cells were then diluted in 50 ml YPD plus 10 μg/ml ethidium bromide in a 250 ml conical flask, and incubated with shaking in the dark for 24 hours at 29^0 C. The culture was further diluted into YPD plus 20 μg/ml ethidium bromide and incubated again with shaking. Samples were withdrawn from the culture after 4 and 8 hours and either washed and plated on solid YPD to give approximately 100 colonies per plate, or subjected to

further sequential treatment with ethidium bromide .

2. Screening of ρ^- Clones

The screening of ρ^- clones was performed as essentially described in "procedure A" by Carignani et al (1979).

3.Restoration Test of *mit*$^-$ mutants by ρ^- clones

The *mit*$^-$ strains of the sensitive strain D27 or other *mit*$^-$ strains like CD24, CD41, mit 175 were grown to late log phase in either YP10 or liquid YPD and a cell suspension was spread onto WO plates (about 2 $x10^7$ cells per plate). ρ^- clones were then cross replica plated from NO master plates to the tester lawn. The mating plates were incubated for 3 days at 29^0 C. The resulting diploid growth was then velveteen replicated on to N3, N301 and N3OS plates and growth was scored after 3 days at 29^0 C.

RESULTS

Isolation of initial ρ^- petites in the Oli-2 Region.

The strain CD40 possessing oligomycin and ossamycin resistivity was constructed from the parental grande strain D27 (Connerton *et al*, 1984). Sequential ethidium bromide treatment of the CD40 strain led to the generation of thousands of ρ^- clones of which over 1000 were screened for their retention and discrimination of genetic markers directed towards the Oli-2 region of the mitochondrial genome. Approximately 26% of the total ρ^- clones screened had detectable functional genetic information. Of these 75% complemented the *mit*$^-$ testers M9-3/L, M9-94/L and M5-16/L deficient in cytochrome oxidase activity (Slonimski & Tzagoloff 1976). The remaining 25% appear to retain equally portions of the COB-box and the Oli-2 regions as ascertained by the restoration of oxidative growth and drug resistivity in mutants deficient in cytochrome b or directed at oligomycin-sensitive ATPase function.

Representatives of the classes of ρ^- clones produced by initial ethidium bromide mutagenesis are given in Table 2.2, showing the *mit*$^-$ and *ant*^{r} x ρ^- restoration crosses in

Table 2.2 Restoration Matrix for the Oli-2 Region

ρ (a)	ρ⁺ *mit*⁻ or *drug*ˢ											
	M9-3/L	M9-94/L	M5-16/L	CD41	CD24	D27-Oli	D27-Oss	M28-81	M6-239/L	mit-175	M17-231	M17-162
CD40	***	***	***	***	***	***	***	***	***	***	***	***
CD40/LPB2				**	***	***	***	***	***	***	***	***
CD40/ICB2											***	***
CD40/ICB9	***	***	**								***	***
CD40/ICD9	**	***	*									
CD40/IC5A6	***	***	**	**	**	***	***	**	**			
CD40/IC2B1			***	*	*	***	**	*	**			
CD40/MR53				***	***	***	***	***	***	***		

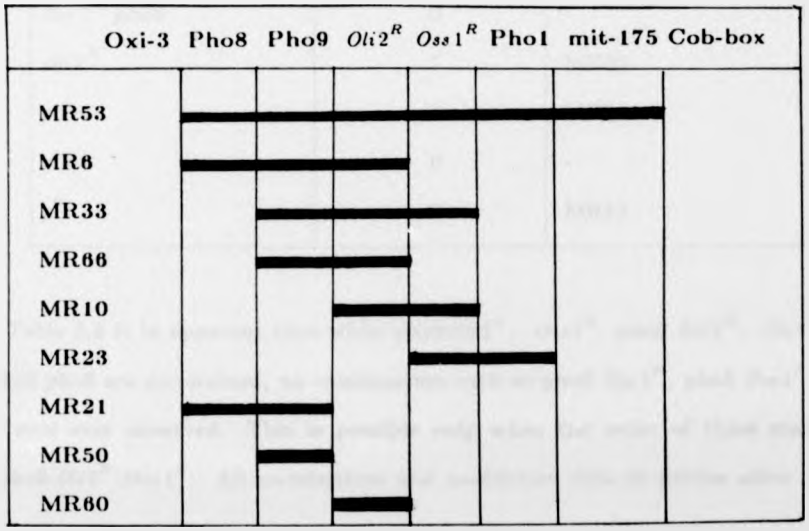
Scoring of the diploids on non-fermentable substrate:
*** = Full and confluent growth of diploids
** = Full but not confluent growth of diploids
* = Full growth but less than 10 diploid colonies

matrix form. From Table 2.2 it is evident that MR53 is a suitable strain for further deletion studies which has retained all genetic markers between Oxi-3 and COB-box. It is important to mention that the strains studied here are in general based on the short mitochondrial genome of D273-10B/A1 (Tzagoloff *et al*, 1975) with the exception of mit-175 which is derived from the grande FF1210-6C (Bolotin-Fukuhara *et al*, 1977). The *mit*⁻ strains pho 9 and pho 8 were originally generated in a nuclear mutant (Op1) of the parental strain D603-3B (Darlison & Lancashire, 1980) and later cytoducted into CD24 and CD41 respectively to possess defined nuclear genomes of opposite mating types (Lancashire & Mattoon, 1979b).

Isolation of discriminative petites from the Oli-2 region by further Ethidium bromide mutagenesis

Subsequent transfer of MR53 strains which have retained genetically all the markers between Oxi-3 (M5-16/L) and Cob-box (M17-231) into ethidium bromide containing culture led to the generation of all ranges of possible petites in this region, representatives of which were shown in Fig 2.1.

Figure 2.1 Representative of discrete petites from the Oli-2 region



The theoretically possible and experimentally produced petites with different genotypes for co-retention and co-deletion studies are shown in Table 2.3 (data of one experiment only).

Table 2.3

Genotypes	Number of finally selected petites	Representatives shown in Fig 2.1
Oli2^R *Oss1*^{R} pho8 pho9	5	MR53
Oli2^R *Oss1*^{R} pho8	0	-
Oli2^R *Oss1*^{R} pho9	5	MR33
Oli2^R pho8 pho9	2	MR06
Oli2^R *Oss1*^{R}	2	MR10
Oli2^R pho9	12	MR66
Oli2^R pho8	0	-
Oss1^R	2	MR23
pho8 pho9	1	MR21
Oss1^R pho9	0	-
Oli2^R	7	MR60
pho9	8	MR50
pho8	0	-
ρ^0	9	MR61

From Table 2.3 it is apparent that while pho9 *Oli2*^{R}, *Oss1*^{R}, pho9 *Oli2*^{R}, *Oli2*^{R} *Oss1*^{R} and pho8 pho9 are co-retained, no combination such as pho9 *Oss1*^{R}, pho8 *Oss1*^{R} or pho8 *Oli2*^{R} were ever observed. This is possible only when the order of these markers are pho8-pho9-*Oli2*^{R}-*Oss1*^{R}. All co-retention and co-deletion data in petites allow a map

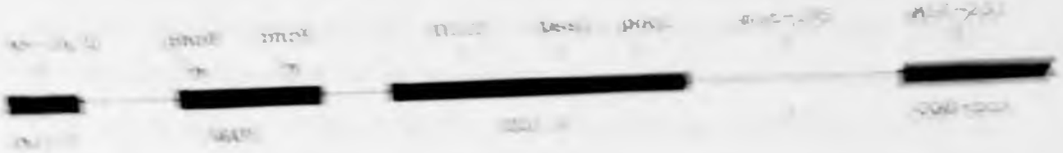
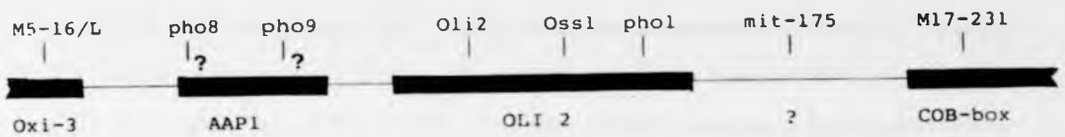


Figure 2.2. Fine structure map of the Oli-2 region. The enclosed boxes represent the recognised genes while the thin lines between the boxes repre-sent the intergenic regions containing hitherto unassigned mutations.



and order to be deduced for the markers which are summarised in Fig 2.2.

It should be borne in mind, that the co-retention and co-deletion of the markers in the ρ clones are only consistent with the assumption that none of the mutants presented here have doubly deleted or genetically rearranged genomes.

3. Localisation of the mit-175 (*mit*$^-$) mutation

Previous study by Bolotin-Fukahara *et al* (1979) has shown that mit-175 is a non conditional *mit* mutation located between the Oli-1 and the Oli-2 regions of the mitochondrial genome. No other attempt has been made to localise this mutation to any,specific region of the mitochondrial genome, so it was interesting to note whether or not this *mit*$^-$ marker maps in the Oli-2 region. Our petite deletion study (see Table 2.2) shows that mit-175 in fact, lies between *Oli*2^R and Cob-box and downstream of the *Oss*1^R marker. To substantiate this result further the mit-175 strain was crossed with a petite strain CDS-14, a cytoductant of DS-14. DS-14 is a discrete well known petite produced in Dr. A. Tzagoloff's laboratory (Macino & Tzagoloff, 1980) whose combined genetic and physical map together with almost all its DNA sequence is known. It has retained the whole of 7th fragment and part of the 6th fragment of the EcoR1 digests of the wild type mitochondrial genome (See Chapter 4). It does not retain any portion of *tRNA*^{gln} gene or Cob-box. The diploid produced by the cross between CDS-14 (similar to DS-14 as checked by restriction enzyme analysis; see next chapter) and mit-175 restored the normal growth on glycerol media. This clearly establishes that the mit-175 locus is situated downstream of the Oli2 and Oss1 loci and probably within the 1.7kb DNA EcoR1 fragment (band 7) found between the Oli2 locus and the Cob-box locus and retained by CDS-14. Unfortunately, no petite was obtained only with the mit-175 marker in this petite deletion study. This could be due to problems with replication in this region of the genome.

DISCUSSION

The data presented here (summarised in Fig. 2.2) represents a genetic dissection of the

mitochondrial genome between Oxi-3 and Cob-box. This has been possible due to the availability of large numbers of mutational sites in this region of the mitochondrial genome. Most of the mutants pho8, pho9, *Oli*2^R and *Oss*1^R have been produced in this laboratory in the past, and some of them are generated in other laboratories (see Table 2.1). The picture which now emerges, is that the pho8 and pho9 mutations are not allelic with the pho1 mutations of M28-81 and M6-239 (Darlison & Lancashire, 1980), the two groups are in fact separated by the Oli2 and Oss1 resistance loci. Petites discriminating the pho8 and pho9 mutations have also been isolated, indicating that these alleles are not homoalleles. This observation is important since the purpose of the present project was to find out the contribution of this part of the mitochondrial genome towards the biogenesis of the mitochondrion or more specifically to that of mitochondrial ATP synthetase.

The Oli2 and Oss1 markers used in this study belong to the unique groups "D" and "E" respectively, as described by Lancashire & Mattoon, (1979). This designates that they are specifically resistant towards these drugs rather than a wide range of cross resistances which fall in to groups "A", "B" and "C". So as far as the aim of this project is concerned, sequence analysis of mt-DNA from discrete ρ clones containing the Oli-2 and Oss-1 resistant loci will help to predict specific drug-protein interactions in relation to oxidative phosphorylation of mitochondria.

The localisation of a new *mit* mutation, mit-175 between Oli-2 and Cob-box is a useful step towards the identification of a product from a hitherto unknown gene. It is anticipated that *Oli*2^R and *Oss*1^R probably lie within the coding sequences for subunit-6 of oligomycin sensitive ATPase (Macino & Tzagoloff, 1980). The pho1 mutations are also likely to be within this structural gene. The location of the pho8, pho9 and mit-175 mutations is open to speculation. Pho8 and Pho9 lie on the Oxi-3 side of Oli-2, while mit-175 lies at Cob-box side of Oli-2. Sequencing data upstream of Oli-2, published recently by Macreadie *et al*, 1983) has revealed a putative reading frame for 48 aminoacids, coding a protein which has been named as AAP1. Recently Velours *et al*, 1984, have

sequenced a 10 KD lipoprotein associated with the O.S.ATPase complex and have shown it to be in perfect homology with predicted aminoacid sequences of AAP1 from DNA sequencing data. So it is anticipated that either pho8 and pho9 will be located within this AAP1 gene (a phosphate binding ATPase associated protein) or in the 5' flanking controlling region of the Oli-2 gene. The location of the mit-175 locus to any other gene product involved in oxidative phosphorylation, or some gene controlling transcription and/or processing transcripts of this region, would also be interesting. DNA sequencing analysis of this region of mitochondrial DNA will rationalise all these alternatives (See Chapter-5).

CHAPTER 3

Effect of the *kar-1* mutation on the transmission and the copy number of the petite mitochondrial genome of *Saccharomyces cerevisiae*

Introduction

Kar-1 is a nuclear fusion mutation (Conde & Fink, 1976) in the yeast *Saccharomyces cerevisiae*, which has been a tool in the genetic research on organelle genome and extra chromosonal inheritance. The general method, called cytoduction (Lancashire & Mattoon, 1979a) utilises this mutation in mitochondrial genetic studies to develop a series of *mit* ,*syn* , $drug^R$ strains in isonuclear backgrounds, so that the biochemical effects of these mutants could be analysed systematically. This also potentially eliminates any arguments against possible nuclear involvement on biochemical characteristics of respective mitochondrially inherited mutations.

To achieve this goal, this laboratory has generated a series of *mit* (pho8,pho9) and $drug^R$ (e.g. $Oli2^R$,$Oss1^R$) mutations in an isonuclear background (*a leu kar-1*).As a basic aim of the present project to study the Oli-2 region (between Cob-box & Oxi-3) of mitochondrial genome in mitochondrial biogenesis, almost all strains used have the above genotypes.Therefore all the ρ strains generated during petite deletion mapping (as described in a previous chapter) have the *kar-1* mutation within the nucleus. When attempts were made to isolate mitochondrial DNA from these petites for physical characterisation, it was found impossible to isolate enough mt-DNA for futher molecular analyses by the available standard techniques (Lang *et al*, 1977; Williamson & Fennell, 1975; Fukuhara, 1969; Bernardi, *et al*, 1972; Hudspeth *et al*, 1980). After recurrent failure to isolate mt-DNA in sufficient quantity for physical mapping by restriction endonucleases and for sequencing of DNA, we undertook a systematic study to look at the effect of the *kar-1* gene on the suppressivity of the petites employed, the amount of mt-DNA and the copy numbers they retained. Interestingly we found that *kar-1* has a profound effect,at least in our petites, both on the copy number of the mt-DNA genome and on its

suppressivity.

MATERIALS & METHODS

1. Yeast Strains

The genotypes and origin of the grande and petite strain of *S.cerevisiae* used in this study are listed in Table 3.1. The prefix CD of the grande strains indicates their cytoductant origin by standard technique (Lancashire & Mattoon, 1979a).

2. Media & Chemicals

Standard media for yeast culture, maintenance and nuclear and mitochondrial genetic analysis were used as described in Chapter 2. The recipes of the media are given in the Appendix. The sources of oligomycin and ossamycin are the same as mentioned in Chapter 2.

3. Basic Cytoduction Procedure

The basic cytoduction procedure was similar to that described by Lancashire & Mattoon (1979). Briefly, complementary cells [*a leu kar-1* ρ^0 and α (ρ ,*mit* or Ant^R)] were precultured separately in NO media for 24 hours. The fresh cells were then mixed together in equal proportions on solid YPD plates or in liquid medium (2 $X10^7$ cells/ml). The mixtures were allowed to incubate at 30^0C for about 4 hours. Sampling for the formation of zygotes were sometimes carried out to get appreciable numbers of zygotes which we found 4 hours in most cases. Then cells were harvested by centrifugation and suspended in sterile distilled water. Then, either the zygotes were micromanipulated on a thin YEP agar slab and the zygotic buds were removed and cultured, as described by Lancashire & Mattoon (1979a), or processed by spreading them onto WO plates supplemented with the respective agent for an auxotrophic nuclear marker to be selected for.In all cases before manipulation of zygotic buds, the plates were incubated in a humidified chamber at 30^0 C. In the later case, the cytoductants with ρ^- or *mit*$^-$ phenotype are easily identifiable from

Table 3.1 Yeast strains used in the study

Strain	Nuclear Genotype	Mitochondrial Genotype	Origin
D273-10B/A1	α met	ρ^+ *Oli2*^{S} *Oss1*^{S}	Macino & Tzagoloff (1980)
D27	α met	ρ^+ *Oli2*^{S} *Oss1*^{S}	Subclone of D273-10B/A1
CD40	a leu kar-1	ρ^+ *Oli2*^{R} *Oss1*^{R}	Dr. Lancashire
CD24	α his kar-1	ρ^+ *mit* pho9	Dr. Lancashire
CD41	α his kar-1	ρ^+ *mit* pho8	Dr. Lancashire
LP81	a leu kar-1	ρ *Oli2*^{R} *Oss1*^{R}	Dr. Lancashire
LPA12	a leu kar-1	ρ pho9	Dr. Lancashire
MR53	a leu kar-1	ρ *Oli2*^{R} *Oss*^{R} pho8 pho9	This thesis
MR60	a leu kar-1	ρ *Oli2*^{R}	This thesis
MR23	a leu kar-1	ρ *Oss1*^{R}	This thesis
MR50	a leu kar-1	ρ_0 pho9	This thesis
MR61	a leu kar-1	ρ^0	This thesis
DS-14	α met	ρ *Oli2*^{R} *Oli4*^{S}	Macino & Tzagoloff(1980)
DS14(a)	a ade2	ρ *Oli2*^{R} *Oli4*^{S}	This thesis
CDS14(α)	α his kar-1	ρ *Oli2*^{R} *Oli4*^{S}	This thesis
CDS-14	a leu kar-1	ρ *Oli2*^{R} *Oli4*^{S}	This thesis

diploids (which form larger colonies). The suspected cytoductants are then tested for their nuclear and mitochondrial markers by standard genetic techniques (Coruzzi *et al*, 1979).

4. Determination of Suppressivity

Suppressivity is the frequency of transmission of a ρ mitochondrial genome in a cross of ρ x ρ'. A parental petite is called highly suppressive if the frequency of petite (ρ) zygotic colonies are much higher than the frequency of spontaneous petite mutants in the ρ parent.A petite is called neutral or zero-suppressive if the frequency of zygotic colonies produced are no higher than the frequency of spontaneous petite generated in the ρ' parent. Accordingly, ρ^0 strains are called zero suppressive or neutral petites. The degree of suppressivity is measured by the equation of Ephrussi and Grandchamp (1965)

$$S = \frac{X - Y}{100 - Y} \times 100$$

where, S = degree of suppressivity (%)

X = percentage of entirely petite zygotic clones

Y = spontaneous frequency of petites observed in grande tester strain.

In practice, strains to be tested for suppressivity were crossed with a grande (ρ') tester strain in a liquid NO media (5 X10^6 cells/ml). The mating media was incubated at 30^0 C with shaking for about 4 hours. The cells were then harvested by centrifugation, washed in sterile distilled water and plated onto solid WO media with proper dilution to get about 100-200 colonies per plate. After 3 days of incubation at 30^0 C, zygotic clones were velveteen replica plated onto NO & N3 solid media and incubated for a further 2 days at 30^0 C. The petites (ρ) were identified and scored by their non-ability to grow on nonfermentative media.

5. Isolation of Mitochondrial DNA (mit-DNA)

Mitochondrial DNA was isolated by bisbenzimide-CsCl buoyant density centrifugation as described by Hudspeth *et al* (1980). Briefly, yeast cells were harvested, washed in sterile distilled water and re-suspended in a breaking buffer (0.25M Mannitol, 10mM EDTA, 50mM Tris-Cl, pH 7.5) with or without 0.1% Bovine serum albumin (BSA) and the suspended cells (1 gm/ml) were transferred to a 250ml bottle containing glass beads of 0.5mm diameter and then broken by the "handshake" procedure (Lang *et al*, 1977) at 4^0 C. The cells were, in fact, shaken three to four times (2 minutes each) with a one minute interval on ice. More than 90% of the cells appeared to be broken, as monitored by light microscopy. The liquid was decanted and the beads were rinsed with five volumes of breakage buffer. The supernatant was then centrifuged at 2000 X g to spin down the cell debris and glass beads which escape during washing. The supernatant was then re-centrifuged at 16000 X g for 30 minutes at 4^0 C. The pelleted mitochondria were washed with MTE buffer (0.25 M Mannitol, 10mM Tris-Cl,pH 7.4,10 mM EDTA) and repelleted at 16000 X g by centrifugation.

The mitochondrial pellets were then lysed in lysis buffer (10mM Tris-Cl, pH 8.0, 10mM EDTA, 100mM NaCl, 2% Sarkosyl or 1% SDS) and extracted with phenol and phenol:chloroform:isoamyl alcohol mixture (24:24:1) repeatedly to remove proteins and other cellular debris. The aqueous phase was collected, made up to 0.3M Na-acetate and DNA was precipitated with 2 Vol. of ethanol (overnight at -20^0 C or 2 hours at -70^0 C). The crude DNA pellet was collected by centrifugation at 10,000 X g, dissolved in TE buffer (10mM Tris, pH 7.5, 1mM EDTA). Then CsCl solution (0.9 gm/ml) was added to the DNA solution to give a refractive index of 1.390. The bisbenzimide (Hoechst 33258) was then added to give a final solution of 100 μg/ml. Centrifugation was then carried out in a 50 Ti rotor (Beckman) at 45 K r.p.m.(at 15^0 C) for 24-42 hrs.

After centrifugation the green fluorescing bands were collected under long-wave UV illumination. The mt-DNA appears at the low density zone, or towards the top of the gradient and the nuclear DNA, r-DNA satellite, 3 μ plasmids are banded sucessively

towards the bottom of the tube. The collected bands were extracted with CsCl saturated isopropanol to remove bisbenzimide and then dialysed against TE buffer (10mM Tris.Cl, pH 7.5, 1mM EDTA overnight). The DNA was precipitated by adjusting the solution to 0.3M Na-acetate and adding 2 volumes of ethanol. Several other methods for mt-DNA isolation (using CsCl-ethidium bromide, CsCl only, CsCl-DAPI gradients) were also employed (see Williamson & Fennel, 1975; Fukuhara, 1969; Bernardi *et al*, 1972).

6. Restriction-endonuclease Analysis and Gel-electrophoresis

Restriction enzymes were bought from commercial sources (from BRL, BCL, PBL). The digestion was carried out as per supplier's instructions. The digests were heat shocked at 65^{0} C for 3-5 minutes before loading onto the gel. The agarose gel (generally 1%) was electrophoresed in 50 mM Tris-Borate, 1mM EDTA, pH 8.3, buffer and stained with 1 μg/ml ethidium bromide solution (for 15-20 minutes) before examination on a UV transilluminator (Fotodyne,UK). Photographs were taken using a Polaroid-665 land camera (Polaroid UK Ltd.) with a Kodak 23A Wratten filter (orange).

7. Dot-hybridization

The double stranded DNA (dsDNA) was denatured in formaldehyde - formamide buffer (50 % Formamide,2 M Formaldehyde, 2.5 mM EDTA, 40 mM Tris-HCl,pH 7.5) and dot-spotted onto a sheet of nitrocellulose filter. The filter was baked at 80^{0} C for 2 hours in a vacuum oven, before hybridization with a specific labelled probe (see below).

8. Southern Hybridization

This was carried out according to the method of Southern (1975). Briefly, the samples of restriction digests were run in agarose gel as described above, visualised on a UV transilluminator after ethidium bromide staining, and photographed. The gel was then shaken in a tray containing 0.5M NaOH, 1M NaCl for 45 minutes at room temperature. The gel was then neutralised in the presence of 50mM Na-phosphate buffer (pH 7.0) by several changes. The gel was rinsed in distilled water before transferring into the neutralising

solution. The gel was then equilibriated in 50mM Tris-HCl pH 7.5, 10 X SSC for 20 minutes. The DNA was then transferred to a nitrocellulose filter overnight, following the same procedure of Southern (1975). The nitrocellulose filter was air-dried and baked at 80° C for 1-2 hours in a vacuum oven. The filter was pre-hybridised in a pre-hybridisation buffer (4 X SSC, 10 X Denhardt's solution, 0.1 % SDS, 0.1 mg/ml heat denatured CT DNA) for 2-4 hours at 65° C in a sealed plastic bag. The pre-hybridisation mixture was squeezed out and the bag was refilled with pre-warmed (65° C) hybridization mix containing probe (the labelled DNA). The hybridisation was carried out overnight at 65° C. The filter was washed successively in 2 X SSC, 1 X SSC, 0.1 X SSC (with or without 0.1% SDS) for 30 minutes each, at 50° C. The filter was then air-dried and exposed with film (Kodak, XOmat) for the desired time, depending on the radioactivity of the filter, as monitored by a hand counter. The autoradiograms were developed and fixed according to standard procedures using Kodak's Universal developer (1:9 dilution) and rapid fix (1:4 dilution).

9. Fluorescent Staining of Yeast Cells with DAPI

The yeast cells were stained with the A+T base preferential DNA-binding dye DAPI to visualise mitochondria or chondriolytes as described by Williamson & Fennell (1975). Briefly, a sample of culture of yeast cells was mixed either directly with 2 volumes of ethanol, or pelleted cells were re-suspended in 70% (V/V) ethanol. After about 30 minutes the cells were washed once in distilled water and re-suspended in a solution of 0.1-0.5 μg DAPI/ml. The cells were examined immediately under a fluorescent microscope and photographed.

RESULTS

Suppressivity of different strains used in the experiment

When the petites with *kar-1* nuclear marker (MR53,MR50,MR60) were crossed to a ρ^+ strain D27 and measured for the suppressivity of the ρ^- genome, it was observed that

they have an average of 15-25% suppressivity (Table 3.2). This low suppressivity, as it was assumed, could be due to absence of the "Ori" sequences in the retained mitochondrial genome which fail to transmit into the buds due to low copy number. ρ petite, DS14 however showed a 45% (with *a ade-2* nuclear background) to 68% (with α *met* nuclear back- ground) suppressivity.

Table 3.2 Suppressivity of various ρ strains

Strain	Nuclear genotype	Suppressivity
MR53	a leu kar-1	23-25%
MR60	a leu kar-1	16-20%
MR50	a leu kar-1	15-20%
MR23	a leu kar-1	17-18%
DS14	α met	68%
DS14(a)	a ade2	45%
CDS14(α)	α his kar-1	22%
CDS14	a leu kar-1	28%

To find out whether *kar-1* has any effect on the suppressivity of this ρ genome, the DS14 m^+-genome was cytoducted into two *kar-1* backgrounds (α *kar-1 his* and *a kar-1 leu*), named as CDS14(α) and CDS14 respectively, and the suppressiveness of the genome was measured (see Table 3.2). In the new *kar-1* background CDS14 mt-genome showed reduced suppressivity value, 22% (in α *his kar-1* background) to 28% (in *a leu kar-1* background). This demonstrates that the kar-1 mutation is somehow affecting the transmission of the ρ mt-DNA.

Isolation of mt-DNA from various petites

Several methods have been employed to isolate mt-DNA from grande and petite strains of the yeast *Saccharomyces cerevisiae*. We found bisbenzimide-CsCl density gradient

they have an average of 15-25% suppressivity (Table 3.2). This low suppressivity, as it was assumed, could be due to absence of the "Ori" sequences in the retained mitochondrial genome which fail to transmit into the buds due to low copy number. ρ petite, DS14 however showed a 45% (with *a ade-2* nuclear background) to 68% (with α *met* nuclear back- ground) suppressivity.

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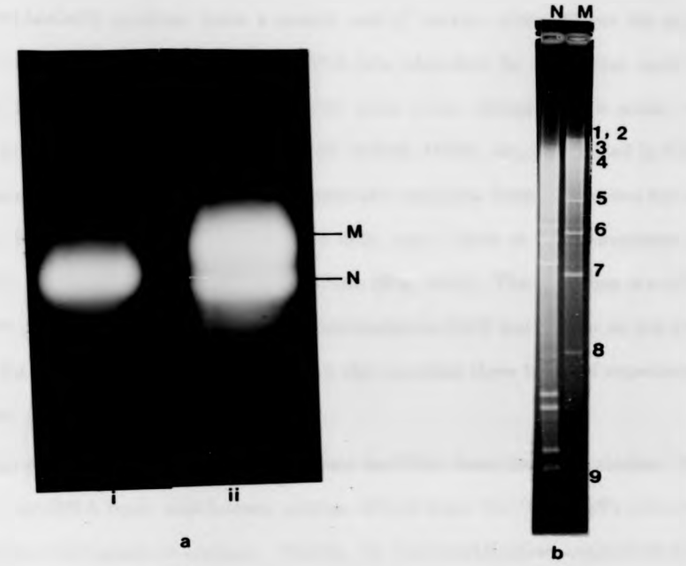
MR23	a leu kar-1	17-18%
DS14	α met	68%
DS14(a)	a ade2	45%
CDS14(α)	α his kar-1	22%
CDS14	a leu kar-1	28%

To find out whether *kar-1* has any effect on the suppressivity of this ρ genome, the DS14 mt-genome was cytoducted into two *kar-1* backgrounds (α *kar-1 his* and *a kar-1 leu*), named as CDS14(α) and CDS14 respectively, and the suppressiveness of the genome was measured (see Table 3.2). In the new *kar-1* background CDS14 mt-genome showed reduced suppressivity value, 22% (in α *his kar-1* background) to 28% (in *a leu kar-1* background). This demonstrates that the kar-1 mutation is somehow affecting the transmission of the ρ mt-DNA.

Isolation of mt-DNA from various petites

Several methods have been employed to isolate mt-DNA from grande and petite strains of the yeast *Saccharomyces cerevisiae*. We found bisbenzimide-CsCl density gradient



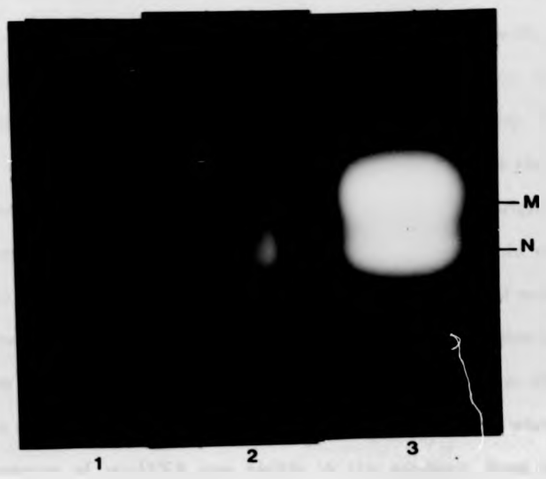
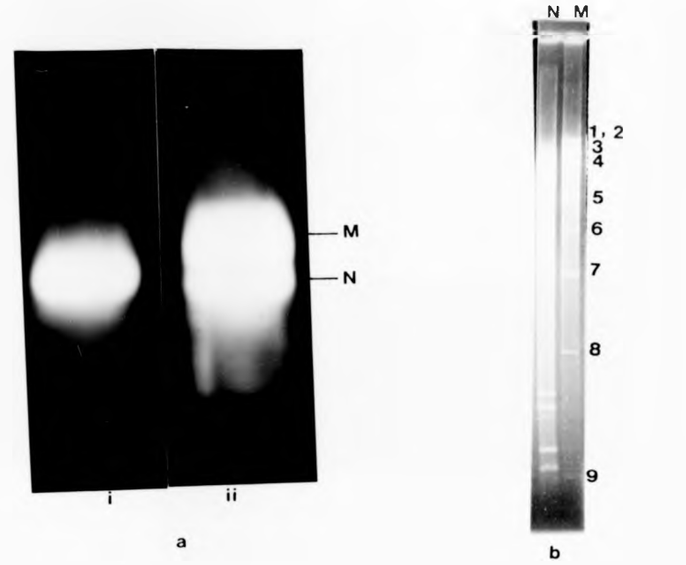


Figure 3.1 Fractionation and isolation of mt-DNAs from various yeast strains. (a) A CsCl bisbenzimide density gradient to show the position of mt-DNA (M) and nuclear DNA (N). (i) is a preparation from ρ° cells and (ii) is the preparation from the grande strain (CD40).

(b) EcoR1 digests of nuclear DNA (N) and mt-DNA (M). The DNA fragments were analysed in 1% Agarose gel electrophoresis. 1, 2, 3, 4, 5, 6, 7, 8, and 9 are various mt-DNA EcoR1 fragments with the molecular sizes ; 32.5, 17.0, 7.10, 5.10, 3.5, 2.55, 1.70, 0.90 and 0.15 Kbp respectively (see Chapter 4).

(c) Analysis of DNAs from ρ^{-} cells with *Kar-1* and without *Kar-1* nuclear marker. (i) From ρ° (MR61). (ii) From ρ^{-} (MR53) and (iii) from ρ^{-} (DS-14). See table 3.1 for genotypes of the strains.



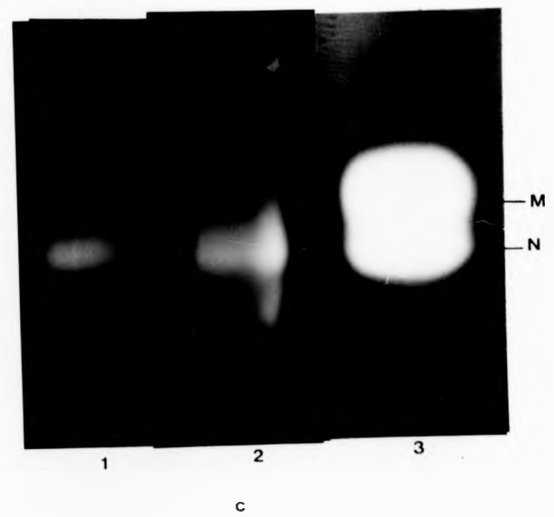
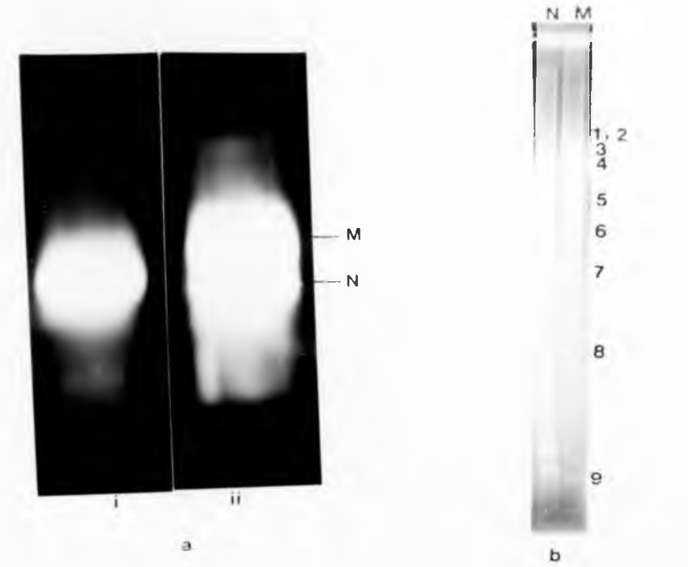


Figure 3.1 Fractionation and isolation of mt-DNAs from various yeast strains. (a) A CsCl bisbenzimide density gradient to show the position of mt-DNA (M) and nuclear DNA (N). (i) is a preparation from ρ^+ cells and (ii) is the preparation from the grande strain (CD40).

(b) EcoR1 digests of nuclear DNA (N) and mt-DNA (M). The DNA fragments were analysed in 1% Agarose gel electrophoresis. 1, 2, 3, 4, 5, 6, 7, 8, and 9 are various mt-DNA EcoR1 fragments with the molecular sizes ; 32.5, 17.0, 7.10, 5.10, 3.5, 2.55, 1.70, 0.90 and 0.15 Kbp respectively (see Chapter 4).

(c) Analysis of DNAs from ρ^- cells with *Kar 1* and without *Kar 1* nuclear marker. (i) From ρ^+ (MR61). (ii) From ρ^- (MR53) and (iii) from ρ^- (DS-11). See table 3.1 for genotypes of the strains.



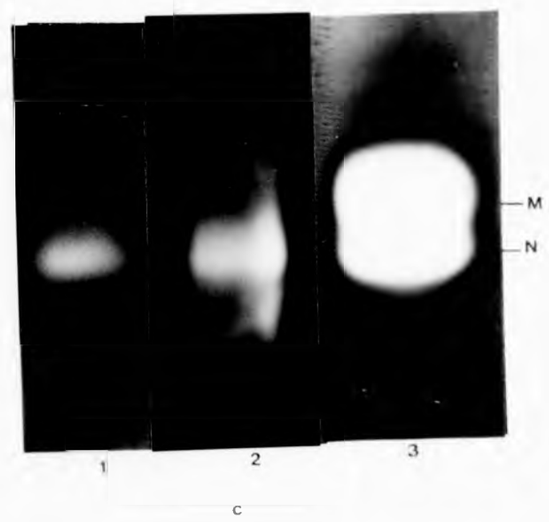


Figure 3.1 Fractionation and isolation of mt-DNAs from various yeast strains. (a) A CsCl bisbenzimide density gradient to show the position of mt-DNA (M) and nuclear DNA (N). (i) is a preparation from ρ^- cells and (ii) is the preparation from the grande strain (D10).

(b) EcoR1 digests of nuclear DNA (N) and mt-DNA (M). The DNA fragments were analysed in 1% Agarose gel electrophoresis. 1, 2, 3, 4, 5, 6, 7, 8, and 9 are various mt-DNA EcoR1 fragments with the molecular sizes; 22.5, 17.0, 7.40, 5.10, 3.5, 2.55, 1.70, 0.90 and 0.45 Kbp respectively (see Chapter 4).

(c) Analysis of DNAs from ρ^- cells with *Kar 1* and without *Kar 1* nuclear marker. (i) From ρ^- (MR61). (ii) From ρ^- (MR53) and (iii) from ρ^- (DS-11). See table 3.1 for genotypes of the strains.

centrifugation to be the best method for this purpose. Fig. 3.1a shows a typical (bisbenzimide-CsCl) gradient from a grande and ρ^0 strains which shows the position of various bands of DNA. Each band of DNA was identified by restriction analysis (Fig. 3.1b) and Southern hybridization. Difficulty arose when attempts were made to isolate mt-DNA from defined ρ^- petites (e.g. MR53, MR50, MR60, etc.,) described in Chapter 2. It was impossible to isolate mt-DNA in analysable amounts from the petites having *kar-1* genotype. Starting with 2 litres of cultured cells, only a little or no fluorescence was visible in the mt-DNA position of the gradient (Fig. 3.1c). The question we asked was whether it was due to difficulty with the bisbenzimide-CsCl method, or to the low abundance of mt-DNA in these petites. To solve this question three types of experiments were performed.

First using other available methods to isolate mt-DNA from the same strains. Secondly, to isolate mt-DNA from well-known petites, DS-14 from Dr. Tzagoloff's laboratory, by bisbenzimide-CsCl gradient method. Thirdly, by dot hybridization analysis of total DNA of the petites with a mt-DNA specific probe.

Using other methods we were not successful in isolating mt-DNA from petites having the *kar-1* nuclear genotype, so it appears that failure was not due to any specific method. When we tried to isolate mt-DNA from DS-14, we found the mt-DNA from this petite gave a good yield, parallel to any grande strain (0.6 μg - 0.8 $\mu g/10^9$ cells). However, when CDS-14 (*a kar-1*, *Oli*2^R), a cytoductant strain of DS-14, was used the yield of mt-DNA decreased to about 0.2 $\mu g/10^9$ cells), a three-fold reduction of the original. This shows that *kar-1* is somehow affecting the amount of mt-DNA in a haploid cell. One interesting aspect of the later experiment was that although the amount of mt-DNA in CDS14 was reduced three-fold due to *kar-1* marker, nevertheless it was possible to isolate mt-DNA from a 2 litres of culture (40-44gm wet weight of cells) for further restriction analysis, etc. This is in contrast to other petites studied here, except MR53 where only a trace amount of fluorescence of mt-DNA was visible in the gradient, from a similar amount of cells, and was not enough for further restriction study. In MR53 however, it

was possible to isolate mt-DNA (Fig. 3.4a), but in lower yield in comparison to CDS14. The reason for this discrepancy is not clear. It is possible that the interaction of *kar-1* with mt-DNA replication and/or copy number is somehow dependent upon the residual DNA sequences of petites. In other words, *kar-1* itself is not affecting mt-DNA copy number and/or replication directly, rather it's action is indirect and depends upon the presence or absence of a certain class of sequences in the mt-DNA.

We also attempted to find out whether or not the petite in the study retains mt-DNA by dot-hybridization analysis. Fig. 3.2 shows the result of such an experiment. All ρ^- strains used in this experiment showed Oli-2 specific hybridizable sequences together with that of the grande strains CD40. MR61, a ρ^0 strain, as expected, did not show any positive hybridizable sequences. This proves conclusively that all the petites described retain their genetic markers as well as respective mt-DNA sequences, but their copy numbers, being low, impose difficulty isolating in large amounts for further analyses.

Copy numbers of mt-DNA in different Petites

From the result of analytical centrifugation of mt-DNA and dot-hybridization analysis, it was possible to calculate the copy numbers of mt-DNA in the different petites used. For wild type strains, a value of 1.7×10^{9} μg mt-DNA per cell can be calculated from the value of 10^{10} daltons DNA per haploid nucleus (Hartwell, 1970; Bicknell & Douglas, 1970), assuming that 10% of the total DNA is mitochondrial and that the amount of nuclear DNA does not vary during the cell cycle. It has also been suggested that a haploid cell contains about 50 copies of mt-DNA molecule (Grimes *et al*, 1974). Based on these values, it could be calculated that 50×10^{9} copies of mt-DNA molecule should give a yield of 1.7 μg DNA. In our experiments, the yield of mt-DNA was about 1.5 μg/10^{9} cells (calculating 0.6 μg DNA/10^{9} cells as 40% recovery in CsCl-bisbenzimide gradient, data not shown; see also Hudspeth *et al*, 1980), which gives a value of 44 copies of mt-DNA molecule per haploid cell both in grande strain and petite DS-14. In CDS-14 the yield decreases about 3-fold and gives rise to about 15 copies per cell. In other petite lines

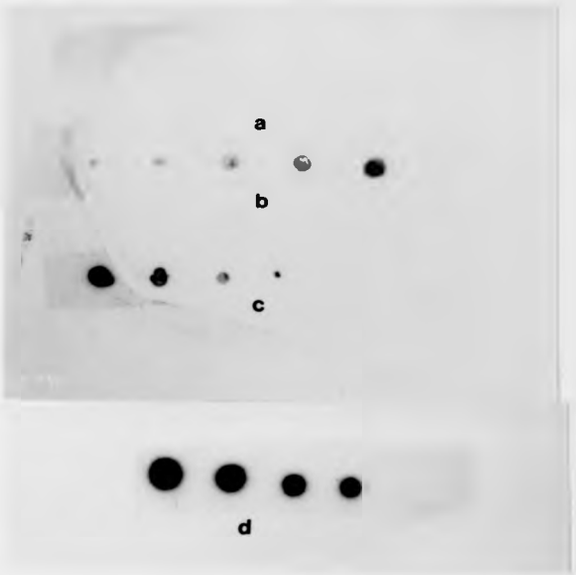


Figure 3.2 Dot hybridization analysis to show the retention of the mt-DNAs in ρ^- petites with *kar-1* nuclear background. The crude DNAs isolated from mitochondrial pellets were spotted onto a sheet of nitrocellulose. Then the filter was probed with DS-14 mt-DNA. (a) ρ° DNA (MR61) (b) ρ^- DNA (MR50), (c) ρ^- DNA (MR53), (d) ρ^+ mt-DNA (CD-40).

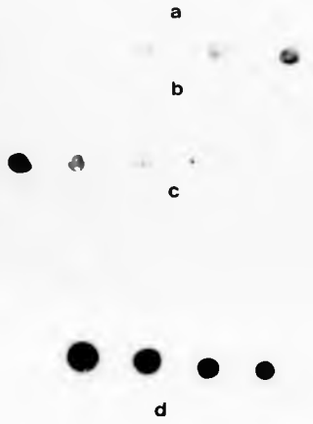


Figure 3.2 Dot hybridization analysis to show the retention of the mt-DNAs in ρ^- petites with *kar 1* nuclear background. The crude DNAs isolated from mitochondrial pellets were spotted onto a sheet of nitrocellulose. Then the filter was probed with DS-14 mt-DNA. (a) ρ^o DNA (MR61) (b) ρ^- DNA (MR50), (c) ρ^- DNA (MR53), (d) ρ' mt-DNA (CD-10).

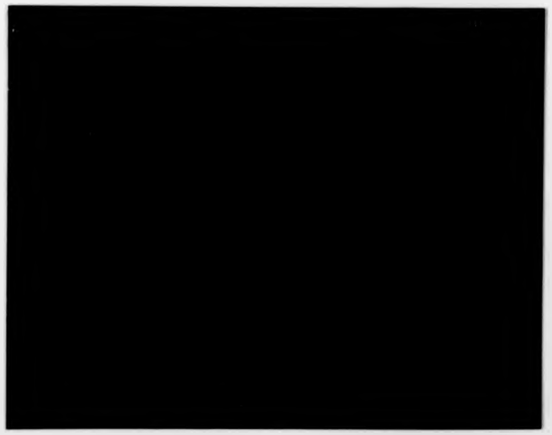
MR53, MR50, MR60, MR51, the yield decreases about 16 to 18 fold which means that copy numbers in these petites are between 2 to 3 per cell. Using total DNA from the same number of cells from various strains and hybridising with a labelled Oli-2 region specific probe (cloned in *E.coli*), it was possible to calculate the relative mt-DNA copy numbers which paralleled with those determined from the yield of mt-DNA by analytical bisbenzimide-CsCl gradient.

Fluorescent microscopy studies of DAPI-stained cells of various strains

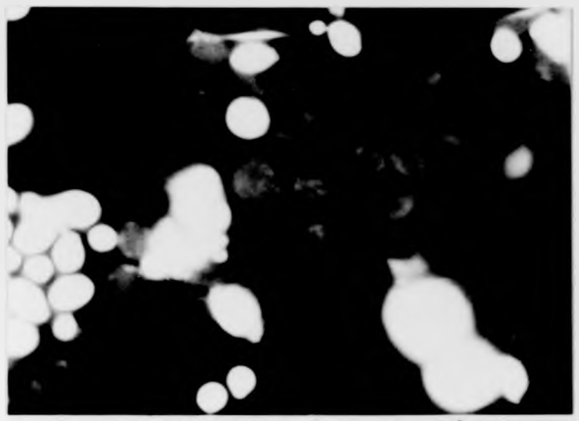
As a more direct approach to visualise the effect of *kar-1* on the state or number of chrondrialytes in various strains of yeast was employed using the fluorescent probe DAPI. Fig. 3.3 shows the fluorescence photomicrographs of an experiment. The photograph displays the nucleus as a single large conspicuous body in the cells. The mt-DNA in the form of condensed chondriolytes are scattered in the cytoplasm, preferably towards the periphery, as minute stained particles which are absent in ρ^0 strains. Both the grande strains and petite DS-14 showed about 25-30 such particles per cell (data not shown). This is comparable to the results obtained by Williamson *et al* (1975) who observed an average of 38 stained mt-DNA aggregates per cell. The number of counts were much less than the supposed, 50 mt-DNA molecules per cell. Presumably the visualisaton of mt-DNA as a stained fluorescent body depends upon the number of mt-DNAs in an aggregate and/or the condensation state of respective mt-DNA molecules. However, other petites like MR53, MR50, MR60, MR51 etc (Fig. 3.3) did not show many stained particles in the cytoplasm. Two or three scattered fluorescent images in the cytoplasm are in fact comparable with the determined copy number of mt-DNA in these petites (2-3 per cell). However some of the cells did have the appearance of ρ^0 cells which means a fraction of cell population may be devoid of mt-DNA.

Southern Hybridization Analysis of Petite mt-DNA

Because of the isolation problem of mt-DNA from the petites an indirect approach was adopted to study the span of petite mitochondrial genomes in comparison to that of wild



a



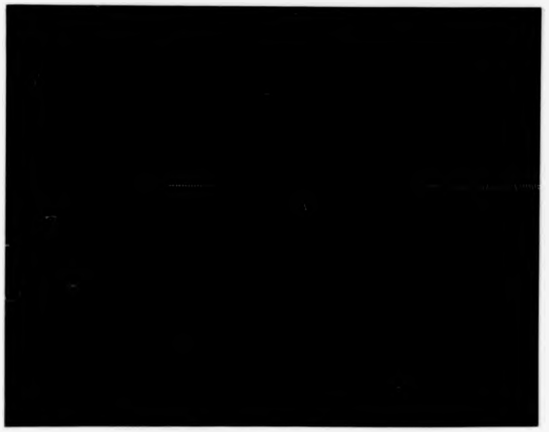
b

Figure 3.3 DAPI stained cells of a ρ^- with *kar-1* and a ρ^0 strain.

(a) ρ^0 cells (MR61)

(b) ρ^- cells (MR53)

Note that the nucleus is the major fluorescing body in the cells and in MR53, 2-3 fluorescing chondriolytes scattered in the cytoplasm.



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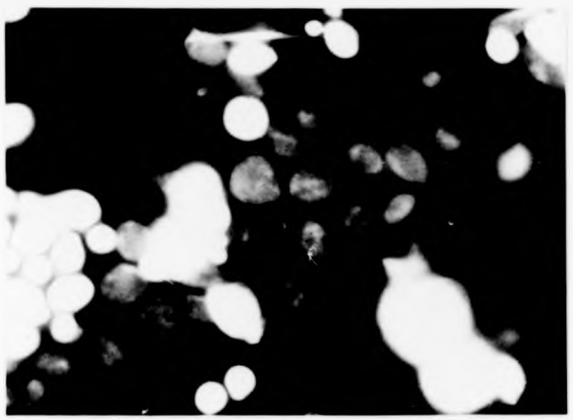


b

Figure 3.3 DAPI stained cells of a ρ^- with *kar-1* and a ρ^0 strain.

(a) ρ^0 cells (MR61)

(b) ρ^- cells (MR53)

Note that the nucleus is the major fluorescing body in the cells and in MR53,2-3 fluorescing chondriolytes scattered in the cytoplasm.

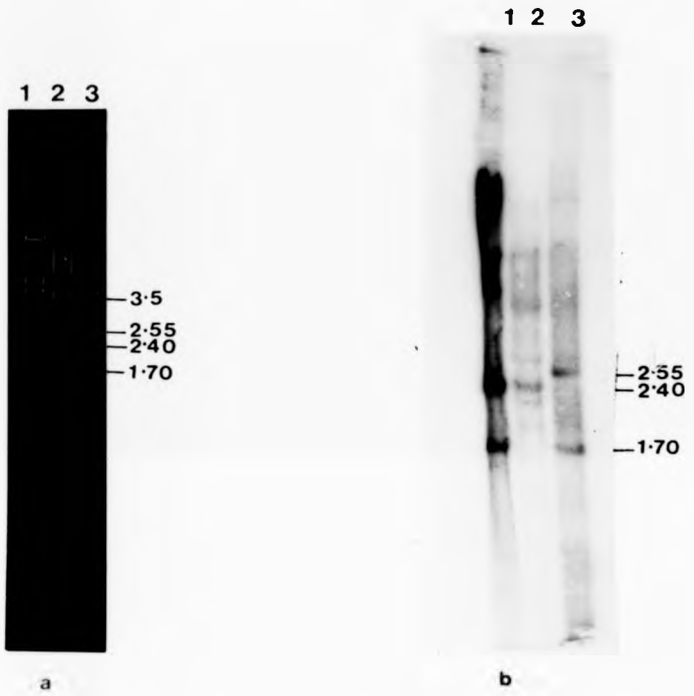
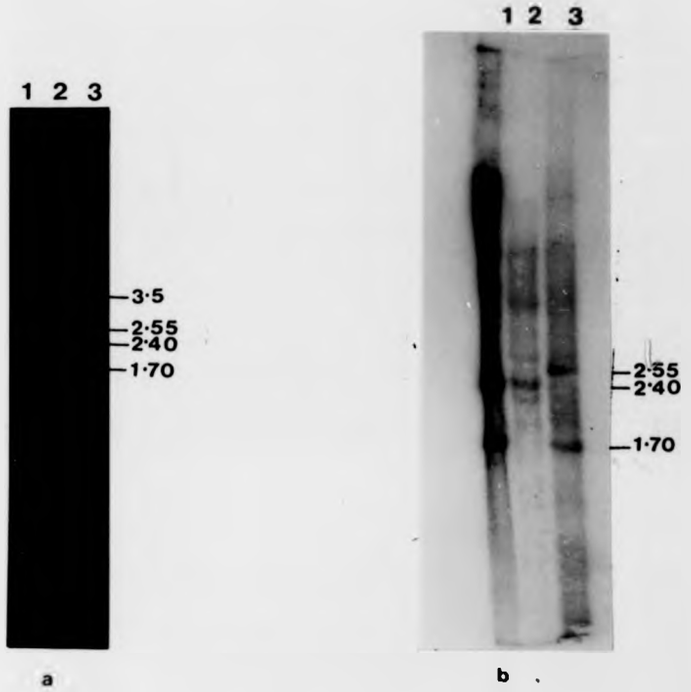


Figure 3.4 Identification of the ρ mt-DNAs from the strains with *kar-1* nuclear background.

(a) A ethidium bromide staining gel. The mt-DNAs isolated from the wild type strain (D273-10B/A1) (Lane 2) and the petite strain (MR53) (Lane 3) were digested with the restriction endonuclease EcoR1 and analysed on a 1% agarose gel. λ DNA (HindIII + EcoR1) digests (Lane 1) was used as the marker DNA. Note that MR53 has retained 2.4 Kbp (mitochondrial EcoR1 fragment-6 which is shorter than the fragment (2.56) of the wild type D273-10B/A1) and 1.7 Kbp (mitochondrial EcoR1 fragment-7) fragments. The size of the marker λ DNA fragments are (in decreasing order): 21.7, 5.15, 5.00, 4.27, 3.48, 1.98, 1.90 1.59, 1.37, 0.94, 0.83 Kbp respectively.

(b) Southern hybridization analysis. The mt-DNAs from the strain D27 (Lane 1), MR60 (Lane 2) and D273-10B/A1 (Lane 3) were digested with EcoR1 and transferred onto a sheet of nitrocellulose filter by Southern blotting. The filter was hybridised first with the unlabelled ρ^0 nuclear DNA to mask the contaminated nuclear DNA on the filter and then rehybridized with the nick translated ^{32}P labelled MR53 DNA. The autoradiogram shows that MR53 mt-genome has retained hybridizable sequences of both the Eco-6 (2.55 kbp) and Eco-7 (1.70) fragments of the wild type mt-DNA while MR60 has retained only the Eco-6 fragment. The strain D27 shows a smaller Eco-6 fragment and the normal Eco-7 fragment.



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type. The technique was to Southern blot the restriction digest of wild type mt-DNA genome onto nitrocellulose and its saturation hybridisation with unlabelled ρ^0 cellular DNA. The filter was then re-hybridized with labelled cellular DNA from the mitochondrial fraction of petites (assuming that this would contain mt-DNA plus contaminating nuclear DNA). Pre-hybridisation of the filter with ρ^0 cellular DNA masked most of the contaminated nuclear DNA in wild type mt-DNA restriction digests. Therefore nuclear DNA in petites which was labelled along with its trace amount of mt-DNA was not able to hybridize nuclear sequences on the filter. Only mt-DNA gave the positive signal showing the extent of the petite mitochondrial genome. Fig. 3.4b shows the results of this indirect hybridization analysis. It appears that the petite MR53 has retained the 6th and 7th bands of *EcoR*1 digests of wild type mt-DNA (strain D273-10B/A1), which was expected to contain the Oli-2 region from the published physical map of the yeast mitochondrial genome (Morimoto & Robinowitz, 1979). The strain MR60 which has retained the $Oli2^R$ allele only shows the positive hybridization signal for the Eco-6 fragment whose mobility is slightly faster. The strain D27 which was used as the second control for ρ^+ grande strain also shows the faster migrating Eco-6 fragment in conjunction with the normal Eco-7 fragment.

DISCUSSION

The observation of reduced suppressiveness of the petite mitochondrial genome in a *kar-1* nuclear background could be attributed to the effect of the *kar-1* gene on the transmission of petite mitochondrial DNA. Sena (1982) for the first time reported that certain ρ^- genomes in a *kar-1* background could not be transferred to another nuclear background by cytoduction. She pointed out that this could be due to

(i) inefficient replication of mitochondrial genome by certain nuclear backgrounds resulting in low input of ρ^- genome and consequent reduced genome transmission frequencies;

(ii) incomplete mixing of parental mitochondrial genetic units leading to zygotic buds receiving mt-DNA from only one parent.

(iii) Differential organelle organisation in different nuclear backgrounds resulting in the modulation of suppressiveness.

(iv) Structural changes associated with the mitochondrial genome during cytoduction might lead to the instability of the ρ^- genome resulting in the production of neutral petites which in turn leads to low suppressiveness in cell-population levels.

Point (iv) is very unlikely as a possible mechanism due to the fact that when the DS-14 mitochondrial genome is transferred to the *kar-1* nuclear background, producing CDS-14, the mt-DNA remains unchanged, as evidenced by restriction analysis (data not shown). The possibility of a change at the single nucleotide level however can not be ruled out.

Copy number analysis by analytical ultracentrifugation and fluorescent microscopic studies of cytoplasmic chondriolytes favours the hypothesis that *kar-1* mediates its effect by copy number determination. This could either be through direct interference with DNA replication, or through mitochondrial sequence specific action. The latter probability is reflected in the copy number variation of CDS-14 and the rest of the petites (MR53, MR50, MR60, MR23) used in this study. In any case, low copy numbers of petite genomes in the *kar-1* nuclear background results in lower suppressiveness through one or all of the first three possible mechanisms stated early in the discussion. In this context it is important to discuss the organisation of mitochondrial DNA within mitochondria. Grimes *et al* (1974), determined that there would be approximately 50 copies of mt-DNA molecule per haploid cell, paralleled by about 10 mitochondria which are doubled in diploids. From these values one may conclude that there would be about 5 mt-DNA molecules per mitochondria or mitochondrial chondriolites. Yet from our data with DAPI-stained haploid yeast cells as well as that of Williamson *et al* (1974) it appears that each chondriolite has only one to two condensed mitochondrial genomes. It is possible that each chondriolite contains one mitochondrial molecule in a condensed form, the lower number of observed chondriolites may be due to their functional state and consequently not condensed to be visible by the DAPI-staining method. Considering Hoffman & Avers' result (1973) of electron microscopy, the section of a yeast cell showing only one branched

mitochondrion, it is probable that chondrialites are a local aggregate of each individual mitochondrial genetic unit.

The evidence for a branched structure of yeast mitochondria also comes from the indirect observation that the isolation of intact mitochondria suitable for oxidative phosphorylation studies is difficult. This ultra-structural organisation can be used to explain the suppressive modulation by *kar-1* of the ρ^- genomes. During zygotic transmission, fragmentation of large mitochondria or biosynthesis of new mitochondria produce functional vesicles in the absence of mt-DNA which may lead to the low suppressiveness among the zygotic clones. In this scenario, *kar-1* probably plays a role in the faulty transmission of the cell's mitochondria into daughter cells by influencing the ultra-structural architecture.

Dot-hybridization and Southern hybridization analyses prove definitely that petites still maintain their respective ρ^- genome which were not detectable at the individual cell level, but at the cell population level. The transfer of these ρ^- genomes by cytoduction to another nuclear background without *kar-1* has not proved to be successful so far. Therefore it remains undetermined whether or not these ρ^- petite genomes in another nuclear background will recover higher suppressivity and consequently higher yields of their mitochondrial DNA.

Recently by complementation analysis, the *kar-1* gene has been cloned and sequenced (Rose & Fink,1984). From the aminoacid sequence deduced from the DNA sequence analysis of cloned DNA, it appears that the product of the gene could be a MAP (Microtubule associated protein). The polypeptide has a hydrophobic domain at one end which might help to anchor the peptide in a lipid bilayer.This later finding is very interesting in the sense that if this protein is involved in the organisational architecture of the mitochondria within the cell, by connecting them with cellular network of microtubules for example, it might affect the transmissability of the organelle (and consequently it's genome) into daughter cells. Mutations in this gene, therefore, might affect suppressivity of a particular mit-genome. But why it is *rho* genome (ρ^-) specific remains unexplainable. The physiological effect of this gene has been reported to be toxic for the cells with high copy number(Rose, M., personal communication).

CHAPTER-4

Cloning of the Oli-2 region from various strains of yeast in *E. coli*

INTRODUCTION

It has been outlined in the previous chapter that the petites generated in a *kar-1* background do not yield mt-DNA in sufficient quantities that can be used for further molecular analyses. Therefore it was desirable as an alternative approach to clone the oli-2 regions of mt-DNA in a suitable cloning vector for their amplification and propagation. In this chapter I will describe the cloning strategies used for cloning the oli-2 region in a high copy number plasmid of *E. coli* and the characterisation of these clones.

1. Plasmids as cloning vehicles

Plasmids are extrachromosomal genetic elements which have the ability to replicate independently of the chromosome. Generally they are double-stranded, covalently closed circular DNA molecules ranging in size from 1 kbp to greater than 200 kbp (Broda, 1979). A range of phenotypes (e.g. resistance to antibiotics, production of antibiotics, degradation of organic compounds, production of enterotoxins, production of restriction and modification enzymes, etc.) are conferred by different plasmids on their host cells. On the basis of their copy number per cell they could be categorised as relaxed plasmids (higher copy number, 10-200) or stringent plasmids (low and limited copy number, 1-3) (see Novick *et.al*, 1976)

Any useful plasmid cloning vector should have the following properties:

(i) It should be relatively small and should replicate in a relaxed fashion.

(ii) It should carry one or more selectable markers to allow identification of transformants and to maintain them in the host cell.

(iii) It should contain a single recognition site for one or more restriction enzymes in regions of the plasmids that are not essential for replication and which could be used for insertion of foreign DNA.

2. Use of pAT153 for primary cloning of the Oli-2 region

pAT153, a derivative of pBR 322 (Bolivar *et.al*, 1977) is a useful cloning vector possessing all the criteria mentioned above. It was constructed by Twigg and Sherratt (1980) by deleting the HaeII B and G fragments, which span a region of the plasmid genomes involved in control of copy number. Its copy number per cell is 1.5 to 3 times more than that of of pBR 322. Fig.4.1 describes the physical and genetic map of pAT153 which has been used as a primary cloning vector of the Oli-2 region of the mitochondrial genome.

MATERIALS and METHODS

1. Materials

All chemicals used are of analytical grade unless otherwise stated. The restriction enzymes and ligase are from commercial sources, (BRL, BCL). Aha III was a gift from P&S Biochemicals. Ligase was either bought from BRL or BCL or was a gift from Anglian Biotechnology. When lyophilized restriction endonucleases (Lyphozymes, BRL) were bought, they were reconstituted according to the supplier's recommendations. Bacterial alkaline phosphatase (BAP) and calf thymus intestinal phosphatase (CIP) were bought from Sigma. Ampicillin, Tetracyclin, chloramphenicol were also bought from Sigma. Electrophoretic grade of agarose was purchased either from Sigma or from BRL. One particular batch was bought from Serva which was found to be inferior to the former two. Low melting point agarose was bought from BRL. Acrylamide and bis-acrylamide were bought either from Sigma or BDH. Ethidium bromide, bis-benzimide (Hoechst 33258) and DAPI were bought from Sigma. Caesium chloride (CsCl) was purchased from Fisons. Nitrocellulose sheets were bought from Schleicher & Schull.

2. Growth Media and Culture of Strains

The media and culture of yeast were performed as described in Chapter 2. *E. coli* strains were grown in LB media with or without antibiotics. Detailed recipes are given in the Appendix. The stock of yeast strains was maintained as described in Chapter 2. The

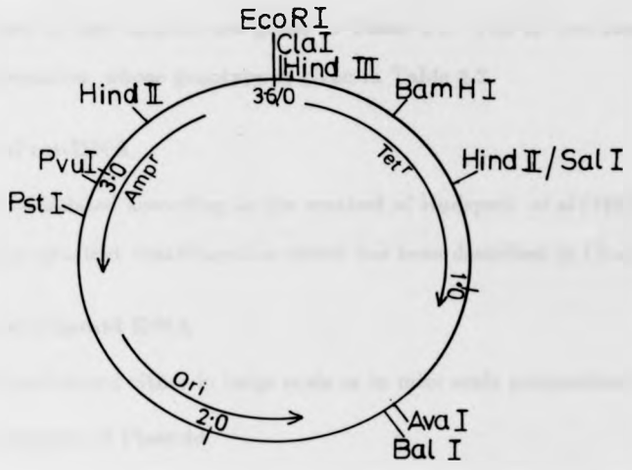

Figure 4.1 Physical and genetic map of pAT 153. The map has been drawn after Maniatis *et al*, 1982. The size of pAT 153 is 3.6Kbp with the selective markers *Amp*^{r} and *Tet*^{r}. The single restriction sites (AvaI, PstI, BamH1, ClaI, SalI, EcoR1 and HindIII) have been shown on the map. Insertion at PstI site inactivates the *Amp*^{r} gene. Insertion in the BamH1, HindIII and SalI site inactivates the *Tet*^{r} gene Insertion at HindIII site is variable. The plasmid was developed by Twigg & Sherratt (1980) by deletion of two HaeII restriction fragments of the plasmid pBR322. For comparison with pBR322 map see Appendix.

stock of *E. coli* strains were either maintained on LB plates by monthly streaking or in 7.5% DMSO at - 70° C.

3. Strains used

Yeast strains used in this chapter are given in Table 4.1. The *E. coli* strain HB101 was used for transformation, whose genotype is given in Table 4.2.

4. Isolation of mt-DNA

The mt-DNA was isolated according to the method of Hudspeth *et al* (1980) by CsCl: bis-benzimide density gradient centrifugation which has been described in Chapter 3.

5. Isolation of Plasmid DNA

Plasmid DNA was isolated either in large scale or in mini scale preparations.

Large scale preparation of Plasmid

Plasmid DNAs were isolated in large scale according to the method of Katz *et al* (1973). 15-20 ml of LB medium containing the appropriate antibiotics (Ampicillin 100 μg/ml or Tetracyclin 12.5 μg/ml) was inoculated with a single bacterial colony. They were grown overnight with vigorous shaking at 37°C. Next morning, this overnight late log culture were inoculated into one litre of LB medium with the proper antibiotic (with or without 0.5% glucose) and shaken for 4-6 hours at 37° C (The $O.D._{600}$ of the culture would be approximately 0.4). Then 5ml of a solution of chloramphenicol (34mg/ml in ethanol) was added to it (final concentration of chloramphenicol was 170 μg/ml) and incubated at 37° C with vigorous shaking for a further 12-16 hours (overnight). The cells were harvested by centrifugation at 5000rpm for 10 minutes at 4° C (Sorvall GSA rotor) and washed with 20ml of 10 mM Tris-Cl, 1mM EDTA, pH 8.0. The cells were resuspended in 8.4ml of 25% sucrose, 50mM Tris-Cl, pH 8.0 and transferred to an Erlenmeyer flask. Then 1.4ml of lysozyme (10mg/ml in 50mM Tris-Cl,pH 8.0) was added to it and kept in ice for 5 minutes with gentle swirling. 4.6ml of 0.25mM EDTA (pH 8.0) was added to it slowly and kept on ice for another 10 minutes with gentle swirling. Then 9.6ml of lysis mix

Table 4.1 Yeast strains

Strain	Nuclear genotype	Mitochondrial genotype	Source
D273-10B/A1	α met	ρ^+	Dr. Tzagoloff
D27	α met	ρ^+	Subclone of D273-10B/A1
CD40	a leu kar-1	ρ^+ *Oli*2^R *Oss*1^R	Dr.Lancashire
CD24	α his kar-1	ρ^+ *mit* pho9	"
CD41	α his kar-1	ρ^+ *mit* pho8	"
D27/76	α met	ρ^+ *Oli*2^R	"
D27/92	α met	ρ^+ *Oss*1^R	"
mit-175	α ura	ρ^+ *mit*	Bolotin-Fukuhara et al(1979)
D22	a ade2	ρ^+	Dr. Wilkie
DS-14	α met	ρ^- *Oli*2^R *Oli*4^s	Dr. Tzagoloff

Table 4.2 *E. coli* strain

strain	Genotype	Reference
HB101	*F* , hsd S20 (*rB* , *mB*), rec A13, ara-14, proA_2, lac Y1, gal K2, rps L20 (*Sm*^{r}), xyl-5, mtl-1, SupE44, λ^-	Bolivar & Backman (1979)

(50ml 20% Triton-X, 125ml 0.25mM EDTA, 25ml 1M Tris-Cl,pH 8.0 and 300ml water) was added to it slowly with swirling. After 10 to 15 minutes the chromosomal DNA was spun out either at 19000 r.p.m. (Sorvall SS34 rotor) for 1 hour or at 30,000 r.p.m.(Ti50 rotor,Beckman) for 45 minutes. The supernatant was extracted with phenol two or three times and adjusted to 0.3M Na-acetate before the addition of two volumes of cold ethanol. The DNA was spun down at 10,000 r.p.m. for 10 minutes at 4^0 C and redissolved in TE Buffer (10mM Tris-Cl, pH 7.5, 1mM EDTA) About 10gm of CsCl was dissolved into the DNA solution and the final volume was made to 11 ml (a refractive index of 1.39). Ethidium bromide was added into it to a final concentration of about 200 to 300 μg/ml (from a 10mg/ml stock solution). The solution was centrifuged at 45,000 r.p.m. for 36-40 hours in a Beckman 50 Ti or 65 rotor at 15^0 C. The plasmid bands were collected either with a fine tipped Pasteur pipette, or with the help of a syringe. Ethidium bromide was extracted (three times) with isoamyl alcohol (saturated with CsCl) from the collected fractions which was then dialysed against TE buffer (two or three changes) at 4^0 C for overnight. DNA was precipitated with 2 vol. of ethanol and 0.3M Na-acetate (final conc.) at - 70^0 C for 3 hrs. or at - 20^0 C overnight.

Mini-scale preparation of plasmid ("Mini-lysate" method)

This procedure was generally followed to screen a large number of transformants at a time. The method is more or less similar to that described by Birnboim & Doly (1979). Small shaking overnight cultures (about 1ml each) were centrifuged in a 1.5ml microfuge tube (Eppendorf tube) for 2 to 3 minutes. The supernatant was discarded and the cells were resuspended in 0.1ml lysis solution (25mM Tris-Cl,pH 8.0, 10mM EDTA, 50mM or 1% glucose, 2mg/ml lysozyme) by vortexing and left on ice for 30 minutes. Then 0.2ml of alkaline SDS solution (0.2M NaOH, 1% SDS) was added and the tubes were left on ice for 5 minutes with occasional mixing by inversion. The suspensions should become viscous, then 0.15ml of high salt solution (3M Na-acetate,pH 5.0) is added, mixed and the tubes are left on ice for another 60 minutes. A heavy white precipitate should form which is removed by centrifuging for 10 minutes at room temperature.

The supernatants were transferred to new microfuge tubes. 1ml of ethanol was added to each tube and placed at - 20° C for 30 minutes. They were centrifuged again in the microfuge for 3-5 minutes. The pellets were then dissolved in 0.1ml of 0.1M Na-acetate (pH 6.0) and 2.0ml of cold ethanol was added to them and left at - 20° C for 10 minutes. They were centrifuged again for 5 minutes and the DNA pellets were dried under vacuum. The dried DNA pellets were dissolved in 20-50 µl of TE Buffer which could be digested with most restriction enzymes without further purification.

6. Restriction enzyme digestion.

All restriction buffers were made at 10 times concentrated solution so that mt-DNA, or plasmid DNA with lower concentration (i.e larger volume of DNA solution) could be digested with minimal increase in the volume. Assay buffer and conditions were according to supplier's recommendations. Occasionally core buffer was used to digest a sample of DNA with more than one enzyme. A list of buffers for commonly used enzymes are given in the appendix. During double or triple enzyme digestion, enzymes requiring low salt and low pH were used first, followed by the enzymes with a high salt or higher pH (adjusted by adding an appropriate amount of concentrated salt solution or buffer of higher pH). All enzymes were inactivated by heat shock (65° C for 5 minutes) or where needed, by phenol extraction. A loading buffer of a 10 times concentrated solution (50% glycerol, 25mM EDTA,0.1% Bromophenol Blue) was added to the restriction digests before their analysis by gel electrophoresis.

7. Analysis of Restriction Fragments.

Restriction digests were analysed either by agarose gel (0.7% to 1.5%) electrophoresis or by polyacrylamide gel electrophoresis (3% to 4%) depending upon the size of the expected restriction fragments (See Maniatis *et al*, 1982). For screening purposes a "mini-gel" apparatus was used. Agarose gel electrophoresis were done generally in submerged condition whereas polyacrylamide gel electrophoresis were done in a vertical gel apparatus. Electrophoresis was carried out in 50mM Tris-borate (pH 8.3), 1mM EDTA,

(electrophoretic buffer). The voltage used was generally 5-6 Volts/cm of gel length and in this condition it takes about 3-4 hours (in a 20 x 20 cm gel of 0.3cm thickness).

Gels were stained in 1 μg/ml ethidium bromide solution (in 50mM Tris-borate,pH 8.3, 1mM EDTA) for 15-20 minutes and were observed on a 300nm UV trans-illuminator (Fotodyne, UK). The photographs were taken using a Polaroid type 665 (positive-negative film) camera (Polaroid UK Ltd.) with the help of a Kodak 23A wratten filter.

8. Labelling DNA

DNAs were labelled either by nick translation or by end labelling.

Nick translation

The method is the similar to one described by Rigby *et al* (1977). In a typical reaction mixture of 50 μl volume the following are mixed:

5 μl of 10 x nick translation buffer (See Appendix)
1 μg of DNA to be labelled
1 nmole of each of unlabelled dNTPs (1 μl of a 1mM solution)
100 pmoles of [$\alpha - ^{32}$P]-dNTP
$H_2$0 to make 45 μl.

The mixture was chilled to 0° C and 5 μl of DNA polymerase I and DNase I mix (5 units of DNA polymerase I and 0.5 μl of 0.1 μg/ml DNaseI) was added to it and incubated at 15° C for 60 minutes.The reaction was stopped by addition of 5 μl of 0.25M EDTA,pH 8.0. The unincorporated dNTPs were separated by ethanol reprecipitation in the presence of ammonium acetate (2.5M final conc.). Occasionally nick translation was carried out using a commercially available nick translation kit (BRL).

End-labelling of DNA:

(i) Filling in of 3'- ends with Klenow fragments.

For some experiments DNAs were end labelled by filling in the recessed 3' ends of double stranded DNA, with the help of large fragments of DNA Polymerase I (Klenow fragment). The method is similar to one that has been described by Maniatis *et al* (1982). In brief, 1 μg of DNA was digested with the desired restriction enzyme in 25 μl of the appropriate

buffer. 2.0 μCi of the appropriate [α−^{32}P]-dNTP and 1.0 unit of the Klenow fragment of *E. coli* DNA Polymerase were then added to it and incubated for 30 minutes at 30° C. The DNAs were either precipitated with ethanol in the presence of 0.3M Na-acetate or, when needed loaded directly on to gel for electrophoresis. The labelled DNA fragments were extracted from the gel for further analysis.

(ii) 5' end-labelling of DNA by Polynucleotide Kinase

This was done according to Maxam & Gilbert (1977). Dephosphorylated DNA fragments at their 5'-ends (by BAP treatment, see below) were taken in siliconised eppendorf tube with the following reaction mixture:

5 μl	Dephosphorylated DNA (1-50 pmoles 5' ends)
35 μl	distilled water
5 μl	10 X PNK buffer (See Appendix)
5 μl	γ[^{32}P]-ATP(>1000 Ci/nmole)
1 μl	T4 Polynucleotide Kinase (20 units)

The mixture was incubated at 37°C for 30 mins. and the reaction was stopped with the addition of 200 μl 2.5 M Ammonium acetate, 1 μl tRNA (1 mg/ml), 750 μl of 95% ethanol. They were mixed thoroughly and chilled at - 70° C (dry ice-methanol bath) for 5 mins and centrifuged at 1200 X g for 5 mins.(at 4° C).After removal of the supernatant the pellet was dissolved in 250 μl of 0.3 M Na-acetate. The DNA were reprecipitated with 3 Volumes of cold ethanol (- 70° C). DNA pellets were finally dried and dissolved in TE buffer.

9. Ligation

(i) BAP or CIP digestion of vector DNA

The plasmid DNAs, (pAT153 in most cases) was cut with EcoRI and checked for complete digestion in a "mini gel" apparatus. The enzyme was inactivated by incubating the tubes at 65° C for 10 minutes. The pH of the *EcoR*1 buffer was raised to pH 8.0. Then about 0.1 unit of bacterial alkaline phosphatase (BAP) or calf intestinal phosphatase (CIP) was added to the reaction mix (generally 100 μl) and incubated at 37° C for 1 hr. During CIP digestion, a final concentration of 10 mM $CaCl_2$ was used in the reaction mixture by

adding an appropriate amount of 1M $CaCl_2$ solution. The reaction was stopped by adding either EDTA or EGTA (Final Concentration 20mM) and incubating at 70° C for 10 minutes.

The reaction mix was first extracted with equal volumes of phenol (twice) and then with equal volumes of either chloroform or di-ethyl ether (once), successively. The solution was then made 0.3M Na-acetate and DNAs were precipitated with 2.5 volumes of ethanol at - 70° C for 1-2 hours or at - 20° C overnight. The DNAs were spun down at 10,000 r.p.m. at 4° C, washed once with 95% ethanol and vacuum dried. The dried DNAs were dissolved finally in TE buffer (10 mM Tris-Cl, 0.1 mM EDTA, pH 7.5). (When DNAs were digested with restriction enzymes requiring higher pH (e.g. pH 8.0), as in the case of *Hind*III, BAP or CIP were added directly to the reaction mix).

ii) Restriction Enzyme Digestion of Target DNAs

Parallel with restriction enzyme digestions and BAP or CIP treatment of vector DNAs, the target DNAs were digested with cloning site compatible enzymes. During cloning of *EcoR*1 fragments, mt-DNAs were digested with *EcoR*1 and checked in minigel for proper digestion. The reaction was stopped at 65° C and extracted with phenol. The DNAs were precipitated with ethanol and dried under vaccum. The dried DNAs were disolved in TE buffer.

iii) Set up of Ligation Reaction

Typical Ligation reaction was carried out in a 20 µl reaction volume as stated below:

Vector DNA	0.1 µg
Target DNA	0.1 µg
H_2O to make	15.0 µl
10 x ligation buffer (See Appendix)	2.0 µl
10 mM ATP	2.0 µl
Ligase (1 unit/µl)	1.0 µl
Total	20.0 µl

The reaction mix was incubated at 15° C for 12-16 hours (overnight) by which time more than 90% ligations are achieved.

10. Transformation

Transformation was carried out by the calcium chloride procedure described by Mandel & Higa (1970). *E. coli* strain HB101 was grown up in LB medium overnight at 37° C. The overnight bacterial culture was diluted 100 times in fresh LB (e.g. 0.1ml in a 10ml fresh culture) and grown at 37° C with vigorous shaking to a density of ~ 5 x 10^7 cells/ml. This usually takes three and a half to four hours (2.0 $O.D._{550}$ is equivalent to ~ 5 x 10^7 cells/ml). The culture was chilled on ice for 10 minutes and the cells were harvested by centrifugation at 4000g for 5 minutes. The cells were resuspended in half of the original culture volume of an ice-cold sterile solution of 50mM $CaCl_2$,10mM Tris-Cl (pH 8.0) and kept in an ice bath for 15-20 minutes. The cells were re-centrifuged at 4000g for 5 minutes and resuspended in 1/15 of the original volume of an ice cold sterile solution of 50mM $CaCl_2$,10mM Tris-Cl, pH 8.0.

0.2ml of these competent cells were aliquoted into pre-chilled Eppendorf tubes. Transformation was carried out immediately or within 24 hours by adding ligation mix directly to aliquoted competent cells and the mixtures kept on ice for 30-40 minutes. The cells were heat shocked at 42° C for 2 minutes and 1.0ml of L broth was then added and the mixture incubated at 37° for 30 minutes (tetracycline selection) or 1 hour (ampicillin selection) without shaking. For ampicillin selection, the recovered cells were spread directly onto ampicillin containing LB plates, 200 µl onto each plate. For tetracycline selection cells were spun down and resuspended in 100 µl of sterile water and spread onto a single tetracycline plate. The plates were kept inverted at 37° C for 10-12 hours.

11. Colony Hybridization

Colony hybridization was carried out using the method of Grunstein & Hogness (1975). A nitrocellulose filter was placed onto an agar plate containing the selective antibiotic. Then, using sterile toothpicks each individual bacterial colony to be screened was transferred onto the filter and then onto a master agar plate,which also contains selective antibiotic. Each colony was streaked in an identical position on both plates. About 150

to 200 colonies were streaked on each 85 mm plate. The plates were kept inverted and incubated at 37^{0} C for overnight. The master plate was then transferred to 4^{0} C for storage and the nitrocellulose filter was transferred onto another agar plate containing chloramphenicol (10 μg/ml) and incubated for 8-12 hours at 37^{0} C. After this amplification step, the filter was peeled off from the chloramphenicol plate, and placed colony side up onto a SDS-impregnated Whatman 3MM paper for 3 minutes. The filter was then transferred onto a second sheet of 3MM paper saturated with denaturing solution (0.5M NaOH,1.5M NaCl) and kept for 5 minutes. The filter was then neutralised by transferring it onto another 3MM paper saturated with neutralising solution (1.5M NaCl, 0.5M Tris-Cl, pH 8.0) for 5 minutes. The filter was transferred finally onto a fourth sheet of 3MM paper saturated with 2 x SSPE (0.36M NaCl,20mM NaH_2PO_4, pH 7.4,2mM EDTA, pH 7.4). The filter was dried at room temperature on a dry 3MM paper for 30-60 minutes and then baked at 80^{0} C in a vacuum oven for 2 hours. The baked filters were floated on the surface of a tray of 6 x SSC until they had become thoroughly wet from beneath. The filters were then kept submerged for 5 minutes. The filters were pre-washed in a pre-washing solution (50mM Tris-Cl pH 8.0, 1M NaCl,1mM EDTA, 0.1% SDS) by incubation for 1-2 hours at 42^{0} C, with shaking when more than two filters were used in the same container. The filters were then pre-hybridized and hybridized using the same conditions as described for Southern hybridisation in Chapter 3.

12. Isolation and purification of DNA from Gel

DNA fragments were isolated from gel either by electro elution or by extraction from low melting point agarose.

Electro-elution method

Eletro-elution was done either in a dialysis sac as described by McDonnel *et al* (1977) or by an apparatus (Fig. 4.2) designed in this laboratory.

A. Electro-elution into a dialysis sac

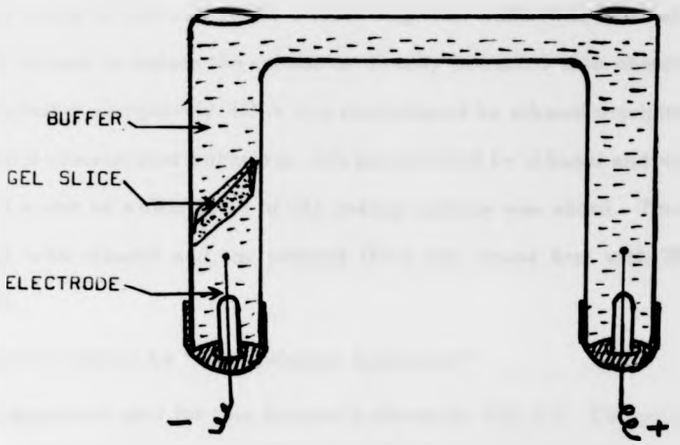

Figure 4.2 Apparatus for electro-elution of DNA fragments from gel.

The DNA band of interest was cut out as a slice of the gel with the help of a sharp scalpel. The gel slice was then put inside a dialysis bag (preboiled in the presence of EDTA for 10-15 mins.) containing 50mM Tris borate, 1mM EDTA buffer. Then the bag was tied at the ends with a minimum volume of buffer inside. The bag was immersed in a shallow layer of TBE electrophoretic buffer in an electrophoretic tank. Current was passed for 1-2 hrs. at 150 V during which time most of the DNAs is electroeluted into the buffer surrounding the gel. Then the polarity of the current is reversed for 1-2 mins. to release the DNA bound to the wall of the dialysis bag. The buffer was collected and either extracted with butanol to reduce the volume or directly extracted with phenol-chloroform (1:1) and diethylether successively. DNA was precipitated by ethanol precipitation. The DNA from butanol concentrated buffer was also precipitated by ethanol and was resuspended in 200 µl of water to which 25 µl of 3M sodium acetate was added. The DNA were reprecipitated with ethanol and the pelleted DNA was rinsed first with 70% ethanol and then dried.

B. Electro-elution by "Electroelution Apparatus"

The apparatus used for this purpose is shown in Fig. 4.2. The gel slices were placed into one of the chambers containing the negative electrode as shown in the figure. The DNA fragments are driven from the gel by electrophoresis into the surrounding diluted buffer (20 mM Tris borate, 1 mM EDTA, pH 8.3) and accumulated into the other chamber containing the positive electrode. The buffer was collected and the volume was reduced by butanol extraction.. The solution was then extracted with phenol/chloroform and chloroform successively. The DNA is precipitated with cold ethanol and rinsed with 70% ethanol. Finally, the DNA pellet is dried and dissolved in TE buffer.

C. Extraction of DNA from low melting point agarose (LMP agarose)

Employing the same conditions which has been described for normal gel electrophoresis, DNA digests were run in low melting point agarose gel but at 4^{0} C. The bands of interest were excised out from the gel with a sharp scalpel under long wave length UV. The LMP

agarose containing the DNA fragments were dissolved by heating at 65° C for 5 minutes and extracted with an equal valume of phenol at room temperature. The aqueous phase was collected after centrifugation and re-extracted with phenol and finally with diethyl ether. The DNAs were recovered by ethanol precipitation (2 hrs. at - 70° C) and centrifugation. Finally, DNAs were dried under vaccum, and re-dissolved either in water or in TE buffer.

13. Mapping of the recombinant plasmids

Mapping of the recombinant plasmids was achieved by:

(i) Direct restriction digest analyses.

(ii) Birnstiel's method (Smith & Birnstiel, 1976) of partial digestion of end labelled DNAs.

(iii) The Bal-31 method of mapping (Legerski *et al*, 1978).

RESULTS

Strain Specific Polymorphism of mt-DNA in *Saccharomyces cerevisiae*

The physical mapping of mt-DNA from *S. cerevisiae* has established that various strains generally possess either of two forms of the molecule, which although similar genetically differ in physical size (Prunell *et al*, 1977; Sanders *et al*, 1977; Morimoto & Robinowitz, 1979). Based on restriction enzyme data the molecules have been sized of approximately 70 and 76 kbp, these being termed the "Short" and "Long" forms respectively. The extra length of one of these molecules has proved to be the result of an increased number of introns within the split genes of the parental strain (see Chapter-1).

Before proceeding to clone the Oli-2 region of mt-DNA, it was therefore necessary to know whether there are any difference among the strains used in the present study. Diagnostically *EcoR*1 digestion yields ten fragments with long strains, for example MH41-7B and only nine with the short strains, for example those that are D273-10B based (see Morimoto & Rabinowitz, 1979). The majority of strains studied in the present work fall into

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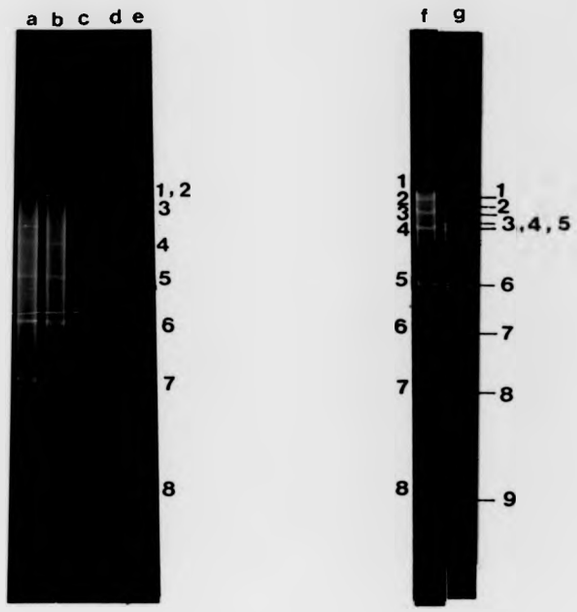

Figure 4.3 EcoR1 digests of the mt-DNAs from the ρ⁻ grande strains.

Purified mt-DNAs from CD40 (Lane a), CD24 (Lane b), CD41 (Lane c), D603-3B (Lane d), D273-10B/AI (Lane e), D22 (Lane f) and mit-175 (Lane g) were digested with Eco R1 and analysed on a 0.9% Agarose gel.

The molecular size of the restriction fragments of lane a through to e corresponds to 1 (32.50Kbp), 2 (17.0Kbp), 3 (7.10Kbp), 4 (5.10Kbp), 5 (3.50Kbp), 6 (2.55Kbp), 7 (1.70Kbp) and 8 (0.90Kbp). The largest fragment (32.5Kbp) is not quite visible and the smallest fragment (0.150Kbp) has run out of the gel.

The molecular weight size of the restricton fragments in lane f (strain D22) corresponds to: 1 (26.0Kbp), 2 (24.2Kbp), 3 (10.10Kbp), 4 (7.80Kbp), 5 (3.50Kbp), 6 (2.55Kbp), 7 (1.70Kbp), 8 (0.90Kbp). However, in lane g (strain mit-175) the fragment sizes are: 1 (24.20Kbp), 2 (17.0Kbp), 3 (10.10Kbp), 4 (8.30Kbp), 5 (7.80Kbp), 6 (3.50Kbp), 7 (2.55Kbp), 8 (1.70Kbp), 9 (0.90Kbp). The fragments 3, 4, and 5 of lane g have been labelled on the same line. The smallest fragment (0.150Kbp) in all cases is not visible. The molecular weight of the fragments have been obtained by co-electrophoresis of the samples with λ DNA digested with HindIII or HindIII with EcoR1 or by external calibration on the slab gel.

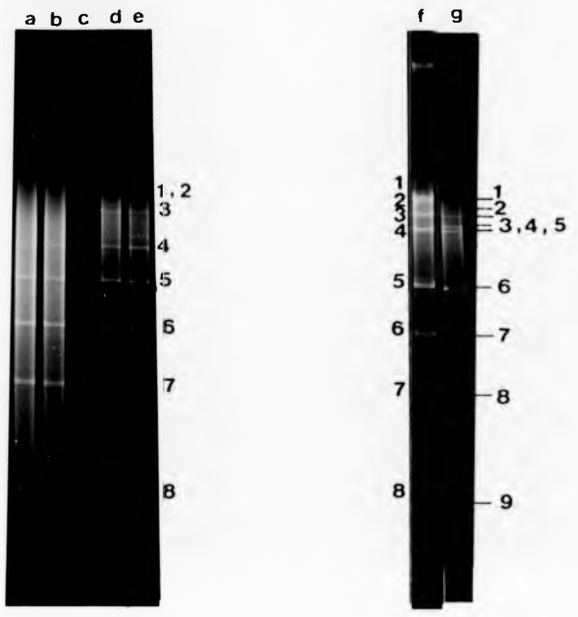

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Table 4.3 EcoR1 digested fragments of mt-DNA from various strains

Fragment No.	D273-10B/A1	MH41-7B†	mit-175	D22
1	32.50	24.20	24.20	26.00
2	17.00	17.00	17.00	24.20
3	7.10	10.1	10.10	10.10
4	5.10	8.30	8.30	
5	3.50	7.80	7.80	7.80
6	2.55	3.50	3.50	3.50
7	1.70	2.55	2.55	2.55
8	0.90	1.70	1.70	1.70
9	0.15	0.90	0.90	0.90
10		0.15	0.15	0.15
TOTAL	70.50	76.20	76.20	76.90

† Data from Morimoto *et al* (1977) and Morimoto & Rabinowitz (1979).
sizes of the fragments are in Kbp.

the latter category, D273-10B/A1, D27, CD40, D603-33B, CD24 and CD41 all generating nine *EcoR*1 fragments (see Fig. 4.3). The strain mit-175 in contrast yields ten *EcoR*1 fragments and hence belongs to the group of long strains. However the strain D22, which has in previous studies been used as the parental sensitive type to many antibiotic resistant screens (Lancashire & Griffiths, 1975a, 1975b) yields aberrant molecular weight *EcoR*1 fragments. This strain produces nine *EcoR*1 fragments, which when tabulated suggest it possesses a genome size of nearly 77 kbp (Table 4.3), a little longer than the long strains. The unexpectedly large genome of D22 may harbour yet further differences in structure and organisation of yeast mitochondrial genes, especially when considering spliced genes.

It is apparent from the *EcoR*1 digests (Fig.4.3) that the strains used in the present study do not differ much in the *EcoR*1 fragments of lower molecular weights (3.50 kbp, 2.55 kbp, 1.70 kbp, 0.90 kbp, 0.15 kbp). The *EcoR*1 fragments 5, 6, 7, 8, 9 of short forms (D273-10B/A1, CD40, D603-3B, CD24, CD41) correspond to the *EcoR*1 fragments 6, 7, 8, 9, and 10 of the long forms mit-175 and D22. It has been established that the *EcoR*1 fragments 3 (7.10 kbp) and 4 (5.10 kbp) of the short forms correspond, in respect to the genetic map, to the *EcoR*1 fragments 3 (10.10 kbp) and 5 (7.80 kbp) of the long forms, but the formers being shorter by 3.0 kbp and 2.7kbp respectively (Morimoto *et al*, 1977; Morimoto & Rabinowitz, 1979) due to the occurrence of extra introns in Cob-box and Oxi-3 respectively. *EcoR*1 fragment 1 (32.5 kbp) of the short form gives rise to two fragments in long the form, namely fragment 1 (24.2 kbp) and fragment 4 (8.3 kbp) due to the development of a new *EcoR*1 site within the fragment. *EcoR*1 fragment 2 (17.1 kbp) is similar in both forms. However in the strain D22, this fragment and the fragment 4 (8.30 kbp) of the long form are missing. When these data are compared with the results of Morimoto *et al* (1979) it appears (Fig. 4.4) that the generation of a new fragment (26.0 kbp) in D22 is related to the disappearance of the *EcoR*1 site between the fragments 2 and 4 which should produce a combined fragment of molecular weight 25.30 kbp. A little higher molecular weight (26.0 kbp) is either due to the acquisition of an insertion of 0.70 kbp or due to the low resolution of the size of fragments in the high molecular weight

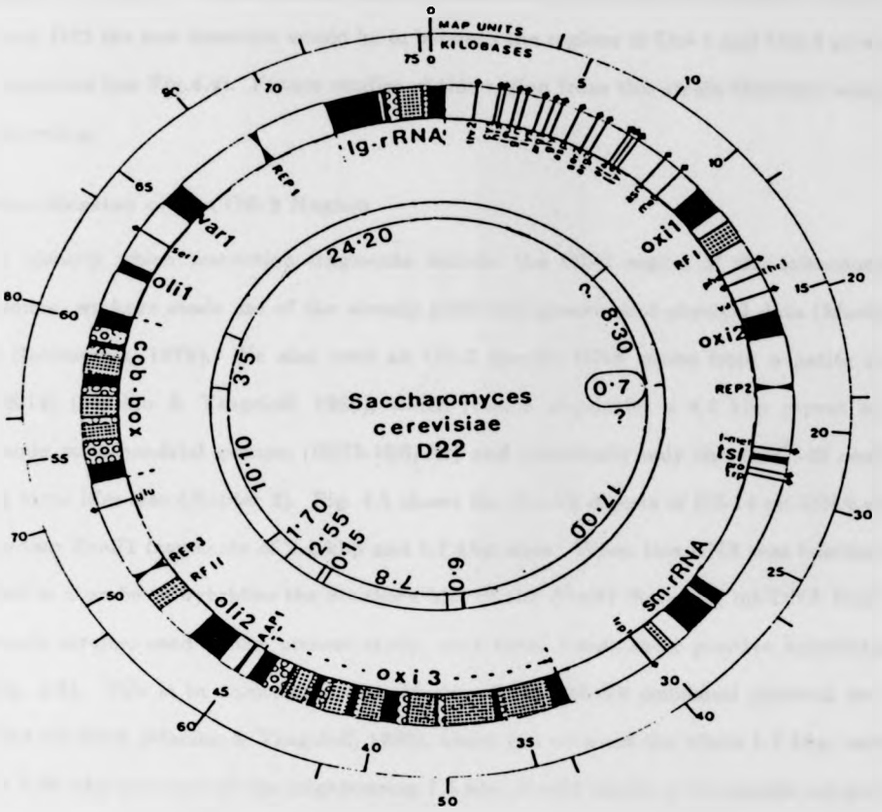


Figure 4.4 Correlation of the physical map of D22 mt-DNA with the physical and genetic map of a long strain (KL14-4A).

The map for KL14-4A has been adapted from Dujon (1983). Only the EcoR1 physical map has been shown (inner ring). The outer ring is the genetic map drawn for KL14-4A. A possible region for extra sequences (0.7Kbp) on the D22 mt-DNA has been shown by either a question mark (?) or a loop.

region of the gel. If the molecular weight determined in this study is correct, then in the strain D22 the new insertion would be in between the regions of Oxi-1 and Oxi-2 or within themselves (see Fig.4.4). Future studies of this region from this strain therefore would be interesting.

Identification of the Oli-2 Region

To identify which restriction fragments contain the Oli-2 region of the mitochondrial genome, we have made use of the already published genetic and physical data (Marimoto & Robinowitz, 1979). We also used an Oli-2 specific DNA probe from a petite strain (DS-14) (Macino & Tzagoloff, 1980), which retains physically a 4.1 kbp repeat of the grande mitochondrial genome (D273-10B/A1) and genetically only the $Oli2^{R}$-48 containing locus (See also Chapter 2). Fig. 4.5 shows the *EcoR*1 digests of DS-14 mt-DNA which has two *EcoR*1 fragments of 2.4 kbp and 1.7 kbp sizes. When this DNA was labelled and used as a probe to hybridize the Southern blot of the *EcoR*1 digests of mt-DNA from the grande strains, used in the present study, only three bands show positive hybridization (Fig. 4.6). This is in accordance with Macino & Tzagoloff's published physical map of DS14 mt-DNA (Macino & Tzagoloff, 1980), which has retained the whole 1.7 kbp, most of the 2.56 kbp and part of the neighbouring 7.1 kbp *EcoR*1 bands of the grande mt-genome (See also Marimoto & Robinowitz, 1979).

Fig. 4.7 shows the comparative physical map for DS14 and wild type mt-DNA, showing the position of the Oli2 gene and the gene for subunit 1 of cytochrome oxidase (Oxi-3). In the realisation that Eco-6 and Eco-7 contain the whole expanded Oli-2 region we proceeded to clone these two fragments from various strains (wild type and mutants, mentioned in Table 4.1), into pAT153.

Cloning of Oli-2 region (Eco-6 and Eco-7 bands)

The initial strategy to clone the Eco-6 and Eco-7 fragments of the grande mitochondrial genome was to ligate total *EcoR*1 digests of mt-DNA with *EcoR*1 digests of the vector, pAT153, and to use the total ligation mix to transform the *E. coli* strain HB101. This

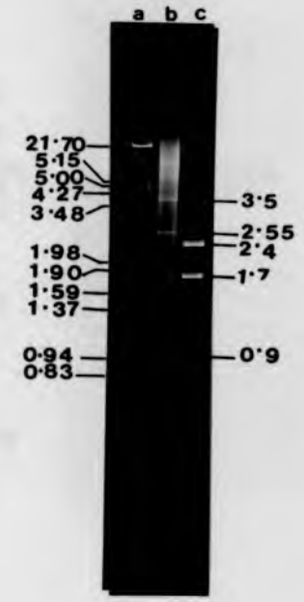


Figure 4.5 EcoR1 digests of DS-14 mt-DNA and a comparison with its grande strain (D273-10B/A1).

Purified mt-DNAs were digested with EcoR1 and analysed on 1% Agarose gel. Lane a, λ (HindIII and EcoR1) digests (marker DNA); Lane b, D273-10B/A1 mt-DNA and Lane c, DS-14 mt-DNA. The molecular size of the fragments (in Kbp) has been indicated.

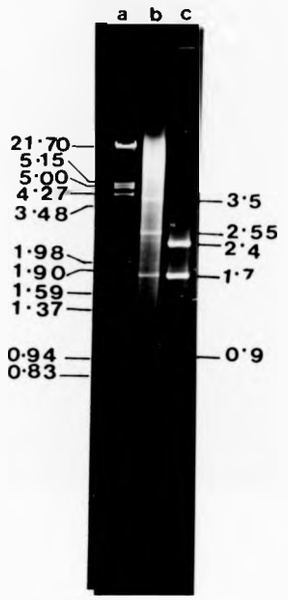


Figure 4.5 EcoR1 digests of DS-14 mt-DNA and a comparison with its grande strain (D273-10B/A1).

Purified mt-DNAs were digested with EcoR1 and analysed on 1% Agarose gel. Lane a, λ (HindIII and EcoR1) digests (marker DNA); Lane b, D273-10B/A1 mt-DNA and Lane c, DS-14 mt-DNA. The molecular size of the fragments (in Kbp) has been indicated.

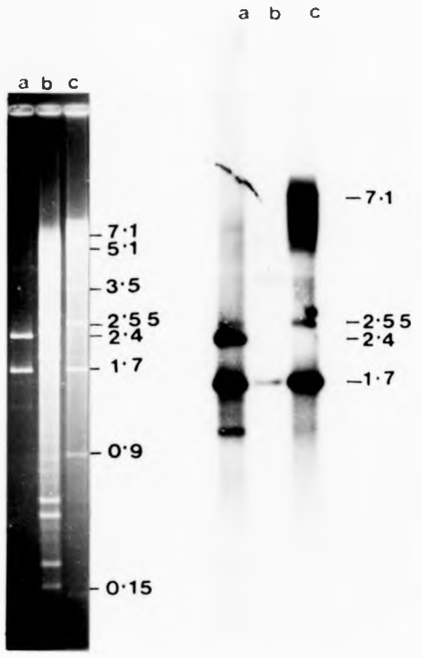


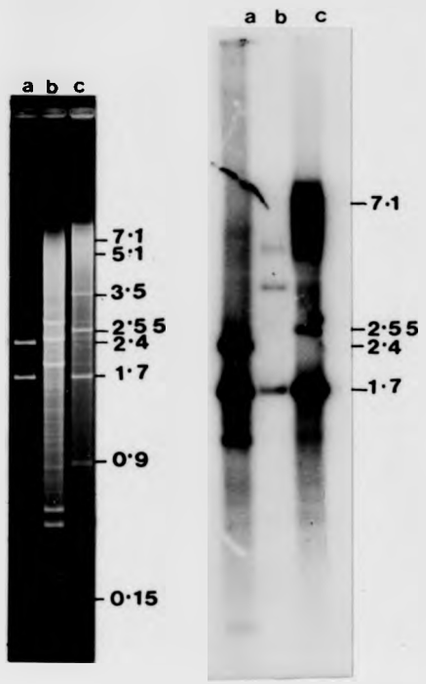
Figure 4.6 Identification of the restriction fragments of wild type mt-genome which share common sequences to DS-14 mt-DNA.

DS-14 mt-DNA was labelled by nick translation and was used as a probe to hybridize EcoR1 digests of wild type (D273-10B/A1) mt-DNA, transferred to a nitrocellulose filter.

(A) Ethidium bromide staining gel.

(B) Autoradiogram of the nitrocellulosefilter.

(a) EcoR1 digests of DS-14 mt-DNA. (b) EcoR1 digests of the mt-DNA from a ρ^- clone (not characterised in the present study). (c) EcoR1 digest of D273-10B/A1 mt-DNA.



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(A) Ethidium bromide staining gel.

(B) Autoradiogram of the nitrocellulose filter.

(a) EcoR1 digests of DS-14 mt-DNA, (b) EcoR1 digests of the mt-DNA from a ρ^- clone (not characterised in the present study). (c) EcoR1 digest of D273-10B/A1 mt-DNA.

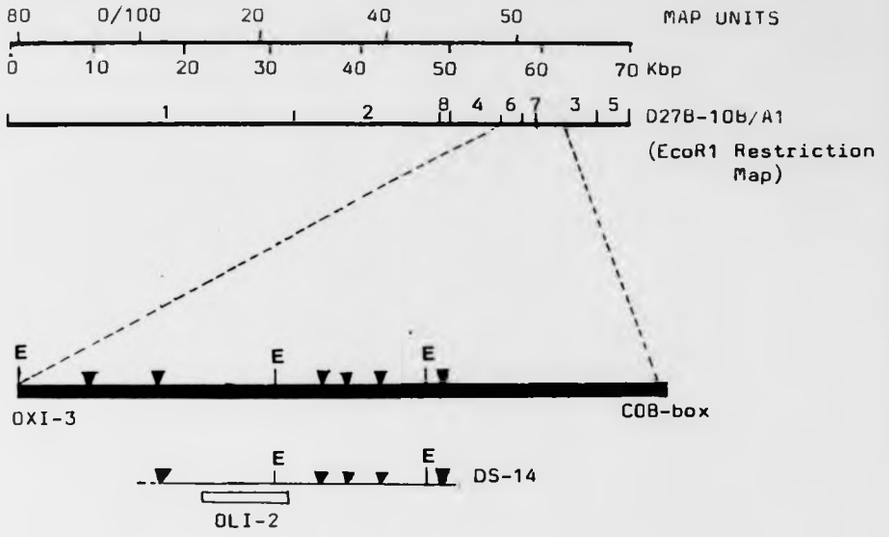

Figure 4.7 Comparative restriction maps of DS-14 and wild type mt-DNA with the relative position of the Oli-2 gene.

The linear *EcoR*1 restriction map of D273-10B/A1 mt-DNA has been aligned with genetic map units (top). The restriction map of DS-14 mt-DNA has been aligned with the enlarged relevant portion of the wild type map to show the position of the Oli2 locus or the gene for subunit-6 of the O.S. ATPase. E = *EcoR*1 restriction site. ▼ = *Hpa*II restricion site. The dotted line of DS-14 mt-DNA indicates the undetermined sequences of the DNA (Macino & Tzagoloff, 1980). 1, 2, 3, 4, 5, 6, 7 and 8 are various *EcoR*1 fragments as described in the text (see also Fig. 4.3).

Note that the wild type map shown here was originally drawn for the short strain D273-10B/A1 (Morimoto & Rabinowitz, 1979) and hence, does not correspond to the map shown in Fig. 4.4 for the long strain (KL14-14A).

seemed to work well. When such transformants were transferred to nitrocellulose and hybridized *in situ* with labelled DS-14 mt-DNA, it was found that on average 12.5%-20% were positive clones. Fig. 4.8 shows an autoradiogram of a typical colony hybridisation filter. The positive clones were picked up from the master plate and screened for their insert(s). For the latter purpose plasmids from all the positive clones were isolated by the minilysate method and digested with *EcoR*1 to compare them with *EcoR*1 digests of mt-DNA from grande strains by agarose gel electrophoresis. Fig. 4.9 demonstrates a typical ethidium bromide staining gel for such screening. It is clear from the figure that occurrence of the 1.7 kbp insert is much greater than that of 2.56 kbp inserts among the positive clones. This is quite expected as it is well known that small inserts preferably recombine with the vector and the transformation rate of these are much higher than those with larger inserts.

It is also apparent that some recombinants have picked up some undesirable DNA fragments contaminated with mt-DNA. Such recombinants were generally avoided. Only those strains which have retained only the Eco-6 and Eco-7 bands were picked up. In some cases desirable clones were made by secondary cloning of initial "complex" clones (retaining one of the desirable Eco-6 and Eco-7 bands, and one or two other undesirable bands). The method was to isolate total plasmid DNA from such "complex" clones, then its digestion with *EcoR*1 enzymes and religation of an aliquot of such *EcoR*1 digests to transform the *E. coli* strain, HB101. It has been found that in such secondary cloning about 25% - 50% of are desirable clones.

The positive oli2 recombinants were named as pSC"X" ME6 and pSC"X" ME7: SC stands for *S. cerevisiae*, "X" denotes the strain used (e.g. from CD40 it is named as pSC40ME6 or pSC40ME7), ME 6 and 7 stands for mitochondrial EcoRI fragments 6 and 7, respectively. Throughout this thesis, henceforth the recombinants will be mentioned in this way. The clones derived from different yeast strains with different mitochondrial markers are shown in Table 4.4. It should be pointed out that the recombinant clones from the strain mit-175 have also been named as pSC175ME6 and pSC175ME7 for convenience's

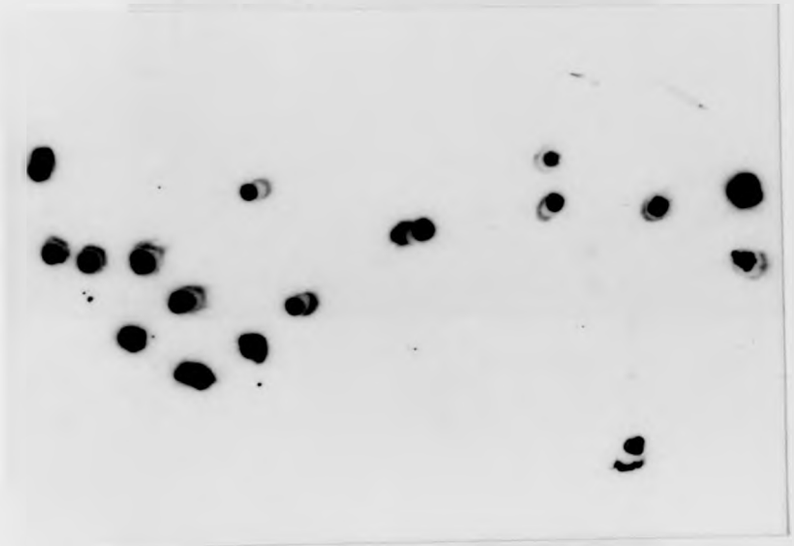

Figure 4.8 Autoradiogram of colony hybridization filter for the Oli-2 recombinants. The E.coli strain HB101 was transformed with ligated pAT 153 and mt-DNA (both were digested with Eco RI before ligation). The transformants (selected by ampicillin resistivity) were replicaplated onto a nitrocellulose filter and probed with radioactive DS-14 mt-DNA probe. The dark spots are positive clones for the Oli-2 region of mt-DNA.

Figure 4.8 Autoradiogram of colony hybridization filter for the Oli-2 recombinants.
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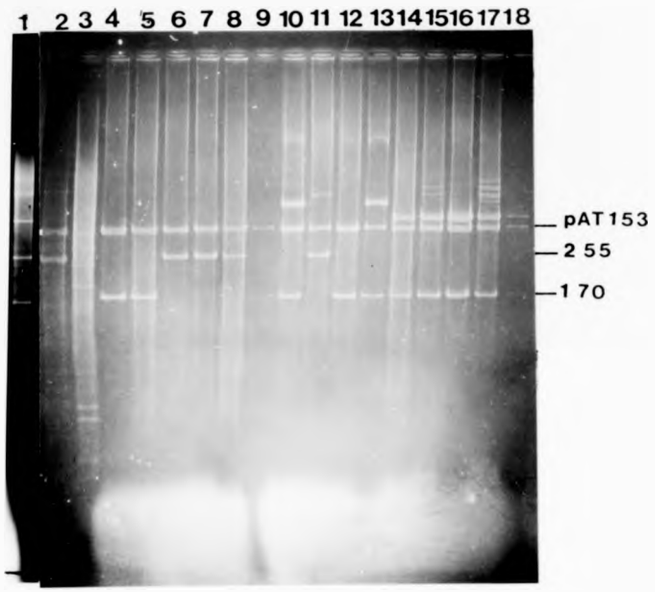

Figure 4.9 Screening for the inserts of the Oli-2 recombinant clones.

The recombinant plasmids were isolated from DS-14 hybridizable positive clones by the minilysate method of Birnboim & Doly (1979). These were digested with the restriction enzymes EcoR1 and analysed on a 1% Agarose gel, using D273-10B/A1 mt-DNA EcoR1 digest (Lane 1) as comparable marker.

Samples in lane 2, 6, 7, 8 and 11 have the 2.55 Kbp insert (Eco-6 fragment) and those in lanes 4, 5, 9, 10, 12, 13, 14, 15, 16, 17 and 18 have the 1.7Kbp insert (Eco-7 fragment). Lane c is the EcoR1 digest of D273-10B/A1 nuclear DNA. The clones with 2.55Kbp insert were named as pSCME6 and those with 1.7Kbp insert were named as pSCME7. Note that the pAT 153 has a molecular weight of 3.6Kbp.

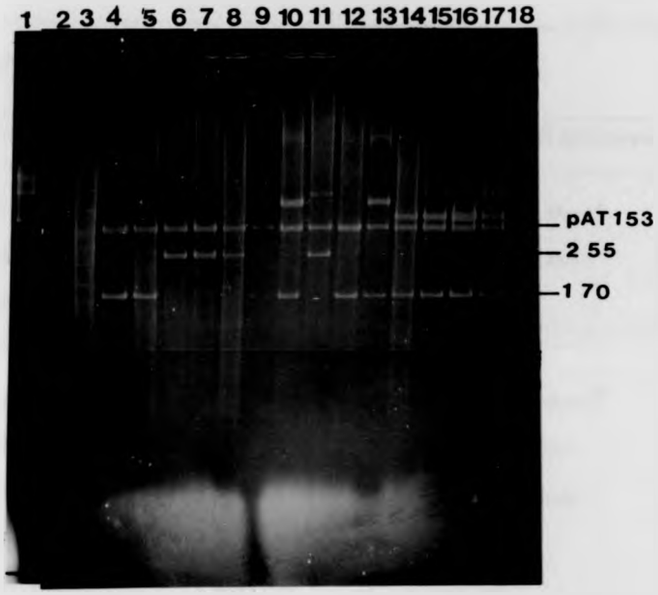

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Table 4.4 Recombinant *E. coli* clones and the parental yeast strains.

Name of the recombinants	Parental yeast strain	Mitochondrial genotype
pSC273ME6 & pSC273ME7	D273-10B/A1	ρ^+ *Oli*2^S *Oss*1^S
pSC27ME6 & pSC27ME7	D27	ρ^+ *Oli*2^S *Oss*1^S
pSC603ME6 & pSC603ME7	D603-3B	ρ^+
pSC22ME6 & pSC22ME7	D22	ρ^+
pSC40ME6 & pSC40ME7	CD40	ρ^+ *Oli*2^R *Oss*1^R
pSC24ME6 & pSC24ME7	CD24	*mit* pho9
pSC41ME7	CD41	*mit* pho8
pSC175ME6 & pSC175ME7	mit-175	*mit*
pSC76ME6 & pSC76ME7	D27/76	ρ^+ *Oli*2^R *Oss*1^S
pSC92ME7	D27/92	ρ^+ *Oli*2^S *Oss*1^R
pSC14ME6 & pSC14ME7	DS14	ρ^- *Oli*2^R

shake though these recombinant clones contain Eco-7 and Eco-8 fragment respectively due to it's long form origin.

Characterisation of pSCME6 Recombinants

For sequencing purposes it was absolutely necessary to characterise physically the cloned fragments. For this purpose two methods have been employed:

i) Direct Restriction analysis by restriction endonuclease digestion (single or double) of each individual isolated band from agarose gel.

ii) by the Birnstiel method using partial digests of end labelled restriction fragments.

When all the Eco-6 bands from different strains were compared it was found that the Eco-6 band from the strain D27 was shorter than the rest as was also apparent in its total mitochondrial *EcoR*1 digest (data not shown). This may be due to the possession of another *EcoR*1 site very close to the COOH end of cytochrome oxidase subunit 1 gene (Oxi-3 locus) (see Fig. 4.7) or, due to an internal deletion. DNA sequence analysis should rationalise these alternatives.

When each individual isolated Eco-6 band was digested with *Sau3A*, it was found that the Eco-6 band from CD40, CD24, CD41 and D603-3B had similar distributions of Sau 3A recognition sites whereas those from D27 and D22 were different (Fig. 4.10a). Eco-6 from D27 yielded only three *Sau3A* fragments in comparison to 5 fragments from the others. Presumably, strain D27 has undergone some point mutation within two *Sau3A* recognition sequences, thereby altering the number of fragments. On the other hand strain D22 has all five *Sau3A* bands in its Eco-6 fragment, but differs in the molecular weight of the lowest band. Therefore it was presumed that in this strain a new *Sau3A* site might have been generated by point mutation very close to either the *EcoR*1 site or to another *Sau3A* site, thereby producing a very small fragment not detectable in 1.5% agarose gels. This has been shown to be correct from the sequence analyses of the Eco-6 band from this strain. A new *Sau3A* site site has been generated by point mutation, towards the COOH end of the Oli2 gene (see Fig.5.11a & 11b, Chapter-5) and very close to the *EcoR*1 site.

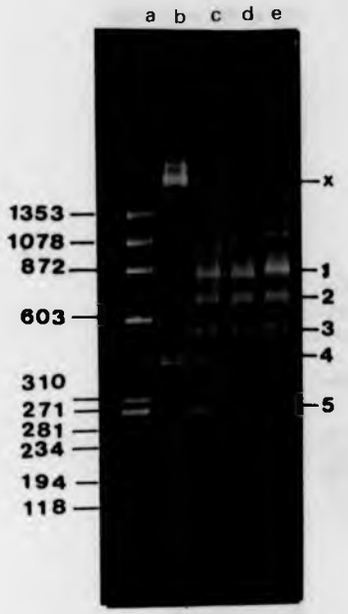


Figure 4.10a Sau3A digest of the Eco-6 fragments from various clones.

Eco-6 fragments were purified from various pSCME6 clones and digested with the restriction enzyme Sau3A. The fragments were analysed on a 1.5% Agarose gel.

Lane (a) ϕX174RF HaeIII marker DNA, (b) D27 Eco-6, (c) D273-10B/A1 Eco-6, (d) D603-3B Eco-6 and (e) D22 Eco-6. Five Sau3A fragments have the molecular sizes of (1) 790bp, (2) 620bp, (3) 500 bp, (4) 400bp and (5) 260bp. The fragment marked as x is the high molecular weight fragment (1.80Kbp) from strain D27. Note that the fourth Sau3A fragment of strain D22 (lane f) has a slightly higher mobility in the gel.

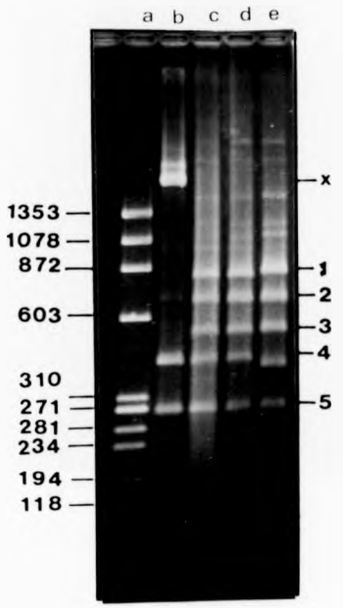


Figure 4.10a Sau3A digest of the Eco-6 fragments from various clones.

Eco-6 fragments were purified from various pSCME6 clones and digested with the restriction enzyme Sau3A. The fragments were analysed on a 1.5% Agarose gel.

Lane (a) ϕX174RF HaeIII marker DNA, (b) D27 Eco-6, (c) D273-10B/A1 Eco-6, (d) D603-3B Eco-6 and (e) D22 Eco-6. Five Sau3A fragments have the molecular sizes of (1) 790bp, (2) 620bp, (3) 500 bp, (4) 400bp and (5) 260bp. The fragment marked as x is the high molecular weight fragment (1.80Kbp) from strain D27. Note that the fourth Sau3A fragment of strain D22 (lane f) has a slightly higher mobility in the gel.

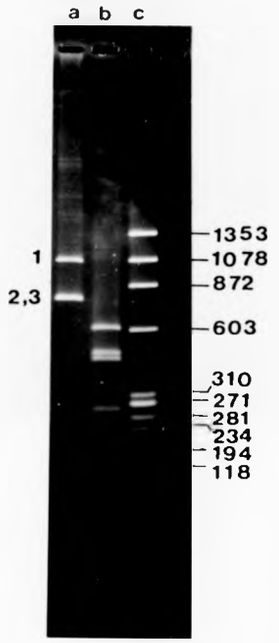


Figure 4.10b Hpa II digests of the Eco-6 and Eco-7 fragments.

Purified Eco-6 and Eco-7 fragments from the recombinant plasmids (pSCME6 and pSCME7) were digested with Hpa II and analysed on 1.5% Agarose gel.

Eco-6 fragments from pSCME273ME6, pSC40ME6, pSC603ME6, pSC22ME6, pSC24ME6 were all the same. The representative shown here (lane a) is that from the clone psc40ME6. Note that the second and third Hpa II fragments move to the same place in the gel, which was proved by densitometer scanning of the negative. The HpaII generated fragments have the molecular sizes of 1.1Kb (fragment 1) and 0.7Kbp (fragment 2 and fragment 3).

Hpa II digests of the Eco-7 fragments from various clones (pSC40ME7, pSC273ME7, pSC22ME7, pSC603ME7, pSC175ME7, pSC41ME7, pSC24ME7) show the same pattern as have been shown in lane b. The molecular sizes of the four Hpa II fragments are (in decreasing order): 620bp, 470bp, 450bp and 250bp respectively. Lane c has the ϕX174RF (Hae III digests) markers.

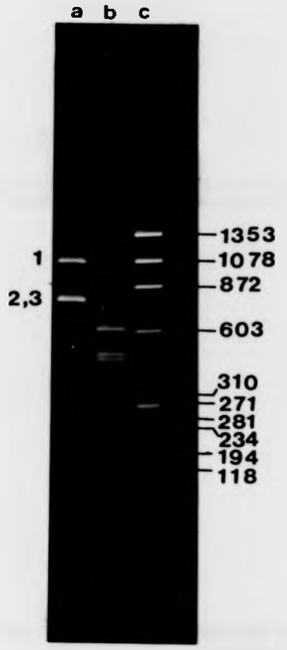


Figure 4.10b Hpa II digests of the Eco-6 and Eco-7 fragments.

Purified Eco-6 and Eco-7 fragments from the recombinant plasmids (pSCME6 and pSCME7) were digested with Hpa II and analysed on 1.5% Agarose gel.

Eco-6 fragments from pSCME273ME6, pSC40ME6, pSC603ME6, pSC22ME6, pSC24ME6 were all the same. The representative shown here (lane a) is that from the clone psc40ME6. Note that the second and third Hpa II fragments move to the same place in the gel, which was proved by densitometer scanning of the negative. The HpaII generated fragments have the molecular sizes of 1.1Kb (fragment 1) and 0.7Kbp (fragment 2 and fragment 3).

Hpa II digests of the Eco-7 fragments from various clones (pSC40ME7, pSC273ME7, pSC22ME7, pSC603ME7, pSC175ME7, pSC41ME7, pSC24ME7) show the same pattern as have been shown in lane b. The molecular sizes of the four Hpa II fragments are (in decreasing order): 620bp, 470bp, 450bp and 250bp respectively. Lane c has the ϕX174RF (Hae III digests) markers.

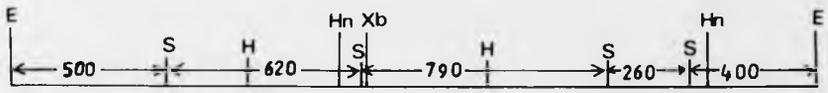


Figure 4.11 Restriction map of the Eco-6 DNA fragment from various pSCME6 clones.

The map is similar in all cases except the strain pSC22ME6 where an extra Sau3A site lies very close to the cloning EcoR1 site, which has been proved by DNA sequence analysis (see Chapter 5).

E, EcoR1 site; H, Hpa II site; Hn, HinfI site; S, Sau3A site and Xb, XbaI site. The map order has been determined by a combination of data of restriction analyses of individual fragments, Birnstiel mapping (Smith & Birnstiel, 1976), and by Bal-31 deletion mapping (Lagerski *et al*, 1978).

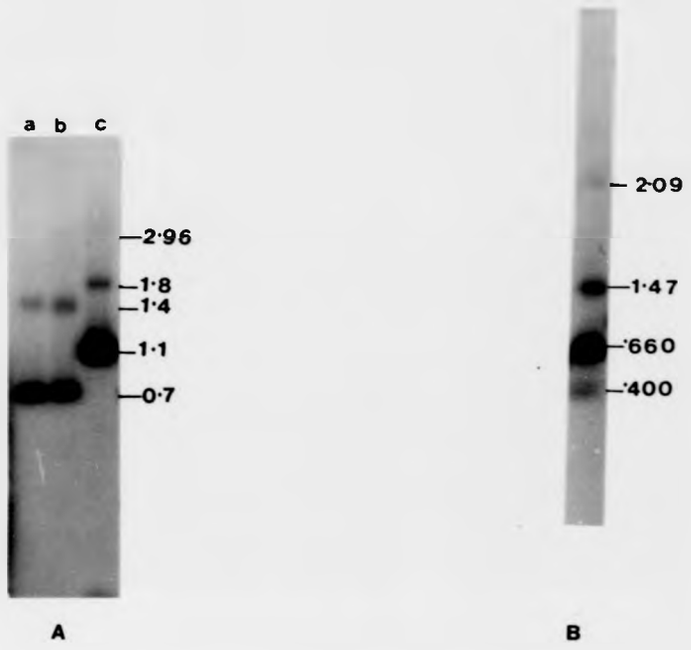


Figure 4.12 Birnstiel mapping of HpaII and Sau3A sites of pSCME6 clones.

The pSCME6 clones were digested with HindIII (this unique restriction site of pAT 153 is situated 30bp away from the EcoR1 cloning site). The DNA ends were labelled with (^{32}P)-dCTP in the presence of Klenow by filling in. Then the DNAs were digested with PstI and run in a 1% low melting point Agarose gel. Of the two fragments generated, the smaller molecular weight fragment (2.85Kbp) contains the pAT 153 DNA and the higher molecular weight fragment (3.310Kbp) contains the 2.55Kbp Eco-6 fragment and 750bp of pAT 153 DNA. The latter fragments, where one end was labelled, were purified from the gel and digested with either HpaII or Sau3A for different time periods for the generation of partial fragments. The samples of different time periods were mixed together and run on 0.8% to 1.5% Agarose gel. The gel was dried and autoradiographed. A flow chart for these experiments is represented diagramatically in Fig. 12a.

A) HpaII generated partial fragments. The molecular size of the fragments have been shown in Kbp. Lane **a, pSC40ME6; Lane b**, pSC22ME6; Lane **c**, pSC24ME6.

B) Sau3A generated partial fragments. fragment sizes are given in Kbp. The sample shown here is that from the pSC24ME6.

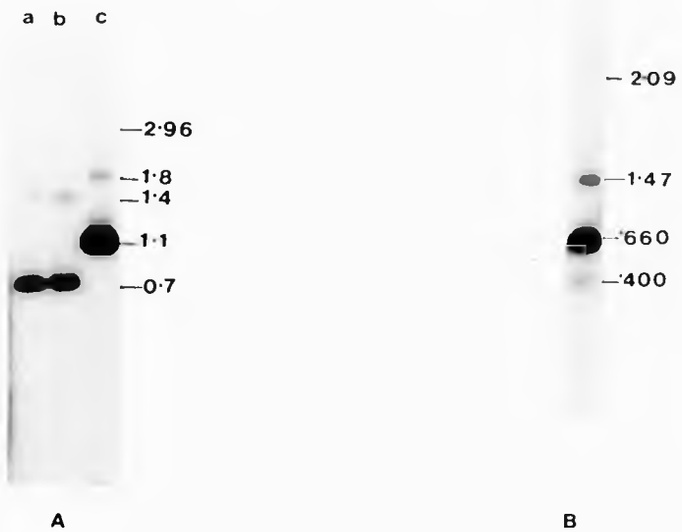


Figure 4.12 Birnstiel mapping of HpaII and Sau3A sites of pSCME6 clones.

The pSCME6 clones were digested with HindIII (this unique restriction site of pAT 153 is situated 30bp away from the EcoR1 cloning site). The DNA ends were labelled with (^{32}P)-dCTP in the presence of Klenow by filling in. Then the DNAs were digested with PstI and run in a 1% low melting point Agarose gel. Of the two fragments generated, the smaller molecular weight fragment (2.85Kbp) contains the pAT 153 DNA and the higher molecular weight fragment (3.310Kbp) contains the 2.55Kbp Eco-6 fragment and 750bp of pAT 153 DNA. The latter fragments, where one end was labelled, were purified from the gel and digested with either HpaII or Sau3A for different time periods for the generation of partial fragments. The samples of different time periods were mixed together and run on 0.8% to 1.5% Agarose gel. The gel was dried and autoradiographed. A flow chart for these experiments is represented diagramatically in Fig. 12a.

A) HpaII generated partial fragments. The molecular size of the fragments have been shown in Kbp. Lane **a, pSC40ME6; Lane b**, pSC22ME6; Lane **c**, pSC24ME6.

B) Sau3A generated partial fragments. fragment sizes are given in Kbp. The sample shown here is that from the pSC24ME6.

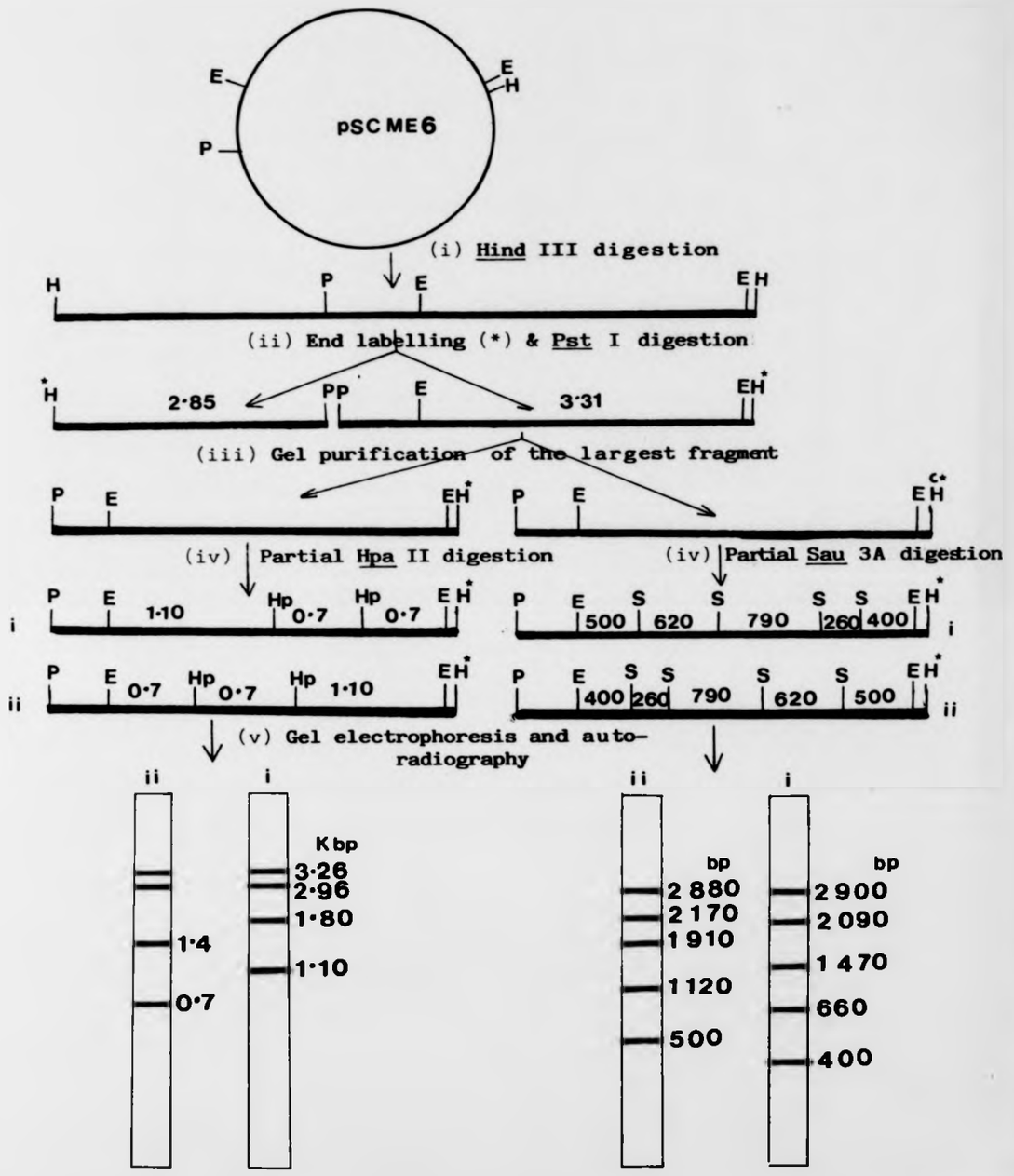


Figure 4.12a Flow chart for Birnstiel mapping of the HpaII and Sau3A sites of the Eco-6 fragment on pSCME6 clones.

Sites for various restriction enzymes have been abbreviated as: E, EcoR1; H, HindIII; P, PstI; Hp, HpaII; S, Sau3A. (i) and (ii) are two possible orientations of the Eco-6 fragment on the vector, pAT153. See Fig. 4.12 for detailed legend.

Restriction enzymes *Xba*I, *Hpa*II (Fig. 4.10b) and *Aha*III were also used to characterise the different pSCME6 clones. On the basis of these restriction analyses a restriction map for the Eco-6 fragment from the pSCME6 clones was derived which is shown in Fig. 4.11.

Orientation of Eco-6 bands in pSCME6 clones

Birnstiel's method (Smith & Birnstiel, 1976) was employed to determine the orientation of Eco-6 bands in pSCME6 clones. The clones were digested with *Hind*III (which is 30 bp away from the cloning site of *EcoR*1) and labelled with $\alpha-^{32}$P-dGTP and $\alpha-^{32}$P-dCTP by filling in the 3' recessed ends in the presence of the large fragment of DNA polymerase I. These end-labelled products were digested with *Sau3A* or *Hpa*II for different time periods (Fig. 4.12a). The digests were then analysed in agarose gels and autoradiographed (Fig. 4.12). These results helped to construct the orientation of the insert in pSCME6 clones which are shown in Fig. 4.13.

Characterisation of the Eco-7 recombinants

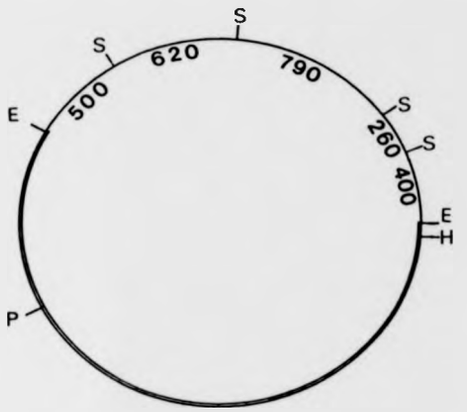
When isolated Eco-7 bands from different clones were digested with enzymes with known restriction sites, it was found to be similar in all strains. *Hpa*II (Fig. 4.10b), *Pst*I and SmaI digests were all similar. *Aha*III, however, showed some differences in the distribution of the recognition sites (Fig. 4.14). *Sau3A*, *Xba*I, *Xho*I, *Sal*I do not have any recognition sequences in this fragment.

Fig. 4.15 shows the restriction maps of pSCME7 plasmids from recombinant clones used in the study and their orientation in respect to each other.

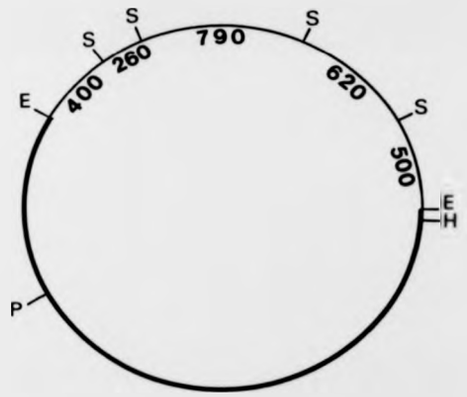
DISCUSSION

Mitochondrial genome of various strains of yeast used in this study

Comparing the restriction digests of grande mitochondrial genomes from various strains of yeast it is found that D22, mit-175 and D273-10B/A1 were all dissimilar, both in the distribution of their restriction sites and their total molecular weights. D603-3B (the



A



B

Figure 4.13 Orientation of the Eco-6 fragment in pSCME6 clones.

The Eco-6 fragment has been cloned into psc ME6 in two different orientations in respect of the vector (pAT 153) HindIII site. (A) In clones pSC40ME6, pSC22ME6 and pSC603ME6, the 400 bp Sau3A fragment is distal to the HindIII site of the plasmid. (B) In pSC24ME6, pSC273ME6 the fragment is proximal to the HindIII site. E, EcoR1 site; S, Sau3A site; P, PstI site.

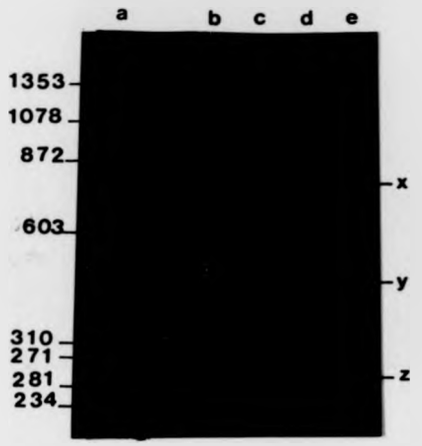


Figure 4.14 Heterogeneity of AhaIII restriction sites (AAA↓TTT) among pSCME7 clones.

Purified pSCME7 plasmids were digested with the restriction enzyme AhaIII and analysed on a 1.5% Agarose gel. Lane a, ϕX174RF HaeIII marker DNA, Lane b, pSC40ME7; lane c, pSC24ME7; lane d, pSC22ME7 and lane e, pSC27ME7. The bands marked with X, Y and Z show the variations in different clones.

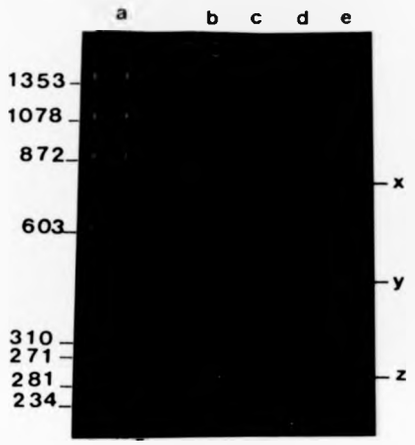


Figure 4.14 Heterogeneity of AhaIII restriction sites (AAA/TTT) among pSCME7 clones.

Purified pSCME7 plasmids were digested with the restriction enzyme AhaIII and analysed on a 1.5% Agarose gel. Lane a, ϕX174RF HaeIII marker DNA, Lane b, pSC40ME7; lane c, pSC24ME7; lane d, pSC22ME7 and lane e, pSC27ME7. The bands marked with X, Y and Z show the variations in different clones.

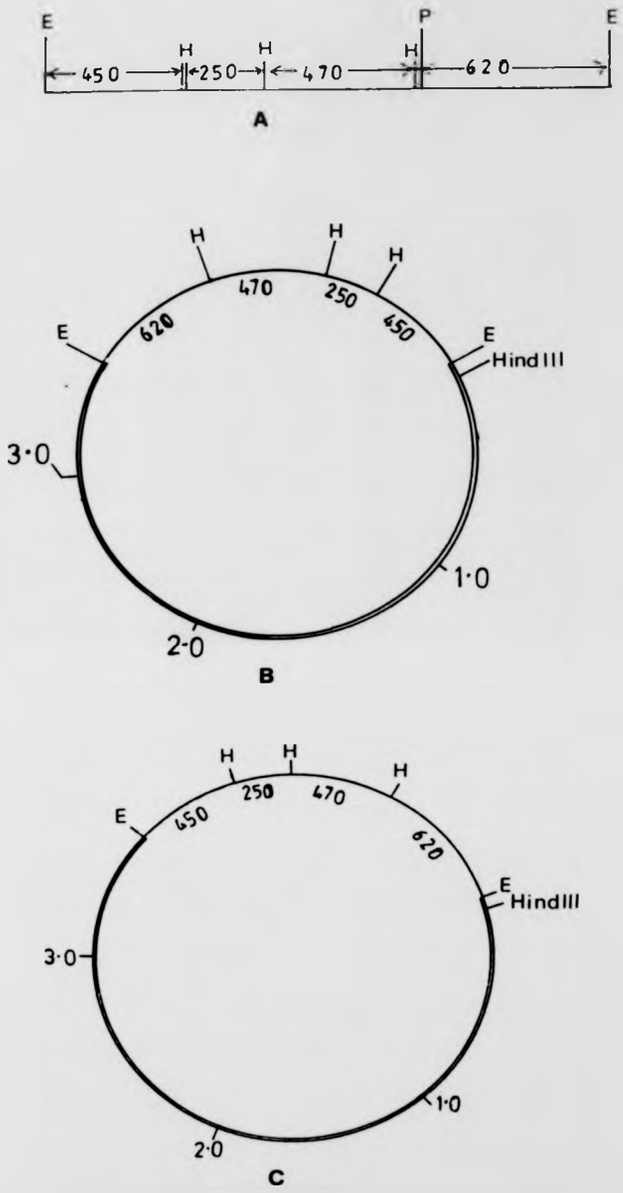


Figure 4.15 Restriction map of pSCME7

The pSCME7 restriction map (A) has been drawn on the basis of HpaII and PstI sites. The distribution of these restriction sites are similar in all pSCME7 clones. Variation on the AhaIII restriction site has not been worked out in relation to these sites and hence, has not been shown on the map. The pSCME7 clones differ from each other by the orientation of the HpaII fragments in relation to the HindIII site on the plasmid. Clone pSC40ME7, pSC22ME7, pSC175ME7 clones have similar orientation (B) i.e., the 450 bp fragment is proximal to the HindIII site of the plasmid, whereas in pSC41ME7, pSC273ME7, pSC24ME7 (C) the fragment is distal to the HindIII site.

E, EcoR1 site; H, HpaII site; P, PstI site.

parental strain of CD24 and CD41) and D273-10B/A1 are similar in their mt-DNA molecular weight (approx. 70.5 kbp), whereas mit-175 mt-DNA has got a molecular weight of 76.2 kbp (similar to the long form of mt-DNA). D22 mt-DNA seems to have a still higher molecular weight, 76.9 kbp (Table 4.3). In the present study the physical map of this strain has not been characterised in detail and therefore, it could not be confirmed as to the nature of any extra insertion or duplication in the D22 mitochondrial genome. However from the analysis of the Oli-2 region from D22, it is clear that this region does not contain any extra insertion. From the comparison of restriction sites of *EcoR*1 (see Fig.4.4) it is suspected that differences may exist within the area of Oxi-2 and Oxi-3 genetic loci or in the (A+T) rich spacer DNA in this region. In the former case, it would be interesting to analyse whether this strain possesses any intron within those genes which have been shown to be intronless in other strains and how they may affect the organisation of the genes and their expression.

Cloning of Eco-6 and Eco-7 fragments of the mitochondrial genome

During cloning of *EcoR*1 digests of mt-DNA into pAT153, it was observed that the Eco-7 fragment (1.7 kbp) was predominantly cloned over Eco-6 (2.56 kbp) band (Fig. 4.9). This is expected because the efficiency of transformation depends on the size of the insert in the vectors. However, one unexpected phenomenon still remains to be explained. In many pSCME7 clones it was found that they have retained a 3.5 kbp *EcoR*1 (Eco-5) fragment (Fig. 4.16). The co-insertions of neighbouring fragments are not unexpected and, in fact, we have found clones with both Eco-6 and Eco-7 fragments together (data not shown). It is also not unexpected in certain clones to have fragments from two different places of the genome, but the frequency of the latter should be much less than the former. This is in contrast to what we found during certain cloning experiments such as from that of CD41. During initial cloning we found all of the pSC41ME7 clones to have co-insertions of Eco-7 and the 3.5 kbp (Eco-5) fragment. However, in the mitochondrial genome of *Saccharomyces cerevisiae* the Eco-8 band (0.9kbp) and Eco-6 (2.56 kbp) lies in between Eco-5 (3.5

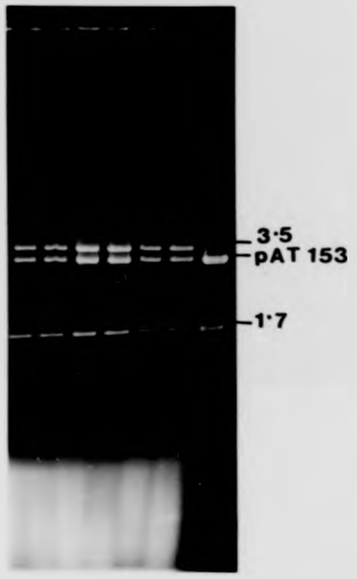
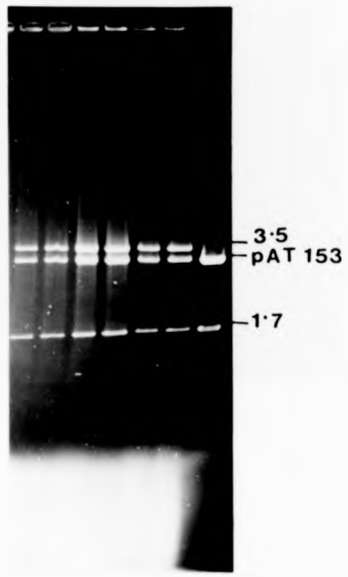


Figure 4.16 Screening of pSC41ME7 clones.

Recombinant plasmids were isolated by the minilysate method of Birnboim & Doly (1979), and digested with the restriction enzyme EcoR1. The samples were analysed on 1% Agarose gel. Note that all the samples from lanes 1 to 6 contain the Eco-5 (3.5 Kbp) fragment along with the Eco-7 (1.7 Kbp) inserts.



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kbp) and Eco-7 (1.7 kbp) (See Fig. 4.7). If this is so then why, in spite of the fact that there is no physical rearrangement, are Eco-5 and Eco-7 fragments cloned predominantly over other fragments. This remains unclear.

Characterisation of the pSCME6 and pSCME7 clones

From the characterisation of different clones with Eco-6 inserts (pSCME6) it appears that there is some minor sequence heterogeneity within from various strains (e.g., pSC27ME6 and pSC22ME6). This is in contrast to clones with the Eco-7 inserts (pSCME7) which are very similar in different strains, as far as restriction sites are concerned except those for *Aha*III.

The heterogeneity observed in Eco-6 fragment does not necessarily mean that the strains involved will have different phenotypic manifestations. Strains D27 and D22, for example, are both wild type with respect to the mitochondrial genotypes but in their Eco-6 fragment, there are more *Sau3A* sites in strain D22. This may be due to conservative change within a reading frame or it could be a random change in the A-T rich spacer sequences. The heterogenity observed in strain D27 is quite unexpected. D27 is a subclone of D273-10B/A1 from Dr. Tzagoloff's laboratory (Macino & Tzagoloff, 1980). Probably during subculture, the former has undergone some point mutations and lost certain region of DNA without affecting the respiratory competency of the cells. Detailed molecular analyses of the Oli-2 region from this strain would be interesting in the future studies.

The restriction site analyses of the cloned fragments were, however, invaluable in terms of sequencing analysis of DNA. As can be seen in the chapter-5 these are very useful for cloning by the "shot-gun" method, into sequencing vector M-13 and for computer analyses of the results obtained from them.

CHAPTER-5

DNA SEQUENCE ANALYSIS OF THE OLI-2 REGION

This chapter will be discussed in two different sections : 5a and 5b. In the section 5a the data will be presented regarding various problems associated with the cloning of the mitochondrial DNA in the sequencing vector M 13 and various strategies used to sequence the mt-DNA fragments. Section 5b will deal with the sequencing analyses of the Oli-2 region from various mutants used in this study.

Section 5a

M 13 Cloning and Sequencing Strategy

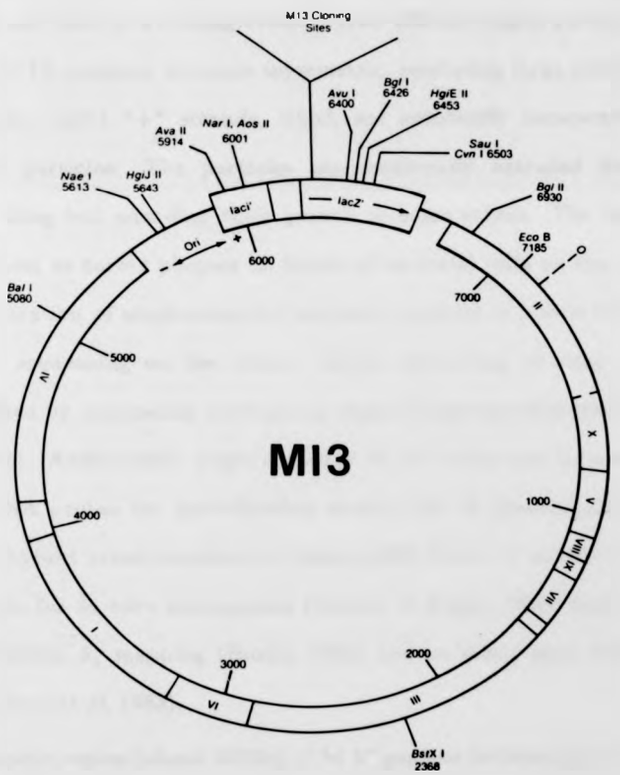
INTRODUCTION

Two types of sequencing methods are currently available to sequence a piece of DNA: one involves the chemical sequencing method (Maxam & Gilbert, 1977) and the other is the enzymatic method of Sanger (Sanger *et al*, 1977). The latter is also called the dideoxy sequencing method because dideoxy nucleotides are used to terminate DNA chains during their enzymatic synthesis which results an array of DNA chains along the DNA fragment to be sequenced. The DNA fragments are then analysed in a highly denaturing urea - polyacrylamide gel. Both methods possess advantages and disadvantages.

Our initial aim was to sequence the petite mt-DNA by Maxam & Gilbert's chemical method, taking advantage of the petite's inherent amplification properties of their mt-DNA (Fukuhara & Wesolowski,1977). Because of the problem described in Chapter 3, a rather different approach has been taken: the relevent portion of the mt-DNA (between Oxi-3 and the Cob-box) from different grande strains has been cloned in the high copy number plasmid, pAT153 for their amplification, and propagation in *E. coli* (described in Chapter-4) and these have been subcloned in M 13 for sequencing by the dideoxy method of Sanger. The advantage of Sanger's method over the other lies in the high degree of base

Figure 5.1 Restriction map of M 13 RF DNA

The map was prepared by BRL, Inc. from the primary sequence data of M 13 (Van Wezenbeek *et al*, 1980) as modified by J. Messing (Messing, 1977). The modifications to M 13 include an insertion of the *lac* gene in the intergenic region between genes IV and II, and single base changes losing a Hpa I site at position 3, a BamH I site at position 5869 and converting an Acc I site to a Bgl II/Sau3A site at position 6096. The remainder of this map is identical to that of wild type M 13. Total length of M 13 mp7 is 7237 nucleotides, mp8 is 7229 nucleotides, mp9 is 7599 nucleotides, mp10/11 is 7245 nucleotides, with position "0" chosen at the unique Hpa I site of M 13. The unique insert within the *lac* Z gene DNA used for DNA cloning/sequencing is given in details in Appendix. Enzymes that cleave M 13 only once are indicated on the map. Positions listed for Pvu I, Bgl I, HgiE II, Cvn I (Mst II), Bgl II, and Eco B are M 13 mp10 locations. Restriction site positions refer to the first nucleotide in the recognition sequence. Relative M 13 gene positions and the origin of replication are also included.



specificity (accuracy) obtained, in the easily controlable enzyme reaction employed and in the significantly lower amount of radioactivity required. In addition, fewer manipulations are needed and there are less hazardous chemicals to deal with.

M 13 as a Cloning Vector

M 13 is a single-stranded filamentous DNA bacteriophage of *E.coli*. It has a closed circular DNA genome approximately 6500 bp in length (See Denhardt *et al*,1978). The phages attach to F-pili of *E.coli* before penetration and are therefore male specific. After infection they proliferate as a double-stranded replicative form (RF), which can be isolated from cells and used as a cloning vector. After 100-200 copies per cell of the RF have accumulated, M 13 synthesis becomes asymmetric, producing large amounts of only one of the two strands, called "+" strands, which are eventually incorporated into mature bacteriophage particles. The particles are continually extruded from the infected cells without killing but retarding their growth to some extent. The latter property helps to identify them as turbid plaques on lawns of bacterial cells on the one hand, and permits rapid preparation of single-stranded template material of cloned DNA suitable for primed "dideoxy" sequencing on the other. Rapid sequencing of longer stretches of DNA is accomplished by sequencing overlapping cloned fragments (Gingeras *et al*, 1979; Anderson *et al*, 1981). Additionally single stranded M 13 clones can be used: to generate strand specific DNA probes for hybridization studies (Hu & Messing,1982); to identify specific clones by hybrid arrest translation (Akam,1983; Ricca *et al*,1982; Chandler, 1982), serve as substrate for *in vitro* mutagenesis (Itakura & Riggs, 1980; Smith & Gillam, 1981), for highly sensitive S_1 mapping (Burke, 1984) and to study gene regulation, structure and function (Artz *et al*, 1983).

The intergenic region (about 507bp) of M 13 genome between gene IV and II (see Fig. 5.1) has been shown to accept inserts of foreign DNA without affecting phage viability (Messing *et al*, 1977). To use M 13 as a cloning vehicle, Messing et al have introduced two types of sequences into this region: First a segment of the *E. coli* lac operon containing the regu-

latory region and the coding information for the first 146 amino acids of the β-galactosidase (Z) gene. The amino terminal portion of the β-galactosidase protein produced in the infected cells is able to complement a defective β-galactosidase gene present on the F episome in the host cell. This complementation, called α-complementation, produces active β-galactosidase, which gives rise to a blue colour when the phage and cells are grown in the presence of an indole containing chromogenic substrate, called X-gal (or BCIG, 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside) with the inducer isopropyl-thiogalactoside (IPTG). Secondly, another sequence of DNA, called "poly-linker", containing several unique restriction sites for cloning has been inserted into the amino terminal portion of the β-galactosidase gene (Messing *et al*, 1981). This latter insertion does not affect α-complementation of host mutant β-galactosidase. However, insertions of additional DNA into the region destroy this complementation and the phage that contain inserts produce colourless plaques, in contrast to the wild type blue plaques when grown in the presence of IPTG and X-gal.

Thus the ease of selection of recombinant phage, together with isolation of single stranded DNA (ssDNA) in large amounts (5-10 μg/ml of culture) and the commercial availability of a "universal primer"complementary to vector sequences have made M 13 a suitable vector for subcloning of DNA fragments and their sequence analyses.

In the course of subcloning of the mt-DNA in the M 13, it has been observed that there are some specific problems associated with the propagation of mt-DNA in M 13. This is partly due to the A+T richness of mt-DNA which is unstable in M 13. The purpose of this section is to discuss these problems and various available methods to circumvent the difficulties.

Strategies used in M 13 cloning

Mainly three methods have been employed for subcloning of DNA fragments in M13 (mp8, mp9, mp10 and mp11) (See Messing, 1983).

i) "Shot-gun" method

Purified Eco-6 and Eco-7 fragments from various pSCME6 & pSCME7 recombinants (described in Chapter 4) were digested with "four-cutter" restriction endonuclease (enzymes which recognise 4 nucleotides for their cleavage on the DNA strand e.g. *Sau*3A, HpaII, etc.) and cloned into the compatible restriction site on the vector DNA. *Sau*3A subfragments were cloned in *BamH*1 digested vector; *Hpa*II generated fragments were cloned into *Acc*1 digested vector, *AhaIII* generated fragments were cloned into *Hinc*II or *Sma*I digested vector.

ii) Directional or "force" cloning method

In this method, vector DNA was digested with two different restriction enzymes and compatible restriction fragments were cloned into it, in such a way that the target DNA (or the DNA to be inserted) can ligate with vector only with one orientation. For example, vector DNA M 13 mp10 RF digested with *Xba*I and *EcoR*1 was used to ligate Eco-6 band digested with *Xba*I. Because *Xba*I site lies first in the "polylinker" site of the vector, the target DNA will be cloned only in the *XbaI-EcoR*1 direction (and not vice versa) so that the sequence could only be read from the *Xba*I site of the target DNA towards the *EcoR*1 end.

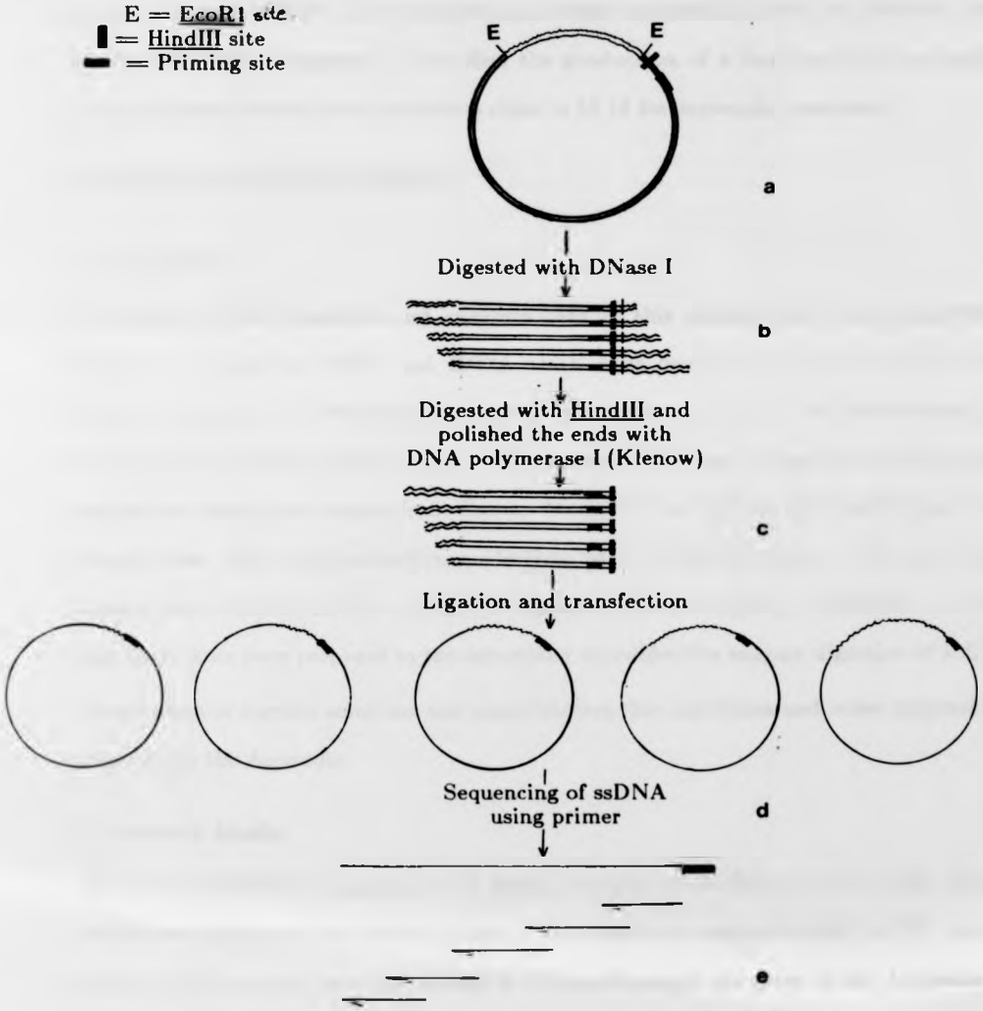
iii) "Systematic" or "non-random" method

In this method the fragments to be sequenced were cloned in such a way that fragments would be cloned into M 13 in an overlapping manner. The overlapping clones thereby would produce overlapping sequence data for their analysis. Hong's method (Hong, 1982) and the Bal-31 method (Poncz *et al*, 1982) were employed for this purpose.

Hong's method

The principle of Hong's method is summarised in Fig. 5.2. The RF from recombinant M 13 vectors are partially digested with DNase I in the presence of Mn^{2+}, which produces double stranded cleavage randomly in the target DNA (see Fig. 5.2b). Breaks outside the target DNA lead to the molecule without an origin and do not yield viable phage. Using a

Figure 5.2 Diagram illustrating Hong's systematic DNA sequencing strategy as employed for the clone 84066, a M 13 mp8 recombinants having the Eco-6 insert from pSC40ME6. The RF from 84066 (a) were isolated as described in the Materials and Methods section and subjected to partial DNaseI digestion in the presence of Mn^{2+} and subsequent HindIII digestion as shown in the figure (b). The ends of the DNA were polished with DNA polymerase I Klenow subfragment in the presence of dNTPs and then ligated with T4 DNA ligase (d). After circularization of the linear RFs, the *E. coli* strain JM103 was transfected with them. They should produce a mixture of clones with insert fixed at one end and sequentially shortened at the other. Sequencing these clones (e) from the priming site (▬) should generate overlapping DNA sequence data of the whole fragment of the the Eco-6.



suitable restriction enzyme one side of the target DNA could be cleared away from after priming site (see Fig. 5.2c) which on ligation (Fig. 5.2d) will produce overlapping subclones suitable for sequencing (Fig. 5.2e).

Bal-31 deletion method

Poncz *et al* (1982) first described this method. The principle is based on the cleavage property of nuclease Bal-31 which removes nucleotides sequentially from both ends of a double stranded DNA fragment. This offers the production of a large array of overlapping DNA fragments which could be used to clone in M 13 for sequencing analyses.

MATERIALS AND METHODS

1. Materials

The sources of all chemicals and enzymes used in this chapter have been described in Chapter 4, except for BCIG and IPTG which were bought either from BRL or from Sigma. Repelcoat (2% Dimethyl dichlorosilane solution in 1, 1, 1, -trichloroethane) was purchased from BDH chemical Ltd., England and Attractant (γ -methyl-acryloxy-propyl trimethoxy silane) was bought from Sigma. Marker DNAs (ϕX174 RF *Hae*III digest) were bought from BRL and radioactive nucleotides ($\alpha -^{32}$P-dGTP and $\alpha -^{32}$P-dCTP were bought from Amersham,UK. λ (*Hind*III digest), λ (*EcoR*1 digest), λ (*Hind*III + *EcoR*1 digest) markers were prepared in the laboratory by respective enzyme digestion of λ DNA. Composition of various solutions and assay bufffers (for restriction and other enzymes) are given in the the Appendix.

2. Growth Media

E.coli strain HB101 was grown in LB media, and that of JM103 in 2 x TY. The stock of JM103 was maintained on minimal plates. The details of recipes for LB, 2 x TY, minimal plate and H-Top-agar, used for plating M 13 transformants are given in the Appendix. All strains were grown at 37° C.

Table 5.1 *E. coli* strain

Strain	Genotype	Remark
HB101	*F* , hsd S20 (*rB* , *mB* , rec A13, ara-14, ProA2, lacY1, galK_2, rps L20 (Sm^r), xyl-5, mtl-1, sup E44, λ	This strain is an hybrid of *E. coli* K12 and *E. coli* B. For details, see Boyer & Rouland-Dussoix (1969); Bolivar & Backman (1979)
JM103	Δ (lac pro), thi, str A, end A, sbcB15, hsd$R^{(\ )}$ 4, sup E, F traD36, proAB, lac I^q, Z ΔM15	A host of K-12 origin, restriction minus but modification plus. See Messing *et al*, 1981 for details.

Table 5.2 M 13 vectors and their cloning sites. See Appendix for the details of the sequences of the cloning sites.

Vector	Cloning site	References
mp7	EcoR1 - BamH1 - SalI/Acc1/HincII- -PstI - SalI - BamH1 - EcoR1	Messing *et al* (1980)
mp8	EcoR1 - SmaI - BamH1 - SalI/Acc1/HincII- -SmaI - HindIII	Messing & Viera (1982)
mp9	HindIII- PstI - SalI/Acc1/HincII- -SmaI - EcoR1	Viera & Messing (1982)
mp10	EcoR1 - SstI - SmaI - BamH1 - XbaI- -SalI/Acc1/HincII - PstI - HindIII	Messing (1983)
mp11	HindIII - PstI - SalI/Acc1/HincII- -XbaI - BamH1 - SmaI - SstI - EcoR1	"

3. Strains Used

The genotype of the strains of *E. coli* are given in Table 5.1. M 13 vectors used in this chapter are given in Table 5.2 with their multiple cloning sites. Recombinants of *E. coli* strains harbouring mt-DNA (pSCME6 and pSCME7) have been described previously in Chapter 4. *E. coli* strain JM103 was always maintained on minimal plate to select against the loss of F factor which complements proline deficiency.

4. Isolation of Plasmid DNA

The methods have been described in Chapter 4.

5. Isolation of M 13 RF

The method employed to isolate replicative form (RF) of M 13 has been described in Katz *et al* (1973). 10ml of 2 x TY medium was inoculated with JM103 (from minimal plate plus glucose) and grown up with shaking at 37° C to the exponential phase ($OD_{550} = 0.6$). This usually takes 3-4 hours. Meanwhile 1ml of 2 x TY was inoculated with a phage plaque and grown with shaking at 37° C. The freshly grown JM103 was then added to 1 litre of 2 x TY and grown at 37° C with shaking. When $OD_{550} = 0.5$, which takes about 4 hours, 1ml of phage was added to it and grown for another 4 hours. The cells were harvested by centrifuging at 5000 rpm for 10 minutes at 4° C. The RF was isolated from the cells with the "clear lysis" method described for the preparation of plasmid in Chapter 4.

6. Isolation of ss DNA from M 13

The ssDNAs were isolated using the procedure described by Sanger *et al* (1980). Briefly, 1/40th dilution of a fresh overnight culture of JM103 in 2 x TY was divided into 1ml aliquots. Phage plaques were inoculated into diluted JM103 with the help of sterile toothpicks. They were incubated at 37° C with shaking for about 7 hours. The culture was poured into 1.5ml microfuge tubes and centrifuged for 10 minutes. The supernatant was transferred into another 1.5ml microfuge tube (Eppendorf) making no effort to transfer completely. The supernatant was either stored at 4° C at this stage, or continued with

for the isolation of ss DNA. In the former case the supernatant must be re-centrifuged to remove any cells which grew during storage before isolation of phage DNA. The pellets were stored at - 20° C with 7.5% DMSO as a stock of the recombinant phage. To the supernatant 200 µl of PEG solution (20% polyethylene glycol (PEG), 2.5M NaCl) was added and left at room temperature for 30 minutes. The viral pellets were collected by centrifugation for 5 min. in a microfuge. The supernatant was discarded and any trace amount of supernatant was wiped out with a kim-wipe or removed by a drawn out pasteur pipette. To the white phage pellets 100 µl of TE (10mM Tris,pH 7.5, 0.1mM EDTA) were added, and vortexed for 5 seconds to suspend the virus. The suspended virus particles were re-vortexed for 10 seconds in the presence of 50 µl of phenol and kept standing at room temperature for 10 minutes. The suspension was re-vortexed for another 10 seconds and microfuged for 3-4 minutes. The aqueous phase was collected carefully with the help of drawn out pasteur pipettes and transferred to a fresh Eppendorf tube. The aqueous phase was extracted twice, either with 2 volumes of chloroform or 2 volumes of diethyl ether to remove PEG and traces of phenol. 10 µl of 3M Na-acetate and 250 µl of ethanol were added to the finally extracted aqueous solution and placed at - 20° C overnight. Phage DNAs were collected by centrifugation for 10 minutes at 4°C in a Sorvall SS-34 rotor using a suitable adaptor. The DNA pellets were washed with 1ml of cold ethanol in a microfuge at room temperature, dried under vacuum and dissolved in 15 to 20 µl of TES buffer (10mM Tris-Cl, pH 7.5, 10mM NaCl, 1mM EDTA) and stored at - 20° C.

7. Ligation of M 13 RF and Target DNA

i) Preparation of Vector DNAs

For the purpose of sticky end ligation the vector DNA, M 13 RF was cleaved with suitable restriction enzyme(s), compatible with the target DNA ends. (See Appendix for the assay conditions for various restriction enzymes). After two hours the reaction mixture was incubated with BAP or CIP using the same conditions as described in the earlier

chapter for BAP or CIP treatment of plasmid vector. The enzymes were inactivated with EDTA/EGTA (Final conc. 20 mM) and incubated at 65° C to 70° C for 10 minutes. Then the reaction mixture was extracted with phenol and chloroform (or diethyl ether), successively and precipitated with ethanol. The DNA pellet was dried and dissolved in H_2O before use in ligation reactions and if needed, stored at - 20°C.

For the purpose of directional ligation, the vector DNAs were digested with two separate restriction enzymes compatible with two different ends of target DNA. For example to clone *EcoR*1 and *Xba*I generated subfragments of the Eco-6 fragment in M 13, mp10 RF was digested with *EcoR*1 and *Xba*I successively. Generally during double digestion, the enzyme of choice for first digestion is the one which requires low-salt buffer, which is supplemented with high salt for the second enzyme. After digestion was completed, the DNAs were electrophoresed in a low melting point agarose gel. After visualisation of the band on the UV (Long wave length) transilluminator, following ethidium bromide staining, DNA bands were cut out of the gel and extracted, as described in Chapter 4 and purified by phenol extraction and ethanol precipitation.

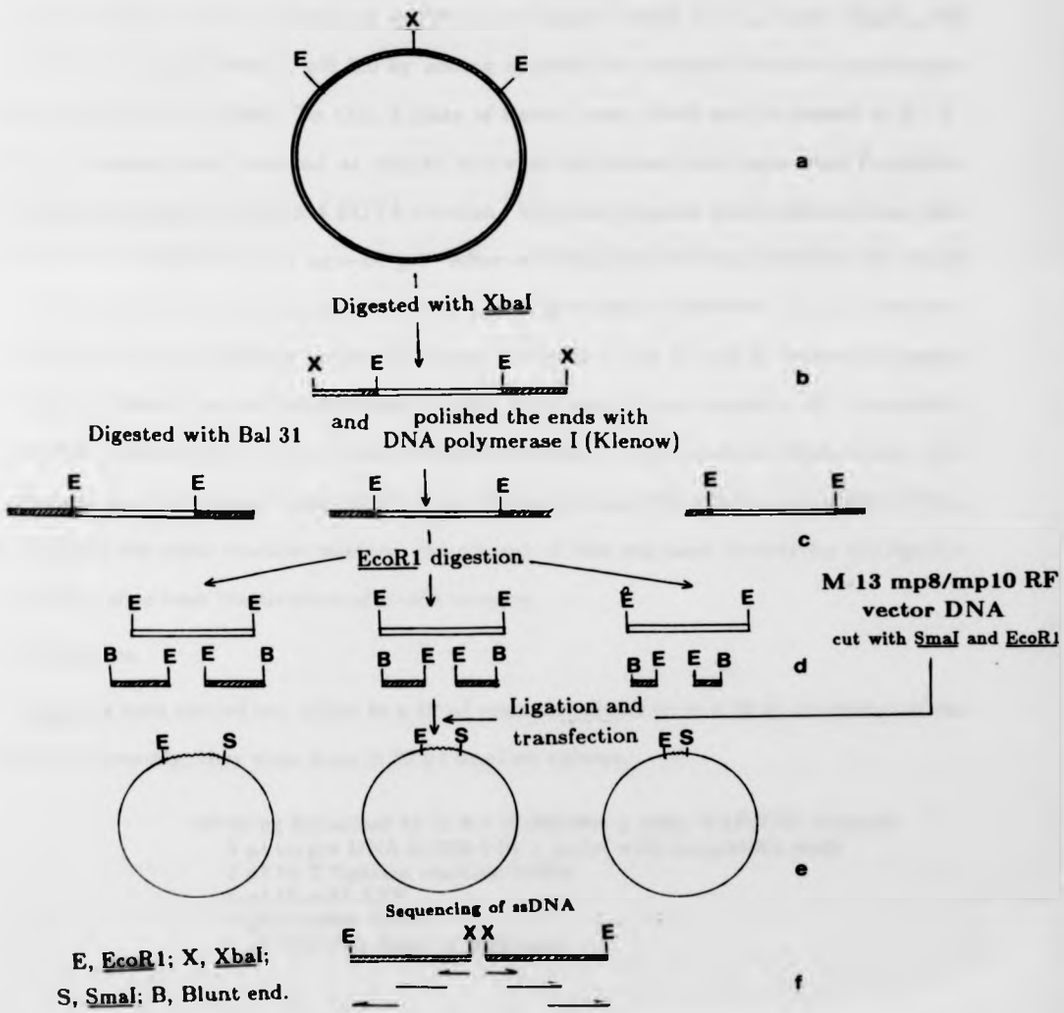
ii) Preparation of the Target DNAs

For the "shot-gun" method, the target DNAs were cloned with "four cutter" restriction enzymes. For example, to sequence the Eco-6 band, the band purified from agarose gel was digested with *Sau*3A which generates compatible sticky ends for the *BamH*1 site of the vector M 13 RF DNA.

For directional cloning, the gel purified band was digested with two different enzymes compatible with the cloning sites of the vector DNA, one after another, depending on the salt requirements of the restriction enzymes concerned. The enzymes were inactivated by heat inactivation at 65° C and used directly for cloning.

For "systematic" cloning using Bal-31, recombinant plasmids (pSCME6 or pSCME7) were digested with a unique enzyme which cuts the plasmid once. The resultant linearised plasmid DNAs were then digested with Bal-31 for different time periods: 0, 1, 2, 3, 5, 7, 9

Figure 5.3 Flow Chart for the strategy used to sequence the Eco-6 DNA fragment from various pSCME6 clones by the Bal-31 method. The purified recombinant plasmids (pSCME6) (a) were digested with the restriction enzyme XbaI, which has only one restriction site on the whole recombinant plasmid and within the Eco-6 fragment. The linearised plasmids (b) were then digested with the nuclease Bal-31 for various time periods (c). The samples were filled in with DNA polymerase I (Klenow fragment) in the presence of dNTPs. Then samples of each time period were digested with the restriction enzyme EcoR1 separately which produced the vector DNA (pAT153) with EcoR1 end at both ends and the target DNA fragments with EcoR1 at one end and blunt end at the other (d). These were ligated with the EcoR1 and SmaI digested M 13 mp10 RF vector DNA, which would allow only the target DNAs to be ligated into the vector (e). The recombinants will have their gradually shortened blunt ends directed towards the priming site which would allow sequencing of the whole fragment by overlapping manner (f).



min. etc. respectively. Here, I will describe the protocol used to subclone the Eco-6 fragment (See Fig. 5.3) taking the opportunity to use a unique *Xba*I site which is absent in the vector pAT153, but present almost at the middle of the Eco-6 fragments.

About 15 μg of pSCME6 was digested with 20 units of *Xba*I in 6mM Tris-Cl (pH 7.4), 6mM $MgCl_2$, 100mM NaCl, at 37° C for 2 hours. The completion of digestion was checked by agarose gel electrophoresis in a mini-gel electrophoretic apparatus. When digestion was complete, the enzyme was inactivated at 65° C for 5 minutes. The volume of the reaction mix was increased to 200 μl and made 12mM $CaCl_2$, 12mM $MgCl_2$, 400 mM NaCl, 20mM Tris-Cl, pH 8.0 by adding appropriate amounts of more concentrated salt solutions and buffer. To this, 2 units of Bal-31 were added and incubated at 37° C. 20 μl aliquots were removed at minute intervals and placed into pre-cooled Eppendorf tubes containing 1 μl of 0.2M EGTA solution. When all aliquots were collected they were run in a low-melting point agarose gel. After staining with ethidium bromide the bands were visualised, cut out and purified. DNAs from each band of different time periods were incubated in an end-filling reaction (because removal of the 3' and 5' termini of duplex DNA by Bal-31 is not synchronous at any one time) in the presence of 4 unlabelled dNTPS (0.2mM each) and 1 unit of Klenow enzyme. This produces blunt ends. The Klenow was then inactivated at 65° C for 5 minutes and the DNAs were digested with *EcoR*1 in the same reaction mixture. An aliquot of this was used directly for the ligation reaction after heat inactivation of *EcoR*1 enzyme.

iii) Ligation

Ligations were carried out either in a 10 μl reaction volume or in a 20 μl reaction volume. More commonly, they were done in 20 μl reaction volume.

10-20 ng linearized M 13 RF (0.002-004 p mole, BAP/CIP treated)
5 μl target DNA (0.002-0.01 p mole) with compatible ends
2 μl 10 X ligation reaction buffer
2 μl 10 mM ATP
H_2O to make 19 μl
1 μl T4 DNA ligase (1 unit/ μl)

Incubation was performed at 15° C overnight. The ligation mixture was directly used for transformation. Blunt end ligations were achieved in the same way, except 2 µl of T4 DNA ligase was used (2 units).

8. Transfection

i) Preparation of Competent Cells

About 2ml of 2 x TY media were inoculated with JM103 from a minimal plate and incubated overnight at 37° C in standing condition. 10ml of 2 x TY, on the following morning, were inoculated with 100 µl of the overnight standing culture and incubated at 37° C with shaking, until the density became about 0.3-0.4 at OD_{600}. This took about 2-3 hours. The cells were kept on ice for 10 minutes and collected by centrifugation at 7000 g for 5 minutes. The pelleted cells were resuspended in 5 ml of 50mM $CaCl_2$ (half of the original growth volume) and left on ice for 20 minutes. The cells were centrifuged again and resuspended in 1 ml (1/10th of growth volume) of 50 mM $CaCl_2$. The resultant competent cells were aliquoted into 300 µl in microfuge tubes and kept on ice.

ii) Plating of M 13 Recombinants

To 0.3ml of competent cells 10 µl or 20 µl of ligation mixture was added directly and kept on ice for 40 minutes. The transformation mixture was heat-shocked for 2 minutes at 42°C and mixed in a tube containing

3 ml of H-Top agar at 45° C
15 µl 20 mg/ml X-Gal
25 µl 25 mg/ml IPTG
0.2 ml fresh, exponentially growing JM103.

(The fresh exponentially growing JM103 are conveniently made by adding 2 ml of 2 x TY directly into the dregs of the container used for growing competent cells). These were mixed properly and poured onto a minimal plate. The plates solidified within 2 minutes after which they were incubated at 37° C overnight in an inverted position. The wild type M 13 generally gave blue plaques while recombinants gave colourless plaques.

9. Dot-hybridization

2 μl of ssDNA isolated from M 13 recombinants were mixed with 2 μl of 20 X SSC and spotted onto a sheet of nitrocellulose. Nitrocellulose filters were first air-dried and then baked at 80°C for 2 hours. The hybridization was carried out in 4 X SSC at 65°C as already described under Southern hybridization conditions (Chapter-3). The filter was washed several times in low salt (0.1 X SSC) at 45° - 50°C and then air-dried and autoradiographed.

10. Southern hybridization and Northern hybridization

Southern hybridization was achieved as has been described in Chapter-3. Northern hybridization was carried out according to the method of Alwine *et al* (1979). In some cases RNAs were transferred on to the nitrocellulose paper according to the method of Thomas (1980).

11. Isolation of mt-RNAs

Mt-RNAs were isolated either according to the method described by Locker (1979) or according to the guanidinium/hot phenol method (Maniatis *et al* 1982).

Briefly, in Locker's method the mitochondrial pellets were suspended in 10mM Tris.Cl, pH 7.4, 1% Sarkosyl, and then extracted with hot phenol (60°C). The aqueous phase was extracted with chloroform. RNAs were recovered from the aqueous phase by ethanol precipitation.

In the guanidinium/hot phenol method, mitochondrial pellets (prepared from 1 litre of yeast culture) were suspended in 2ml of 4M guanidinium isothiocyanate mixture (4M Guanidinium isothiocyanate, 2% Sarkosyl, 1% β-mercaptoethanol). The mixture was brought to 60°C and, while maintaining this temparature, the slimy suspension was passed through an 18-gauge needle fitted into a disposable plastic syringe. This step shears the contaminating chromosomal DNA and mitochondrial DNA. Then an equal volume of preheated phenol (60°C) was added to it and vortexed or shaken vigorously.

Then a half volume of a solution of (100 mM sodium acetate,pH 5.2, 10 mM Tris.Cl,pH 7.4, 1 mM EDTA) and an equal volume of chloroform/isoamyl alcohol (24:1) were added to it and shaken vigorously for 10-15 minutes, keeping the container at 60°C. The mixture was cooled on ice and centrifuged at 2 000 g for 10 minutes at 4° C. The aqueous phase was re-extracted with phenol/chloroform (1:1) once and with chloroform twice. Then two volumes of ethanol were added and the mixture was kept at - 20° C for 1-2 hours. RNAs were recovered by centrifugation at 12 000 g for 20 minutes at 4° C. The pellet was dissolved in 1 ml of 0.1 M Tris.Cl,pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.2% SDS and proteinase K was added to it to a final concentration of 200 μg /ml. Incubation was carried out for 1-2 hours at 37° C. The solution was then heated to 60° C and 0.5 vol. of hot phenol (60° C) was added to it and mixed thoroughly. Then 0.5 vol. of chloroform was added and again shaken vigorously. The mixture was cooled down to 4° C for 10 minutes and centrifuged at 2 000 g for 10 minutes. RNAs were precipitated from the aqueous phase by two volumes of cold ethanol at - 20° C and were then collected by centrifugation.

12. Electrophoresis of DNA

Nondenaturing agarose and acrylamide gel electrophoresis were carried out for analysing DNAs as described in Chapter 3 and Chapter 4.

13. Electrophoresis of RNAs

RNAs were analysed either by agarose-urea gel (Locker,1979) or by the glyoxal and dimethylsulfoxide method of McMaster & Carmichael (1977).

Agarose-Urea gel:

Agarose was dissolved in 6M urea, 15 mM iodoacetate with electrophoretic buffer (Tris-phosphate or Tris-acetate) and poured while hot onto a glassplate (20 X 20 X 0.4 cm) for slab gels. Gels were then cooled to room temperature and subsequently placed at 4° C for 2-3 hours. Gels were run either in Tris-phosphate (40 mM Tris, 36 mM NaH_2PO_4, 1 mM

EDTA, pH 7.4) or in Tris-acetate (0.4 M Tris, 20 mM sodium acetate, 33 mM acetic acid, 1mM EDTA, pH 7.4) buffer. Electrophoresis was carried out at room temperature (5 V /cm) for 3-6 hours. Gels were stained for 30 minutes in ethidium bromide (0.5 μg/ml).

Glyoxal and Dimethylsulfoxide (DMSO) method

In an Eppendorf tube RNAs were denatured in a glyoxal/dimethylsulfoxide solution as follos:

6 M glyoxal	2.7 μl
DMSO	8.0 μl
NaH_2PO_4,pH 7.0 (0.1 M)	1.6 μl
RNA (up to 20 μg)	3.7 μl
Total	16.0 μl

The tube was incubated at 50°C for 60 minutes in a tightly closed condition. While the RNA incubation was carried out, a 1% - 1.5% agarose gel was made ready. The gel was made in 10 mM NaH_2PO_4 (pH 7.0) buffer. After the incubation of RNA was over, the samples were cooled down to room temperature and mixed with 1/5 th volume of loading buffer (50% glycerol, 10 mM NaH_2PO_4, pH 7.0, 0.1% bromophenol blue). The gel was run submerged in 10 mM NaH_2PO_4, pH 7.0, buffer at 3-4 V/cm. Constant recirculation of the buffer is required to maintain the pH. Generally at the end of the run one half of the gel was stained with ethidium bromide (0.5 μ g/ml) for visualisation under UV and the other half was used for transfer to a nitrocellulose filter according to the method of Thomas (1980).

14. Single-lane Screening

This is a method of identification of M 13 recombinants. This method is based upon doing one of the four "dideoxy" sequencing reactions with ssDNAs isolated from suspected recombinants. DNA from a blue plaque is generally used as a control.

In the microfuge tubes of 0.5 ml capacity annealing reactions were set up using 1 μl (250-500 ng) of viral DNA, 0.5 μl (1.0 ng) of 15 bp primer DNA, 0.3 μl of concentrated Polymerase buffer (10X) and 1.2 μl of H_2O (total 3 μl). Tubes were tightly closed and placed in a water-filled test tube which was in a 85-90° C water-bath. They were incubated for 5

minutes and then slowly equilibrated to room temperature for 1 hour which allows the annealing of the primer to the template. Then one of the four dideoxy nucleotide reactions (see next) was chosen for sequencing reaction. The ddT reaction using $\alpha-^{32}$P-dGTP was used most often, as given in the following:

1 µl of (10 µCi) $\alpha-^{32}$P -dGTP
10 µl T° Mix (See Appendix for composition)
10 µl ddT (2mM)
2 µl DNA polymerase,large fragment (1 unit/ µl)

were mixed to set up a pre-reaction mixture (for 10 reactions). To each 3 µl of annealing reaction mixture, 2 µl of this pre-reaction mixture was added and incubated for 15 minutes at 30° C. 1 µl of 0.5mM dGTP was added to each reaction and incubated for a further 15 minutes at 30° C. 5-10 µl of formamide-dye mix were added to each of the tubes at the end of the reaction and analysed on thin sequencing gels, by electrophoresis and autoradiography which is given in the section below.

15. Protocol for "Dideoxy" sequencing (Sanger,1977; Sanger *et al*, 1980)

i) Primer annealing reactions:

The following were added to a microfuge tube:

5 µl (0.5 µg) template DNA
2 µl (04-5 ng) 15 bp primer DNA fragment (see appendix)
1 µl 10 X polymerase reaction buffer
4.5 µl distilled water

12.5 µl Total

The microfuge tubes were sealed and placed in a small beaker which was placed in an 85°-90° C water bath. After 5 minutes incubation, the beaker was placed on the bench at room temperature for 45 minutes to 1 hour. Slow equilibration to room temperature allows proper annealing of the primer to the template.

ii) "Dideoxy" reactions:

All dideoxy sequencing reactions were carried out using $\alpha-^{32}$P-dGTP as radioactive nucleotides. Therefore, the protocol mentioned below is manipulated for use of $\alpha-^{32}$P-dGTP only.

While the DNAs were annealing, four microfuge tubes marked with A, G, T, C respectively, were made ready for the "dideoxy" reaction by adding the following (without mixing):

Tube A: 1 µl A° mix and 1 µl ddA
Tube G: 1 µl G° mix and 1 µl ddG
Tube T: 1 µl T° mix and 1 µl ddT
Tube C: 1 µl C° mix and 1 µl ddC

(Composition of the N° Mix (A°, G°, T° and C°) are given in the Appendix.)

After completion of the annealing incubation, the following were added directly to the microfuge tube with annealing mix:

0.5 - 1 µl (5-10 µCi) $\alpha-^{32}$P-dGTP
1 µl 0.1M DTT
1 µl large fragment, DNA polymerase I (Klenow enzyme)
(1 unit/ µl)

The entire contents (15 µl) of the annealing tube were mixed properly and collected at the bottom of the microfuge tube by a quick (1 sec.) spin in the microfuge. 3 µl aliquots of this were added to each reaction tube, labelled as A, G, T and C. These were mixed well and incubated at 30^0 C for 15 minutes. 1 µl of 0.5mM cold dGTP (i.e. unlabelled) was added to each of the four reaction tubes, mixed and incubated for another 15 minutes at 30^0C. This chase serves to extend chains which are prematurely terminated due to low dGTP concentration. The reactions were stopped by adding 10 µl of formamide-dye mix and kept on ice or at - 20^0 C.

iii) Gel electrophoresis and Autoradiography:

The high resolution denaturing gels used for dideoxy sequencing were 8% or 6% polyacrylamide gels containing 8M urea and 100 mM TBE buffer,pH 8.3. The gel size used routinely was 33 X 40 X 0.02 - 0.04 cm. A typical 8% gel (33 X 40 X 0.3 cm) solution had:

24 gms. Urea
10 ml 40% Acrylamide/bis acrylamide solution
5 ml concentrated TBE buffer,pH 8.3 (10 X)
0.5 ml 10% Ammonium per sulphate (APS)
30 µl TEMED
H_2O to make 50 ml

Before addition of TEMED in the above mixture the acylamide/bisacrylamide solutions were deaerated and the glass plates were kept ready for pouring the solution into it. For this the front plate (i.e. the notched plate) was siliconised by using Repelcoat (BDH chemical Ltd.) and the back plate was treated with γ-methyl-acryloxy-propyl-trimethoxy silane (attractant). The plates were treated with these chemicals for at least 30 minutes and then washed with distilled water and rinsed with ethanol. The gel moulding was done within the support of the plates, separated by plasticard strip (0.2 - 0.4 mm). After deaeration of the acrylamide:urea solution, 30 µl TEMED was added to it and mixed thoroughly. The solution was sucked into a 50 ml disposable syringe and injected into the mould of the glass plates. The moulding frame is kept inclined to the surface at an angle of about 45°. In the conditions mentioned above the gel sets within 5 minutes. The gels were however allowed to set properly for another 30 minutes before the pre-electrophoresis was started.

Pre-electrophoresis lasted for at least 15 - 30 minutes at 1700 Volts and 30 mA current. Before loading the sequencing reaction mixture into the wells, they were heat denatured for 5 minutes at 90° - 100° C in a dry heating block. During the denaturing process of the sequencing samples the wells of the gel were flushed and cleaned thoroughly with the help of a syringe or a drawn out Pasteur pipette.

2-3 µl of the denatured sequencing reaction mixture were loaded generally in a order of A, G, T, C from the same clone. After the electrophoresis was over, the front plate was removed and gel was covered with a cling film. The gel was immediately exposed to gel-size X-ray film (Fuji) and placed at - 70° C , after proper wrapping to prevent the penetration of light.

The films were developed after 12-16 hours using Kodak universal developer (1:9 dilution) and Kodak universal fixer (1:4 dilution) for four minutes and five minutes respectively. The autoradiograms were read manually.

RESULTS

Identification of Recombinants

Theoretically, the lack of colour of the phage plaque in the lawn of *E.coli* (JM103) is the method of identification of recombinants which are unable to produce active β-galactosidase due to insertional inactivation. However, spurious development of colourless plaques on the plate are a common phenomena. This can be generally explained in terms of contaminated nonspecific exo- or endo-nucleases within restriction endonuclease enzymes, bacterial alkaline phosphatase (BAP), or calf intestinal phosphatase (CIP). However sometimes, *E.coli* DNA can contaminate the vector DNAs or target DNAs and may integrate into the M 13 RF cloning sites and produce colourless phage plaques. Therefore, to identify the genuine positive recombinants among "pseudo-recombinants" two methods were employed: direct gel electrophoresis and dot-hybridization. Initial screening of the recombinants with foreign inserts was achieved by direct gel electrophoresis of isolated ssDNA. Fig. 5.4 is representative of such a screening method. Samples with retarded mobility in comparison to control M 13 ssDNA have foreign DNA insertions. This method of screening is however, not suitable for insertions of smaller than 300-400 bp which do not show significant change in their mobility (Fig. 5.5). To identify more positively, the ssDNA are dot-blotted onto nitrocellulose and hybridised with a Oli-2 region specific labelled DNA probe (in most cases with DS-14 mt-DNA) (see Chapter 3 & 4). An autoradiogram of typical screening by dot hybridization is represented in the Fig. 5.6 from where positive clones can be easily identified.

Identification of the orientation of the Target-DNA among Positive Clones

Figure 5.4 Screening of ssDNA from M 13 recombinants by agarose gel electrophoresis.

The pSCME6 plasmids were digested with EcoR1 and ligated with the EcoR1 digested M 13 mp9 RF DNA. Recombinant phages were identified on the (X-gal + IPTG) containing plates by their lack of blue colour. ssDNAs were isolated from the phages and analysed on 0.8% agarose gel. "+" strand of ssDNA of the vector mp9 were isolated from a blue plaque and run as a control on the same gel.

Lane 1, mp9 ssDNA; Lane 2-17, ssDNAs isolated from the colourless plaques. The samples in Lane 3, 5, 6, 7, 8, 11, 12, 13, 14, 15 and 17 show retarded mobility in comparison with mp9 ssDNA and are the recombinants.

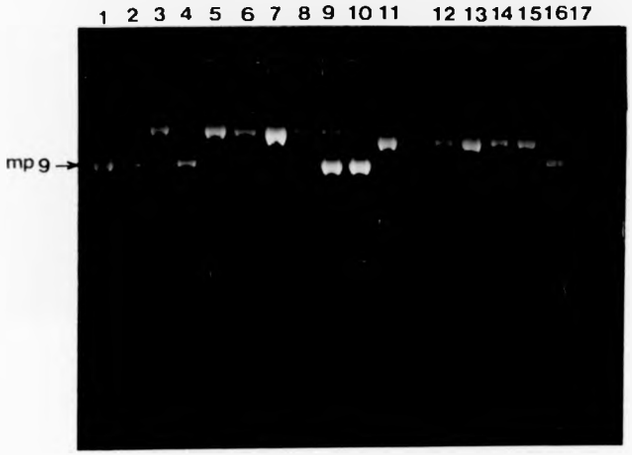


Figure 5.4 Screening of ssDNA from M 13 recombinants by agarose gel electrophoresis.

The pSCME6 plasmids were digested with EcoR1 and ligated with the EcoR1 digested M 13 mp9 RF DNA. Recombinant phages were identified on the (X-gal + IPTG) containing plates by their lack of blue colour. ssDNAs were isolated from the phages and analysed on 0.8% agarose gel. "+" strand of ssDNA of the vector mp9 were isolated from a blue plaque and run as a control on the same gel.

Lane 1, mp9 ssDNA; Lane 2-17, ssDNAs isolated from the colourless plaques. The samples in Lane 3, 5, 6, 7, 8, 11, 12, 13, 14, 15 and 17 show retarded mobility in comparison with mp9 ssDNA and are the recombinants.

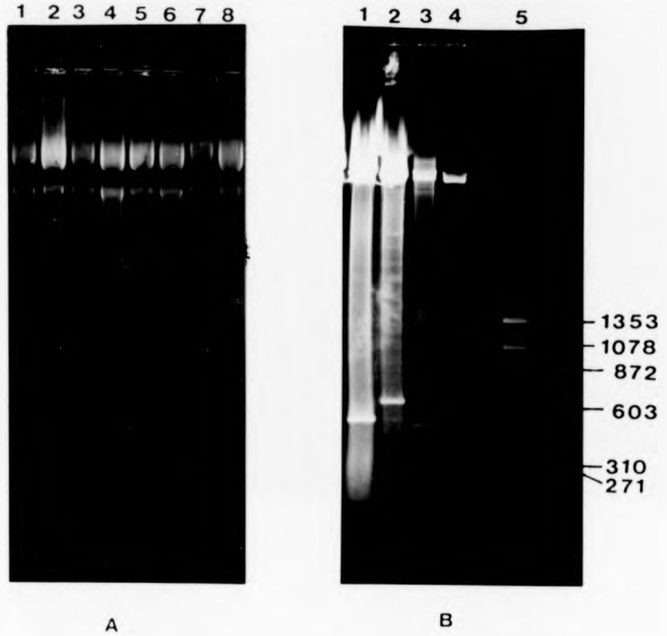


Figure 5.5 Identification problems of the M 13 recombinants with smaller inserts.

The Eco-7 fragment of mt-DNA, isolated from pSC40ME7 clone were digested with HpaII and cloned in the AccI site of the M-13 mp9 vector.

A) Agarose gel (1%) electrophoretic pattern of the ssDNAs, isolated from the colourless plaques. Lane 1, mp9; Lane 2, 9407H1; Lane 3, 9407H2; Lane 4, 9407H3; Lane 5, 9407H4; Lane 6, 9407H5; Lane 7, 9407H6; Lane 8, 9407H8.

B) EcoR1 digests of the RF DNAs isolated of the recombinants.

The RF DNAs were isolated from the recombinant clones 9407H2, 9407H4, 9407H5, 9407H8 and digested with the restriction enzymes, EcoR1 and HindIII. These were analysed on a 1% agarose gel.

Lane 1, 9407H2 showing the 470 bp HpaII fragment insert of the Eco-7 fragment.

Lane 2, 9407H4 showing the 617 bp HpaII fragment insert of the Eco-7 fragment.

Lane 3, 9407H5 showing the 450 bp HpaII fragment insert of the Eco-7 fragment.

Lane 4, 9407H8 showing the 250 bp HpaII fragment insert of the Eco-7 fragment.

Lane 5, ϕX174 RF HaeIII digests as marker DNA.

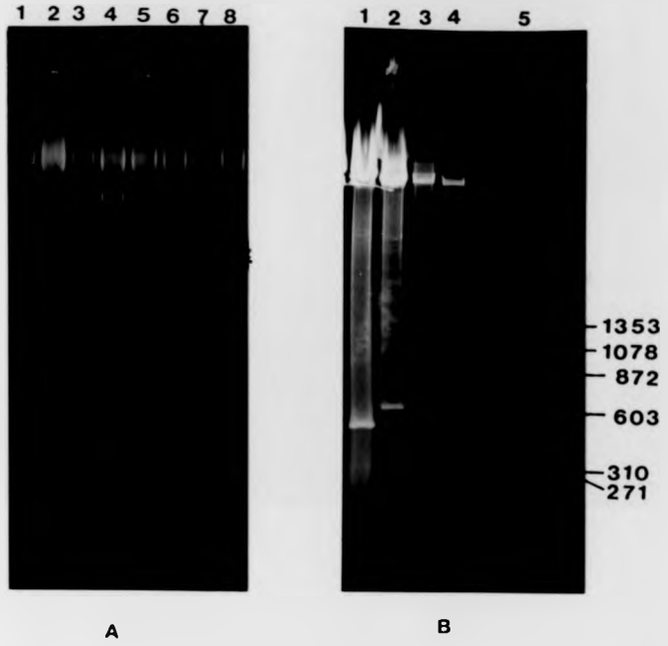


Figure 5.5 Identification problems of the M 13 recombinants with smaller inserts.

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A) Agarose gel (1%) electrophoretic pattern of the ssDNAs, isolated from the colourless plaques. Lane 1, mp9; Lane 2, 9407H1; Lane 3, 9407H2; Lane 4, 9407H3; Lane 5, 9407H4; Lane 6, 9407H5; Lane 7, 9407H6; Lane 8, 9407H8.

B) EcoR1 digests of the RF DNAs isolated of the recombinants.

The RF DNAs were isolated from the recombinant clones 9407H2, 9407H4, 9407H5, 9407H8 and digested with the restriction enzymes, EcoR1 and HindIII. These were analysed on a 1% agarose gel.

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Lane 2, 9407H4 showing the 617 bp HpaII fragment insert of the Eco-7 fragment.

Lane 3, 9407H5 showing the 450 bp HpaII fragment insert of the Eco-7 fragment.

Lane 4, 9407H8 showing the 250 bp HpaII fragment insert of the Eco-7 fragment.

Lane 5, ϕX174 RF HaeIII digests as marker DNA.

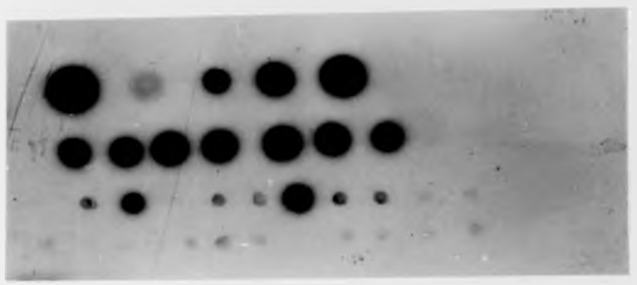

Figure 5.6 Dot hybridization analysis of the M 13 recombinant clones.
The ssDNAs were isolated from the colourless plaques and dot blotted onto a sheet of nitrocellulose filter. The filter were hybridized with ^{32}P labelled DS-14 mt-DNA and autoradiographed. The dark dots were identified as true recombinants.

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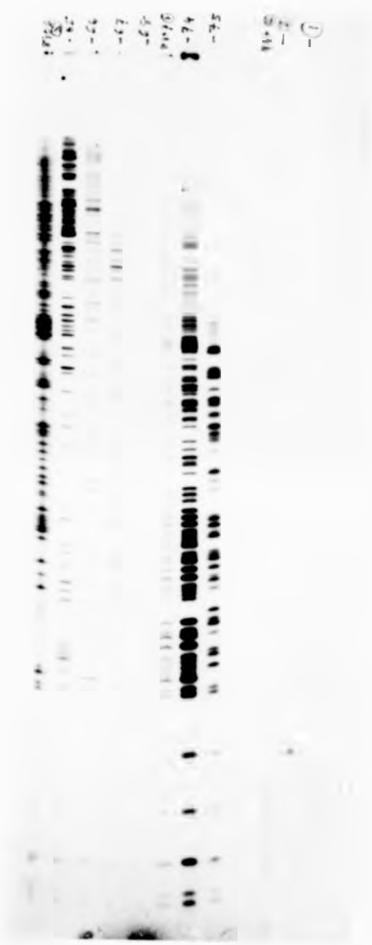
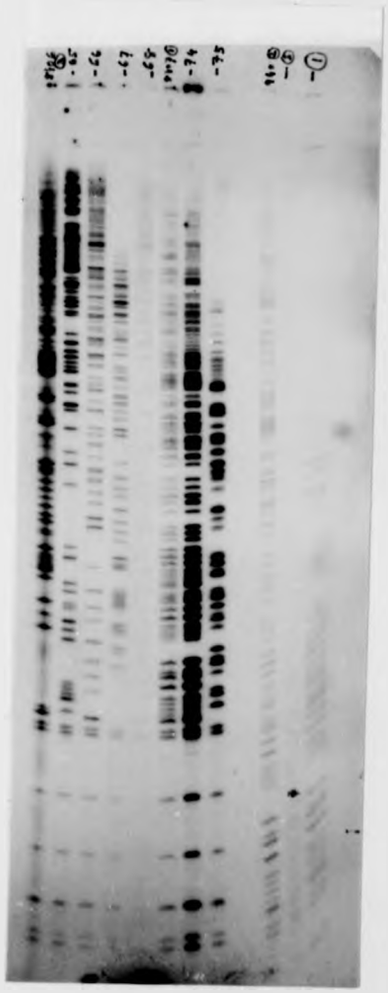


Figure 5.7 Single lane screening for the identification of the M 13 recombinants.

The ssDNAs isolated from the suspected recombinants were sequenced for only the T reaction (T-tracking), as described in the materials and methods section. The samples were analysed on a thin 8% Polyacrylamide-Urea gel and autoradiographed. Similar pattern indicates they are similar clones while dissimilar pattern indicates that they are different clones.



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To identify the orientation of the target DNA among positive clones and to sort out representatives of similar clones, the single-lane screening method has been employed (Sanger *et al*,1980). Identical patterns indicate identical M 13 recombinant clones (Fig. 5.7). From the pattern obtained from the single-lane tracking, the orientation of target DNAs are also identified.

Enzymatic "shot-gun" Method & Directional Cloning in M 13

Enzymatic "shot-gun" method using various "four-cutter" restriction enzymes is very useful when detailed restriction maps are available. Using *Sau*3A (*N*' GATC), *Hpa*II (*C*' CGG), which generate compatible ends with *BamH*1 (*G*' GATCC) and *Acc*1 (*GT*'GGAC, respectively of vector DNA, most of the desirable clones useful for sequencing were isolated. However, the main problem which has been observed in such a procedure, is the preponderence of clones having insertions in one particular orientation. This could be due to a stereo-specific structural constraint exerted by the target DNA and vector DNA. In other words, the vector DNA can recognise some stereo-specific structures similar to its own. This is partly supported by the fact that in one particular experiment, when a total *EcoR*1 digest of pSC40ME6 was used to clone in the *EcoR*1 site of M 13, pAT153 DNA recombined with M 13 more frequently (about 60%) than Eco-6 DNA fragments. The size of pAT153 is 3.6 kbp, as opposed to the 2.56 kbp size of Eco-6. Due to the difference of about 1 kbp, it was thought that M 13 recombinants would have a preferential mt-DNA insert over pAT153. But in the experiment the opposite is found to be true. This may be due to the different structure of mt-DNA which is about 70% (A+T) rich in comparison to pAT153 which has a (A+T) content of 40%. Somehow M 13 can "recognise" DNA structure, similar to its own DNA and can accomodate certain foreign DNAs better than others during the course of their propagation in host cells. This explanation however, needs further rigorous experimental support.

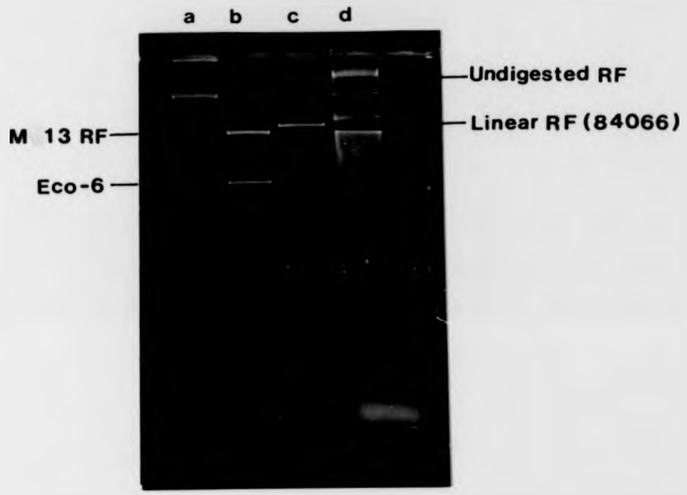
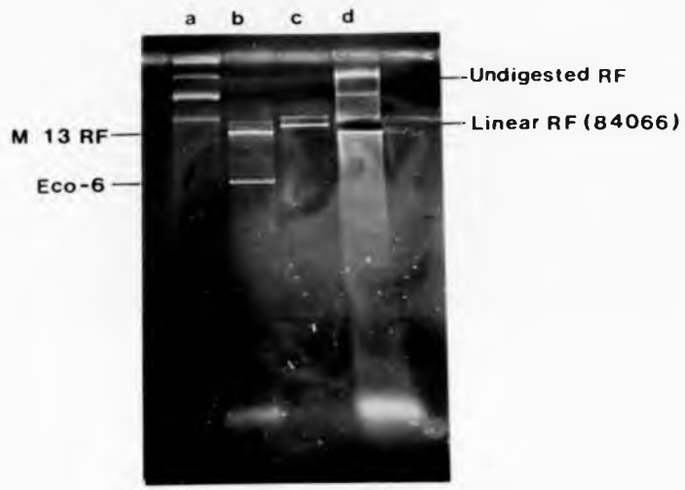


Figure 5.8a. DNase I digestion of the replicative form (RF) DNA of the M 13 recombinants for "systematic" sequencing.

The RF DNA was isolated from the M 13 mp8 recombinant clone, 84066, and digested with DNase I (1.5 pg/μl) in the DNase I buffer (20mM Tris, 1mM $MnCl_2$, pH 7.5, 100 μg/ml BSA) for 3 minutes at room temperature. The reaction was stopped with 20 mM EDTA, 0.01% SDS and loaded onto a 0.9% low melting point (LMP) agarose gel directly.

(a) Undigested 84066 RF DNA, (b) EcoR1 digested 84066 RF DNA, (c) Linear form of 84066 RF DNA (HindIII digest), (d) DNaseIdigested 84066 RF DNA.

The DNA band corresponding to the linear form of 84066 RF was excised out from the Lane with the DNase I digested sample (Lane d) and the DNA was extracted from the gel for subcloning as described in Fig. 5.2.



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Hong's method of obtaining "non-random" clones for sequencing

In principle Hong's method of obtaining non-random clones should produce rapid sequence data. However, experience reveals that the method is not so applicable, mainly for three different reasons: firstly, the isolation of recombinant M 13 RF and secondly, the ligation efficiency and thirdly, the removal of the priming site during the DNase digestion step of the recombinant RF. The first and the last points were major problems in our case. It has been found that M 13 recombinants are unstable with mitochondrial DNA inserts. The reason for this may be due to high A+T content of mt-DNA which exerts steric effects on the conformation of the M 13 genome which is somehow recognised by M 13 and results in the loss of the foreign inserts. Experiments with the 840ME6 clone which is a recombinant of M 13 mp8 with Eco-6 fragment (2.56Kbp) from the CD40 strain, can be cited as an example. After the initial subcloning, the ssDNA from 840ME6 was isolated and about 250-300bp of the DNA sequence was read from the *EcoR*1 ends of the inserted fragment. To produce subclones from this initial 840ME6 (See Fig. 5.2) by Hong's method, clones were grown up for their RF. The RF was digested with DNase I (Fig. 5.8a) and the linearised fragments were isolated from the low melting point agarose gel. DNase I digested RF was subjected to a DNA polymerase catalysed "end filling" reaction and then digested with *Hind*III as described in Fig. 5.2, to produce a series of RF with inserts of different lengths. These were ligated to circularise, to produce overlapping clones. Unfortunately, due to the low efficiency or due to contaminated agarose, ligation did not work and no subclones were produced from them. Most of the initial isolate of RF was spent to standardise DNase I digestion and the various stages described in the Fig. 5.2. When the second attempt was made to isolate the RF from the 840ME6 recombinant stock, it was found that the clone had lost the Eco-6 inserts and when checked on a (X-gal + IPTG) plate, yielded blue plaques. Later it was observed that M 13 recombinants with more than 600 bp mt-DNA inserts always tended to lose their inserts after the first few generations. This poses major problems with the Hong's method, as far as mitochondrial DNA is concerned.

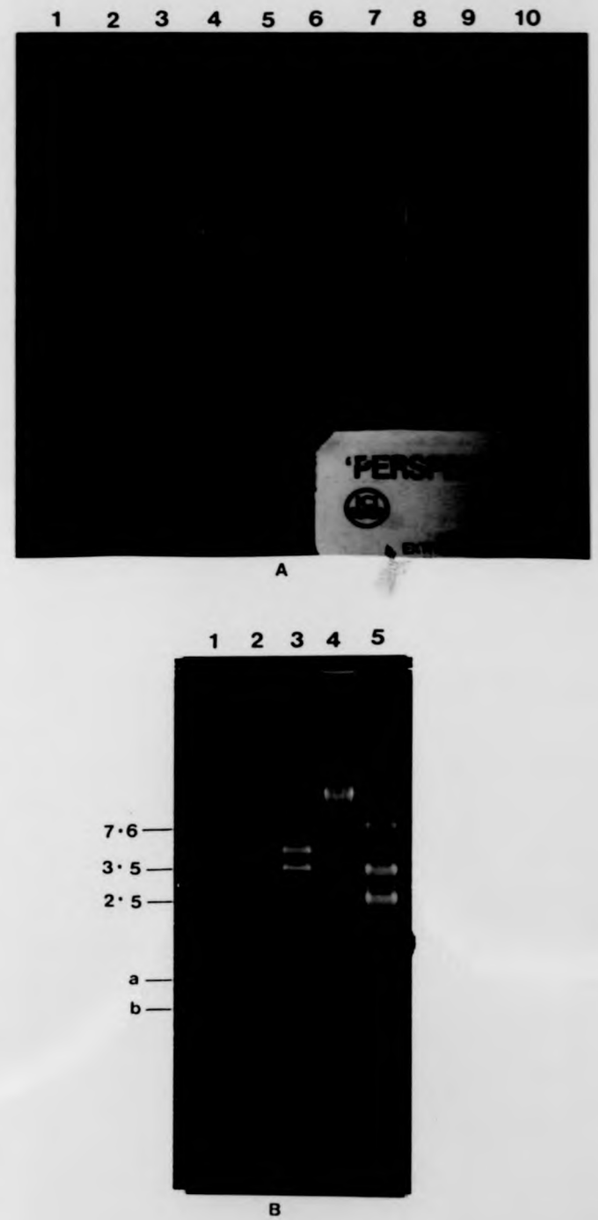


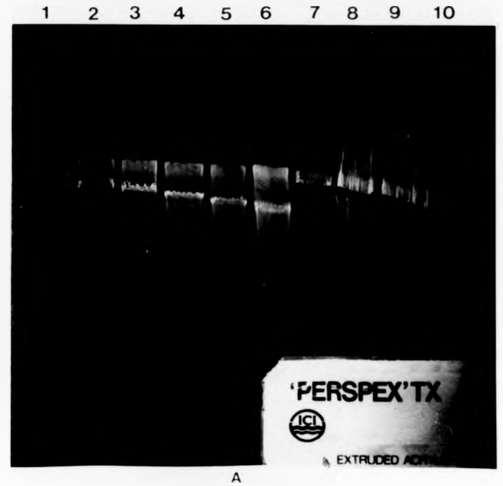
Figure 5.8b Bal-31 deletion of DNA for generation of "non-random" clones for sequencing.

A) Purified plasmid from pSCME6 was linearised with the restriction endonuclease Xba-I (see Fig. 5.3) and digested with the nuclease Bal-31 for 0 min (Lane 1 & 7), 1 min (Lane 2 & 8), 3 min (Lane 3 & 9), 5 min (Lane 4 & 10), 7 min (Lane 5) and 9 min (Lane 6) and directly loaded onto a 1% LMP agarose gel for electrophoresis. After staining in ethidium bromide, the gel was placed on a Perspex sheet which transmits s only long wave length UV (>330 nm) and the DNA bands were excised out (Lane 7, 8, 9, and 10) on a UV transilluminator. DNAs were extracted from the excised bands and subcloned in M 13.

B) The bands isolated from LMP agarose gel (above) was digested with *EcoR*1 to measure the size of the gradually shortened DNA fragments of the target DNA.

Lane 1, Bal-31 digested 0 min. sample; Lane 2, Bal-31 digested 1 min sample; Lane 3, Bal-31 digested 5 min sample; Lane 4, undigested M 13 RF (not relevent to this experiment); Lane 5, pSCME6 *EcoR*1 digest as the marker DNA.

Note that the DNA bands labelled as **a** (1.45 Kbp) and **b** (1.15 Kbp) of Lane 1 have shortened to: 1.30 Kbp and 1.00 Kbp in Lane 2, and 0.90 Kbp and 0.50 Kbp in Lane 3.



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B) The bands isolated from LMP agarose gel (above) was digested with *Eco*R1 to measure the size of the gradually shortened DNA fragments of the target DNA.

Lane 1, Bal-31 digested 0 min. sample; Lane 2, Bal-31 digested 1 min sample; Lane 3, Bal-31 digested 5 min sample; Lane 4, undigested M 13 RF (not relevent to this experiment); Lane 5, pSCME6 *Eco*R1 digest as the marker DNA

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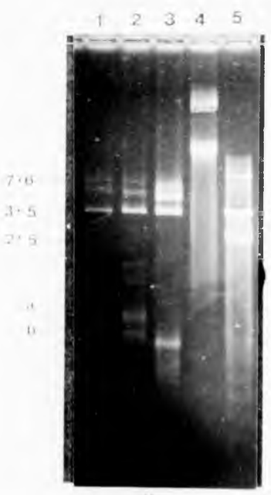
A



B

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B) The bands excised from LMP agarose gel (shown in A) were digested with EcoRI (to release the smaller 2·5 kb fragment of DNA) to remove all the cloned DNA fragments of the cleaved DNA.

Lane 1: Bal-31 digested 0 min control. Lane 2: Bal-31 digested 1 min control. Lane 3: Bal-31 digested 3 min control. Lane 4 undigested M13CBH (a cloning vector) as control for the experiment. Lane 5: pSCME6 digested to release the insert DNA.

Note that the DNA bands released are 7·6 Kbp and 3·5 Kbp in Lane 1, 7·6 Kbp and 3·5 Kbp in Lane 2, 3·30 Kbp and 3·00 Kbp in Lane 3, and 0·90 Kbp and 0·50 Kbp in Lane 4.

Another major drawback has been observed, during DNase digestion the priming site is removed from the recombinant M 13 RF without affecting the viability of the clones. As a result, a lot of subclones can not be sequenced using the commercially available primer. The difficulty mentioned with the ligation efficiency of the blunt ends was overcome. However, it is observed that deletions having smaller inserts always tend to mask the recombinants with larger inserts.

Bal 31 deletion method

A modified method from that of Poncz *et al* (1982) was used in the present study (See Fig. 5.3). From restriction analyses of the recombinant clones (see Chapter 4) it was found that the Eco-6 band had only one *Xba*I site which is absent from pAT153. *Xba*I produces two fragments, upon digestion of the Eco-6 band, of molecular size 1.14 Kbp and 1.42 Kbp, respectively. To avoid the gel purification step, which is always time consuming, the DNAs isolated from pSCME6 clones were digested with *Xba*I and directly digested with Bal-31 for different time periods. Fig. 5.8b demonstrates the gradual decrease of the molecular weight of the plasmid pSC40ME6 at various time periods. In our experience Bal-31 digestion removes 100 to 140 bp/min. After stopping the Bal-31 reaction by phenol extraction and ethanol precipitation, the staggered DNA ends are filled in with dNTPs in the presence of Klenow enzyme. The plasmids were then digested with *EcoR*1 which yielded the pAT153 with two *EcoR*1 ends and the insert DNA with one blunt end and one *EcoR*1 sticky end. This was directly used to clone in M 13 mp10 vector (digested with *EcoR*1 and *Sma*I) by "force" ligation in one particular orientation. The resultant recombinants thus generated had blunt ends directed towards the priming site. The Bal-31 digests of different time periods thereby generated all ranges of insert DNA with overlaps, suitable for sequencing. This method seems very useful as far as mt-DNA sequencing is concerned.

DISCUSSION

Results presented in the above section are not directly relevant to the objectives of the present thesis. However, it was felt that the problems observed with the subcloning of (A+T) rich mitochondrial DNA would be worth mentioning for future reference. It would be interesting to study the steric effect of a particular DNA structure which is dependent on specific nucleotide sequences, during cloning and the recognition of foreign DNA by hosts during their propagation. The genetic barrier observed within the cell (between cellular organelles and nucleus) may not be due to only physical membrane barriers but also due to inherent structural barriers of two different kinds of DNA molecules. It is known that during the divisional state of eukaryotic organisms, nuclear membranes disappear and it has also been postulated that mitochondria fragment into pieces before being transmitted to daughter cells. Therefore, there is a fair chance of mixing the DNA molecules from these two compartments. However, under normal circumstances they do not. A systematic study therefore, how far steric hinderance of DNA molecules can prevent mixing up of two genetic systems would be enlightening.

Section 5b

Interpretation of the DNA Sequences

Based on the combination of the strategies described in the earlier section of this chapter the nucleotide sequences of the Oli-2 region were derived. An overall strategy for sequencing the Eco-6 and the Eco-7 fragments of the Oli-2 region is given in Fig. 5.9. Materials and methods mentioned in the earlier section are common to this section. DNA sequences were analysed using a computer program APP*DNA (Staden, 1980, 1981, 1982).

RESULTS

Nucleotide Sequences of the Oli-2 Region

Three different wild type strains, D273-10B/A1, D603-3B and D22 strains are used to determine the sequences around the Oli-2 region. The clones, pSC273ME6, pSC273ME7, pSC603ME6, pSC603ME7, pSC22ME6 and pSC22ME7, were employed. The characterisation of these clones have been described in Chapter 4. It has been mentioned that the pSC273ME6 and pSC273ME7 clones contain the Oli-2 region of D273-10B/A1, the strain which is the wild type parent of CD40 ($Oli2^R$, $Oss1^R$), D27/92 ($Oss1^R$) and D27/76 ($Oli2^R$). The clones pSC603ME6 & ME7 are derived from the wild type strain D603-3B, a strain containing the OP_1 mutation, from which CD24 (pho9) & CD41 (pho8) were derived (Darlison & Lancashire, 1980). The clones pSC22ME6 and ME7 were derived from a totally unrelated wild type strain D22.

The Oli-2 region was sequenced from the recombinant clones pSC40ME6 & pSC40ME7 almost completely, whereas the region from pSC273ME6 & pSC273ME7, pSC24ME6 and pSC24ME7, pSC22ME6 & pSC22ME7 and pSC603ME6 & pSC603ME7 were sequenced for the relevant portion containing the Oli2 and Aap genes only. The complete nucleotide sequence of Oli-2 locus and its upstream from the strain CD40 (derived from the the recombinant clones, mentioned above) is presented in Fig. 5.10. The sequence spans a 2.88kbp region, starting from the *EcoR*1 site within the COOH terminal end of the Oxi-3

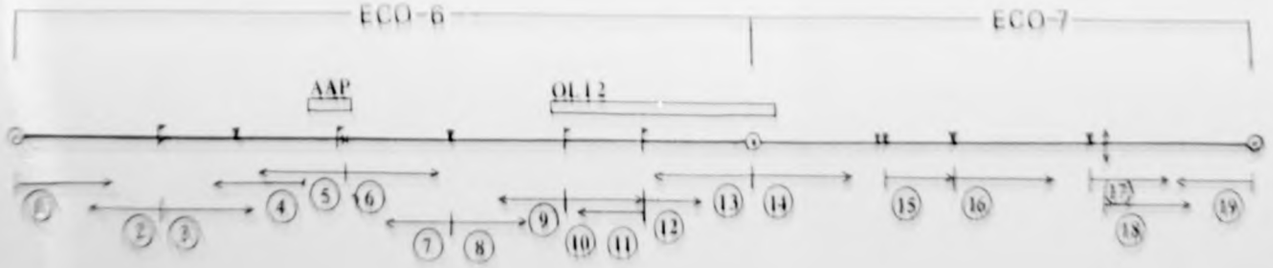


Figure 5.9 Overall DNA sequencing strategy for the Oli-2 region of yeast mitochondrial genome.

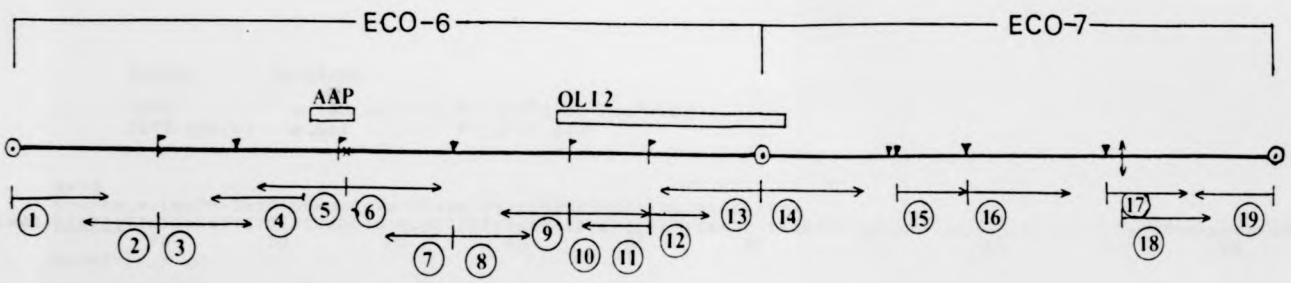
The *Eco-6* and *Eco-7* are two *EcoR*1 fragments spanning the Oli-2 region between the *Oxi-3* and *Cob*-box loci. The solid line represents the *EcoR*1 fragments while arrows beneath it indicate the direction and extent of sequence data. The numbers below the arrows indicate the M13 subclones which have been sequenced. The representative clones are:

(1) 8406E2, 8406E6; (2) 9406HE5; (3) 9406HS10, 9406H8; (4) 10246B4'10; (5) 10406X7, 10246X9, 10603X3; (6) 10406X9, 10246X2, 10603X5; (7) 9406HS6, 9406HS7; (8) 9406HS9, 9406HS11; (9) 8406SH3, 8276S9; (10) 9406S5, 8246S21, 8276S15; (11) 9406S7, 8246S22,8276S10; (12) 8406S27, 8276S3; (13) 84066, 9406S3, 9406S4, 8276E1; (14) 82737, 84077, 9407Ha10, 8175E73, 8603E77; (15) 8277H18, 9407H8; (16) 9407H2, 9407H5; (17) 8407H6, 9407H4; (18) 9407P3; (19) 9407H6, 940E74, 8175E75.

The locations of the Aap gene (subunit-8 gene) and the Oli2 gene (subunit-6 gene) have been indicated. Restriction enzyme cleavage sites are:

⊙, *EcoR*1; ▼, *Hpa*II; ⚑, *Sau3A*; x, *Xba*I; ↕, *Pst*I

Note that the most of the representatives of the M 13 clones shown here have originated from the strain CD40. The DNA sequences from the strains CD24D603-3B, D273-10B/A1 have been partially obtained and only from the regions of Oli2 gene and App gene. Note also, that the sequence analysis of clones 1 - 19 are derived from data of 2-3 autoradiograms per clone and these autoradiograms are available for inspection. (Scale, 1cm = 200 bp).



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Strain	Genotype
CD40	a leu kar 1-1 ρ^+ Oli^R2-76 Oss^R1-92
D273-10B/A1	α met ρ^+ Oli^S Oss^S

```
      0x13
      GluPheLeuLeuThrSerProProAlaValHisSerPheAsnThrProAlaValGlnSer***
CD40  GAATTCTTATTAACTTCTCCACCAGCTGTACACTCATTTAATACACCAGCTGTACAATCTTAAGTTTTAAAATTTAATTATTTACTTAATAATTAAAAAAAAAAAAGTAAATATTATATCT
            10        20        30        40        50        60        70        80        90       100       110       120
      EcoRI

CD40  AAAACTTAATAATATAATAATAATATTCTTATTAAAAAAAAATATATAAAAAAAAAATATATAAAATTTATTAAAATATCTTAATATATTATCCTTTTTGGGAACTATATAAATAAATACTA
           130       140       150       160       170       180       190       200       210       220       230       240
                                                               AhaIII

CD40  ATATAATCCTATTATATATATATATATATAAAATAATATATATATATAATTAATATAAATAATATTTATAATTATTTTTTTAATAATATATATAATTTAATATATTAATTAATATTATAT
           250       260       270       280       290       300       310       320       330       340       350       360

CD40  AATTATTAAATATATTATAATATTATTATTATTTTATAATAAAAATATTTTTAATACTAATTATTATTTATTATTTATAAATATATAAATAGTATGTTTAATATTATTAATACTAAAAAA
           370       380       390       400       410       420       430       440       450       460       470       480

CD40  AAGTATAATTATATTTAGAGATCTACAATACATTTATCTGATAAATATTAATATTAATATTAATATTTATATTAATAAAGGGATTAAATTAATTGTATCCAATTTAATTAAATTATAGAT
           490       500       510       520       530       540       550       560       570       580       590       600
                     Sau3AI

CD40  ATATTATTTATAATATTAATATATTGTTTTATTAAAAAGGTAAAAATAGTTTTTATTTTATTATAAATATGGTATAAATAAATATATTATATATATATTAAGAATATATTTATATTTTAC
           610       620       630       640       650       660       670       680       690       700       710       720

CD40  ATATAATTATTTATAATATAAATATCTCCGCAAAGCCGGTTTAATGTAATTATTTAATAATTTTATTTAATAATTTATTAAAATAAATATTTACATTTAATAATATTTATATTATGTCAG
           730       740       750       760       770       780       790       800       810       820       830       840
                               HpaII

CD40  TTATTTTATATTAATGTTTAATCTATTATAATATTTTTTTTTATAAATATATTATTTATATTAATAAATATATATATTATTTTTATAATATATATATATTTTTATTAAATATTTATTAAA
           850       860       870       880       890       900       910       920       930       940       950       960
                                                               AAPI
                                                               MetProGlnLeuValProPheTyrPheMetAsnGlnLeuThrTyrGlyPheLeuLeuMetIleLeu
CD40  TATTTATTAAATTATTATAATGTTGTTATTAATCTTATTAAAAAATATATATAAAAATGCCACAATTAGTTCCATTTTATTTTATGAATCAATTAACATATGGTTTCTTATTAATGATTC
           970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
                                                                                                              HinfI
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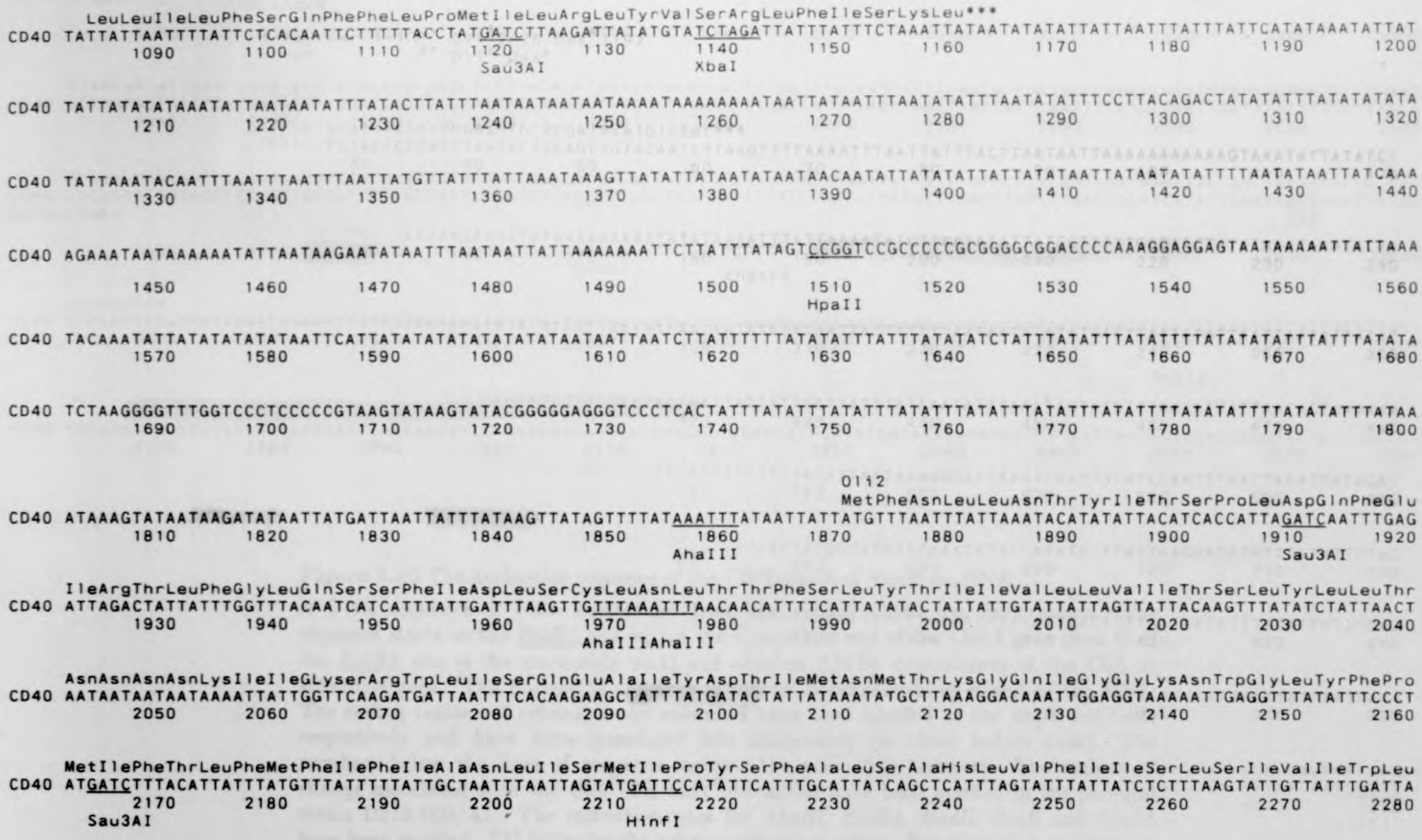
Fig 5.10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1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
Sau3AI
XbaI
CD40 TATTATATATAAATATTAATAATATTTATACTTATTTAATAATAATAATAAAATAAAAAAAATAATTATAATTTAATATATTTAATATATTTCCTTACAGACTATATATTTATATATATA
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
CD40 TATTAAATACAATTTAATTTAATTTAATTATGTTATTTATTAAATAAAGTTATATTATAATATAATAACAATATTATATATTATTATATAATTATAATATATTTTAATATAATTATCAAA
1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
CD40 AGAAATAATAAAAAATATTAATAAGAATATAATTTAATAATTATTAAAAAAATTCTTATTTATAGTCCGGTCCGCCCCCGCGGGGCGGACCCCAAAGGAGGAGTAATAAAAATTATTAAA
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
HpaII
CD40 TACAAATATTATATATATATAATTCATTATATATATATATATATAATAATTAATCTTATTTTTTATATATTTATTTATATATCTATTTATATTTATATTTTATATATATTTATTTATATA
1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
CD40 TCTAAGGGGTTTGGTCCCTCCCCCGTAAGTATAAGTATACGGGGGAGGGTCCCTCACTATTTATATTTATATTTATATTTATATTTATATTTTATATATTTTATATATTTATAA
1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
Oli2
MetPheAsnLeuLeuAsnThrTyrIleThrSerProLeuAspGlnPheGlu
CD40 ATAAAGTATAATAAGATATAATTATGATTAATTATTTATAAGTTATAGTTTTATAAATTTATAATTATTATGTTTAATTTATTAAATACATATATTACATCACCATTAGATCAATTTGAG
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920
AhaIII
Sau3AI
IleArgThrLeuPheGlyLeuGlnSerSerPheIleAspLeuSerCysLeuAsnLeuThrThrPheSerLeuTyrThrIleIleValLeuLeuValIleThrSerLeuTyrLeuLeuThr
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1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040
AhaIIIAhaIII
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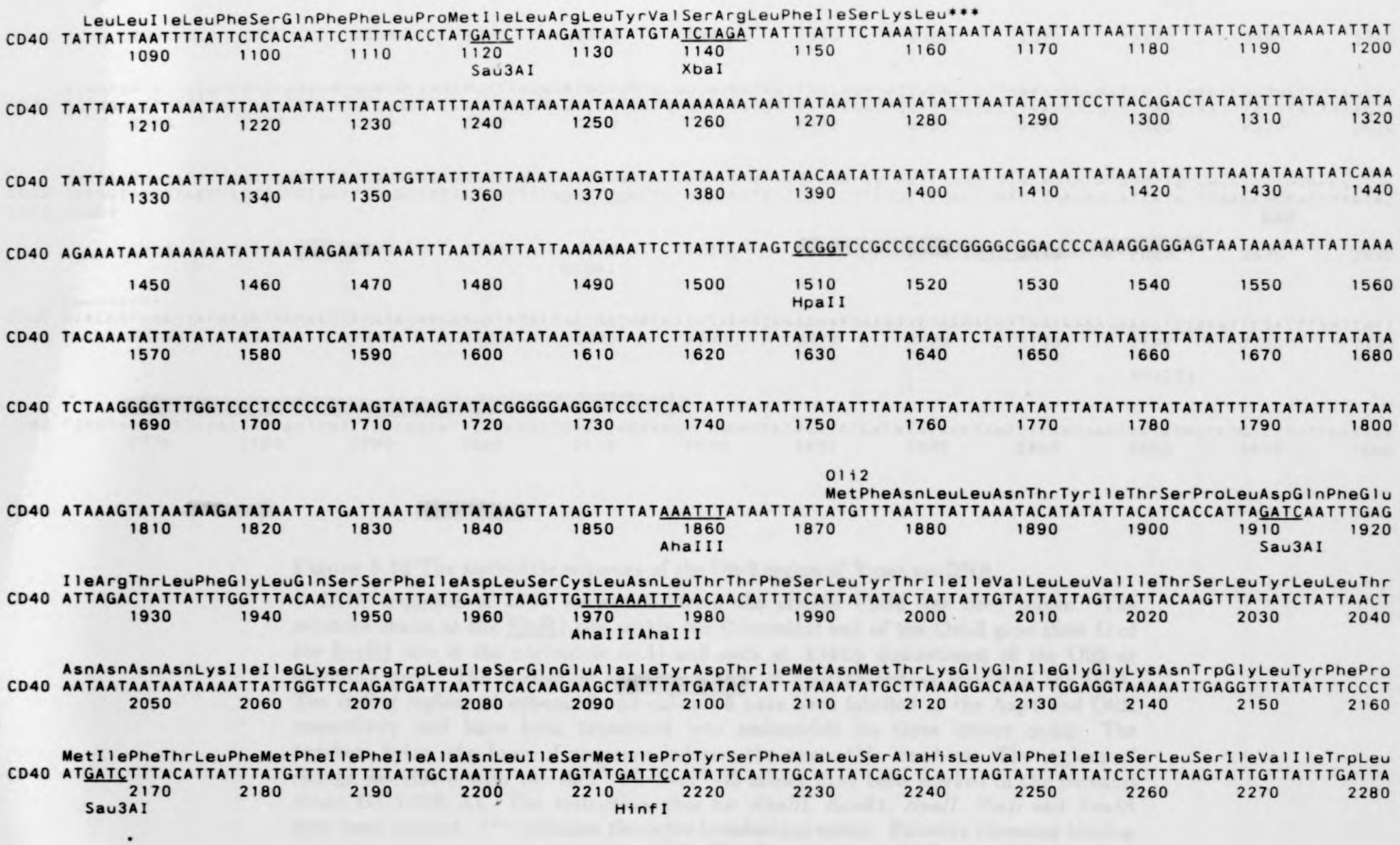
CD40 ATGATCTTTACATTATTTATGTTTATTTTTATTGCTAATTTAATTAGTATGATTCCATATTCATTTGCATTATCAGCTCATTTAGTATTTATTATCTCTTTAAGTATTGTTATTTGATTA
2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280
Sau3AI
HinfI

Fig 5.10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CD40 TATTATTAATTTTATTCTCACAATTCTTTTTACCTATGATCTTAAGATTATATGTATCTAGATTATTTATTTCTAAATTATAATATATATTATTAATTTATTTATTCATATAAATATTAT
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
Sau3AI
XbaI
CD40 TATTATATATAAATATTAATAATATTTATACTTATTTAATAATAATAAAATAAAAAAAATAATTATAATTTAATATATTTAATATATTTCCTTACAGACTATATATTTATATATATA
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
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1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
CD40 AGAAATAATAAAAAATATTAATAAGAATATAATTTAATAATTATTAAAAAAATTCTTATTTATAGTCCGGTCCGCCCCCGCGGGGCGGACCCCAAAGGAGGAGTAATAAAAATTATTAAA
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
HpaII
CD40 TACAAATATTATATATATATAATTCATTATATATATATATATATAATAATTAATCTTATTTTTTATATATTTATTTATATATCTATTTATATTTATATTTTATATATATTTATTTATATA
1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
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1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
Oli2
MetPheAsnLeuLeuAsnThrTyrIleThrSerProLeuAspGlnPheGlu
CD40 ATAAAGTATAATAAGATATAATTATGATTAATTATTTATAAGTTATAGTTTTATAAATTTATAATTATTATGTTTAATTTATTAAATACATATATTACATCACCATTAGATCAATTTGAG
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920
AhaIII
Sau3AI
IleArgThrLeuPheGlyLeuGlnSerSerPheIleAspLeuSerCysLeuAsnLeuThrThrPheSerLeuTyrThrIleIleValLeuLeuValIleThrSerLeuTyrLeuLeuThr
CD40 ATTAGACTATTATTTGGTTTACAATCATCATTTATTGATTTAAGTTGTTTAAATTTAACAACATTTTCATTATATACTATTATTGTATTATTAGTTATTACAAGTTTATATCTATTAACT
1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040
AhaIIIAhaIII
AsnAsnAsnAsnLysIleIleGLyserArgTrpLeuIleSerGlnGluAlaIleTyrAspThrIleMetAsnMetThrLysGlyGlnIleGlyGlyLysAsnTrpGlyLeuTyrPhePro
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2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160
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2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280
Sau3AI
HinfI

Fig. 5.10

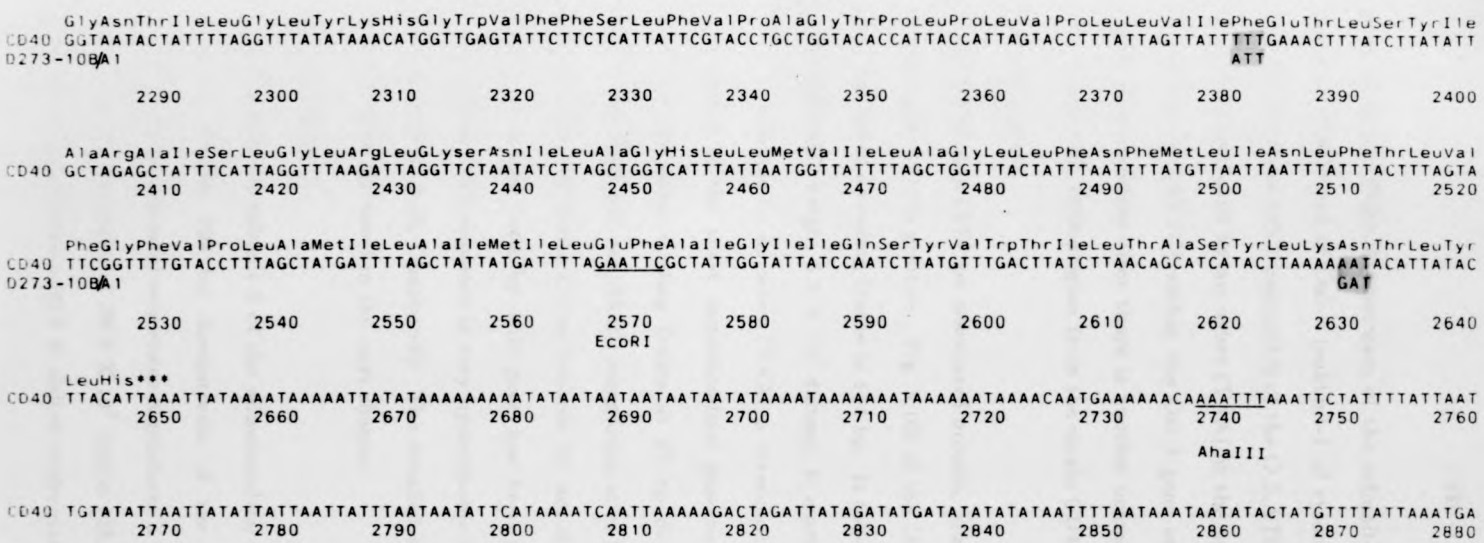


Figure 5.10 The nucleotide sequence of the Oli-2 region of Yeast mt-DNA.

A DNA sequence of 2880 nucleotides from the strains CD40 has been shown. The sequence starts at the EcoR1 site within the C-terminal end of the Oxi-3 gene (first G of the EcoR1 site is the nucleotide no.1) and ends at 230 bp downstream of the Oli2 or subunit-6 gene. The nucleotide sequence is that of nontranscribing strand of the DNA. The coding regions of subunit-8 and subunit-6 have been labelled as the Aap1 and Oli2, respectively and have been translated into aminoacids (in three letters code). The numbers below the lines of sequence indicate the nucleotide numbers. The regions of change associated with the *Oli2*^{R}-76, *Oss1*^{R}-92 alleles have been marked in the parental strain D273-10B/A1. The restriction sites for *AhaIII, EcoR1, HpaII, HinfI* and *Sau3A* have been marked. *** indicates the ochre termination codon. Putative ribosome binding sites, transcripts processing signals and (G+C) rich clusters have been coloured in yellow.

gene to about 230bp downstream of the subunit-6 gene. The predicted polypeptide products of COOH-end of Oxi-3 (subunit-1 of cytochrome oxidase), AAP1 (subunit-8 of the O.S.ATPase) and Oli 2 (subunit-6 of the O.S.ATPase) are shown in the figure. The Oxi-3 region is terminated by an ochre (TAA) at the nucleotide position 63 (assigning G of the *EcoR*1 site (GAATTC) within the Oxi-3 gene as nucleotide No.1). In the same reading frame 30 nucleotides after there is another ochre. The occurrence of different restriction sites within this 2.88kbp region from the strain CD40 has been shown in Fig. 5.10.

Subunit-8 gene

Subunit-8 or Aap (ATPase associated protein, Macreadie *et al.*1983) gene starts at 957 bp downstream (1017th position, Fig. 5.10) of the Oxi-3 or Subunit-1 of cytochrome oxidase gene and spans a reading frame of 144 bp. It codes for a polypeptide of 48 aminoacid residues (molecular weight 5.5 X 10^3 dalton). It starts with the ATG codon for f-methionine and is terminated by an ochre (TAA), a termination codon which has been obtained so far in all genes of the yeast mitochondrial genome. There is another termination codon (ochre) in the same reading frame at 27 bp downstream of the first. Two *Hinf*I, one *Sau*3A, one *Xba*I and one *Aha*III recognition sites lie within the reading frame. The codon used, as expected, shows a bias towards the use of A and T at the third position. The frequency of codons used by this gene has been shown in Table 5.3. The polypeptide, deduced from DNA sequence is very hydrophobic in nature, as evidenced by its hydrophobicity plot (Fig. 6.13, Chapter-6). The detailed secondary and tertiary structure of this protein will be discussed in the next chapter.

Subunit-6 gene

The Oli-2 gene for subunit-6 of the mitochondrial O.S.ATPase is a 777 bp long open reading frame located 709 bp downstream of the Aap gene or Subunit-8 gene. When translated into aminoacid sequences it produces a 259 aminoacid residue long polypeptide with a molecular weight of 28.5 X 10^3 dalton (28.5 K dalton). This is much higher than expected, as it produces a 20.5 K dalton molecular weight protein band in SDS gel. This

Table 5.3 Codon Use by the mitochondrial genes for the O.S.ATPase. The tentative reading frame for mit-175 locus is represented by subunit-x. The data for subunit-9 has been taken from Macino & Tzagoloff (1979).

Aminoacid	Codon	Subunit-6	Subunit-8	Subunit-9	Subunit-x
Ala	GCA	2	0	4	1
	GCT	11	0	5	0
	GCC	0	0	1	0
	GCG	0	0	0	0
Arg	AGA	4	2	1	5
	AGG	0	0	0	0
	CGA	0	0	0	0
	CGT	0	0	0	2
	CGC	0	0	0	0
	CGG	0	0	0	0
Asp	GAT	4	0	0	9
	GAC	0	0	1	0
Asn	AAT	14	1	1	15
	AAC	0	0	1	0
Cys	TGT	1	0	1	0
	TGC	0	0	0	0
Glu	GAA	3	0	1	5
	GAG	1	0	0	0
Gln	CAA	5	3	1	3
	CAG	0	0	0	0
Gly	GGA	2	0	2	1
	GGT	14	1	8	2
	GGC	0	0	0	0
	GGG	0	0	0	0
His	CAT	4	0	0	2
	CAC	0	0	0	0
Ile	ATT	31	3	7	10
	ATC	5	1	2	2
Leu	TTA	47	12	11	12
	TTG	0	0	0	0
Lys	AAA	5	1	2	18
	AAG	0	0	0	0
Met	ATG	9	4	3	0
	ATA	1	0	0	4
Phe	TTT	15	4	1	12

	TTC	6	3	6	0
Pro	CCA	4	2	1	0
	CCT	4	1	1	4
	CCC	0	0	0	0
	CCG	0	0	0	0
Ser	TCA	11	1	5	2
	TCT	5	2	0	4
	TCC	0	0	0	0
	TCG	0	0	0	0
	AGT	3	0	0	1
	AGC	0	0	0	0
Thr	ACA	9	1	2	1
	ACT	6	0	0	1
	ACC	0	0	0	1
	ACG	0	0	0	0
	CTA	3	1	1	1
	CTT	2	0	0	2
	CTC	0	0	0	0
	CTG	0	0	0	0
Trp	TGG	0	0	0	0
	TGA	5	0	0	1
Tyr	TAT	9	3	1	14
	TAC	2	0	0	1
Val	GTA	7	1	5	1
	GTT	5	1	1	4
	GTC	0	0	0	1
	GTG	0	0	0	0
TOTAL		259	48	76	142

anomaly could be due to aberrant mobility of this hydrophobic protein in SDS gel or due to post translational processing.

Within the reading frame there are two *Sau*3A sites (3 in the case of D22), one *Hinf*I site, one *Alu*I site and three *Aha*III sites. The generation of a new *Sau*3A site in the strain D22 (Fig. 5.11a & 5.11b) does not involve any change of aminoacid sequence. The protein (deduced from the DNA sequences) is very hydrophobic in nature, as evidenced by its hydrophobicity plot (Fig. 6.7, Chapter-7). Potentially it can produce seven transmembrane segments. The detailed secondary and tertiary structure of the protein will be discussed in the next chapter.

Changes associated with drug resistance

When the Oli-2 region from CD40 (pSC40ME6 & pSC40ME7) is analysed for DNA sequences, it is found that it has two base changes corresponding to the $Oli2^R$ and $Oss1^R$ mutations. $Oli2^R$ is due to a change of A to T in the 511th nucleotide (Fig. 5.12) of the Subunit-6 reading frame (2380th position in Fig. 5.10 sequences). This leads to a change in the 171st aminoacid residue, isoleucine (ATT) to a phenylalanine (TTT). This isoleucine is a highly conserved residue observed in the Subunit-6 reading frames from various organisms. Similarly a single base change is found for Ossamycin resistivity. A change (G → A) in the 2628th nucleotide position (760th nucleotide position of the reading frame) (Fig. 5.13) alters the aminoacid aspartic acid to an asparagine in the 254th aminoacid residue. This acidic aminoacid is also highly conserved throughout evolution and is probably functionally important.

Localisation of the pho 9 mutation

The DNA sequence analysis of the Oli2 gene (the gene for subunit-6) and the Aap-1 gene (the gene for subunit-8) from the recombinant clones of the pho 9 mutation (pSC24ME6) and of the parental strain (pSC603ME6) does not show any change in the nucleotides within the reading frames of the genes. Therefore the *mit* characteristics of the pho 9 mutant (CD24) can not be attributed to any direct alteration of the genes. The intergenic

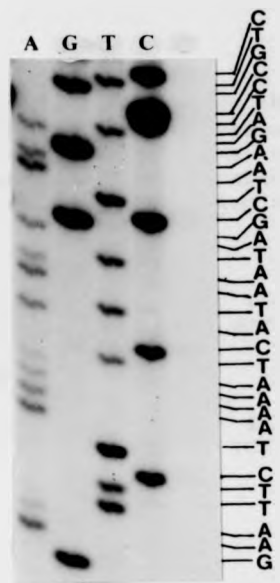
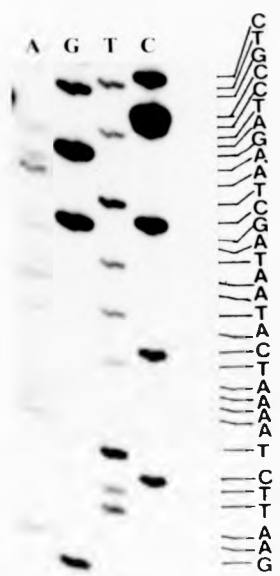


Figure 5.11a Autoradiogram of a part of the DNA sequencing gel of the M 13 recombinant clone, 922S1 to show the generation of a new Sau3A site in the strain D22.

This M 13 recombinant clone possesses the smallest EcoR1-Sau3A subfragment of the Eco-6 mt-DNA fragment of D22. A, G, T, C are four different tracks of the four nucleotides. The sequences reads:

GAATTC TAAAATCATAATAGCTAA GATC CGTC
→ mp9 sequence

EcoR1 Sau 3A



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GAATTC TAAAATCATAATAGCTAA GATC CGTC
→ mp9 sequence

EcoR1 Sau 3A

Figure 5.11b DNA sequence of the gene for the C-terminal end of Subunit-6 from the yeast strains D22. Note the presence of an extra Sau3A site on the 226th codon (I*) of the reading frame. This is due to a change of T to C in the 2547th nucleotide, when compared with D273-10B/A1. Amino acid sequences are shown in single letter amino acid code (see Appendix).

```
  221                                                  Oli4
  V   P   L   A   M   I*  L   A   I   M   I   L   E   F   A   I   G   I   I
GTA CCT TTA GCT ATG ATC TTA GCT ATT ATG ATT TTA GAA TTC GCT ATT GGT ATT ATC
   2535                                          2565
                  Sau3A                                 EcoR1
                                                                Oss1
  Q   S   Y   V   W   T   I   L   T   A   S   Y   L   K   D   T   L   Y   L
CAA TCT TAT GTT TGA CTT ATC TTA ACA GCA TCA TAC TTA AAA GAT ACA TTA TAC TTA

  H  Ochre
CAT TAA
```

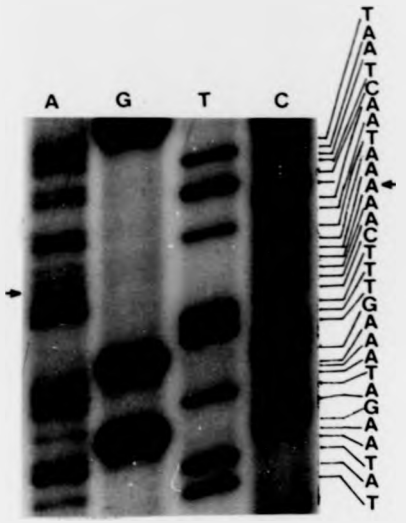
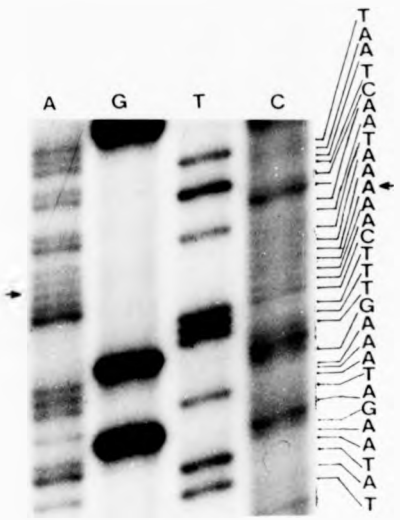


Figure 5.12 Autoradiogram of a part of the DNA sequencing gel of the M 13 mp8 recombinant clone, 84066 to show the change associated with the *Oli*2^R-76 allele.

The clone possesses the whole Eco-6 fragment. The primer proximal EcoR1 site of the vector is the is the EcoRI site within the Oli-2 gene. The sequence is that of "sense" strand and reads from the carboxyl terminal end of the gene towards the amino terminal end. A, G, T and C are four different tracks of the four nucleotides.

The **A** marked with an arrow is a **T** in the wild type strain (D273-10B/A1).



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The **A** marked with an arrow is a **T** in the wild type strain (D273-10B/A1).

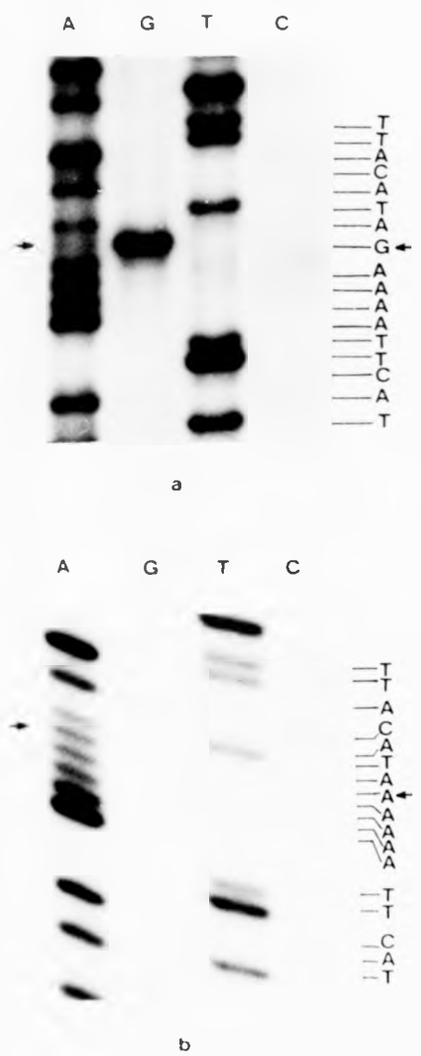


Figure 5.13 Change associated with the Ossamycin resistance.

(a) The autoradiogram shows a part of the DNA sequencing gel of the Oss1 surrounding area from $Oss1^{+}$ strain. The sequence has been derived from the M13 recombinant clone 82737. DNA sequence is that of the nontranscribing strand. The **G** marked with an arrow undergoes change in the $Oss1^{R}$-92 allele (see below).

(b) The autoradiogram shows a part of the DNA sequencing gel of the M13 recombinent clone 84077. The DNA sequence shown is the surrounding area of the Oss1 locus and that of the nontranscribing strand. The **A** marked with an arrow is the **G** in the wild type (D273-10B/A1).

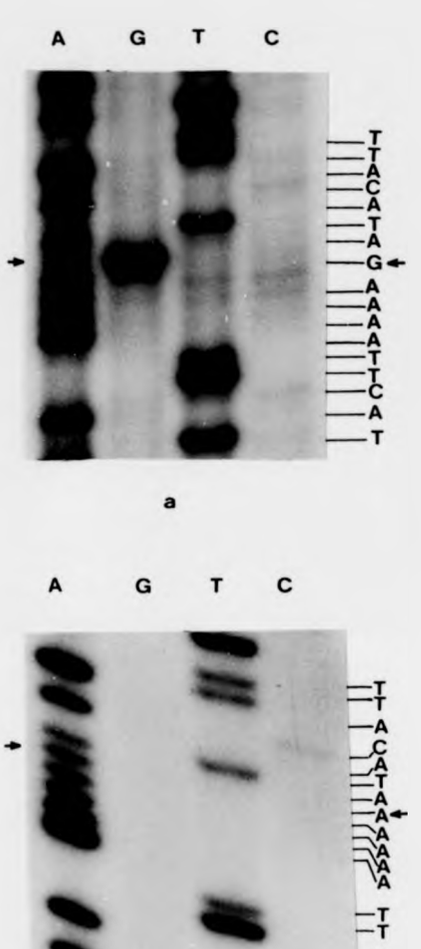


Figure 5.13 Change associated with the Ossamycin resistance.

(a) The autoradiogram shows a part of the DNA sequencing gel of the Oss1 surrounding area from *Oss1*^{s} strain. The sequence has been derived from the M 13 recombinant clone 82737. DNA sequence is that of the nontranscribing strand. The G marked with an arrow undergoes change in the *Oss1*^{R}-92 allele (see below).

(b) The autoradiogram shows a part of the DNA sequencing gel of the M 13 recombinent clone 84077. The DNA sequence shown is the surrounding area of the Oss1 locus and that of the nontranscribing strand. The A marked with an arrow is the G in the wild type (D273-10B/A1).

regions from this strain, however, has not been determined yet completely. It is, therefore possible that the mutation lies somewhere in the intergenic regulatory region of the genes between the Oxi3 and subunit-8 genes or between the subunit-8 and the subunit-6 genes. Absence of these subunits in the O.S. ATPase from this mutant strain (CD24) is very difficult to reconcile at the present.

Prelude sequence of subunit-8 gene

The DNA sequences observed in between the Oxi-3 gene and Aap-1 or subunit-8 gene are typical of other intergenic regions of yeast mt-DNA. This is a highly A+T rich region, as expected, having only 63 (G+C) bases in a string of 956 bp DNA segment (i.e., 93.5% A+T bases). A putative transcriptional initiation site (ATAaAtGTA)†, slightly modified from that of proposed sequence (ATATAAGTA) (Tabak *et al*,1983) is observed 199 bp upstream of the subunit-8 gene.

This is very interesting in the context of proposed co-transcription of the Oxi-3, Aap-1 and the Oli-2 genes (Osinga *et al*, 1984; Christianson & Rabinowitz, 1983; Tabak *et al*, 1983). It is possible that during *in vivo* transcription, the synthesis of mRNA starts either as a co-transcript of Oxi-3, Aap-1 and the Oli-2 genes or only of Aap-1 and Oli-2 genes from this tentative promotor site. No putative ribosome binding site (Tzagoloff, 1982) has been found in the vicinity of the translational initiation codon (AUG). Although a similar sequence, complementary to the 3' end of the mitochondrial 15S rRNA occurs about 309 bp upstream (Fig. 5.15b), it seems unlikely to be a candidate because of its distance from the initiating AUG codon. No remarkable dyad symmetry or structures forming stems and loops are observed in this region.

Prelude sequences of the subunit-6 gene

The prelude sequences of the subunit-6 gene (i.e. the sequences between Aap-1 and Oli-2 genes) are, as expected, highly (A+T) rich as well, with the exception of two (G+C) rich

† a and t have been shown in place of T and A to indicate the differences.

Figure 5.15. Complementarity of the putative mRNA binding sequences in the Yeast 15S rRNA and the sequences upstream of the initiation codon in the subunit-6 and subunit-8 gene. Nucleotide denoted below as "t" is a "T", which is not complementary at the corresponding site.

```
(a)      3'-A C A T T C T T A T A A A T T C T A T A A A T A T T C G -5'

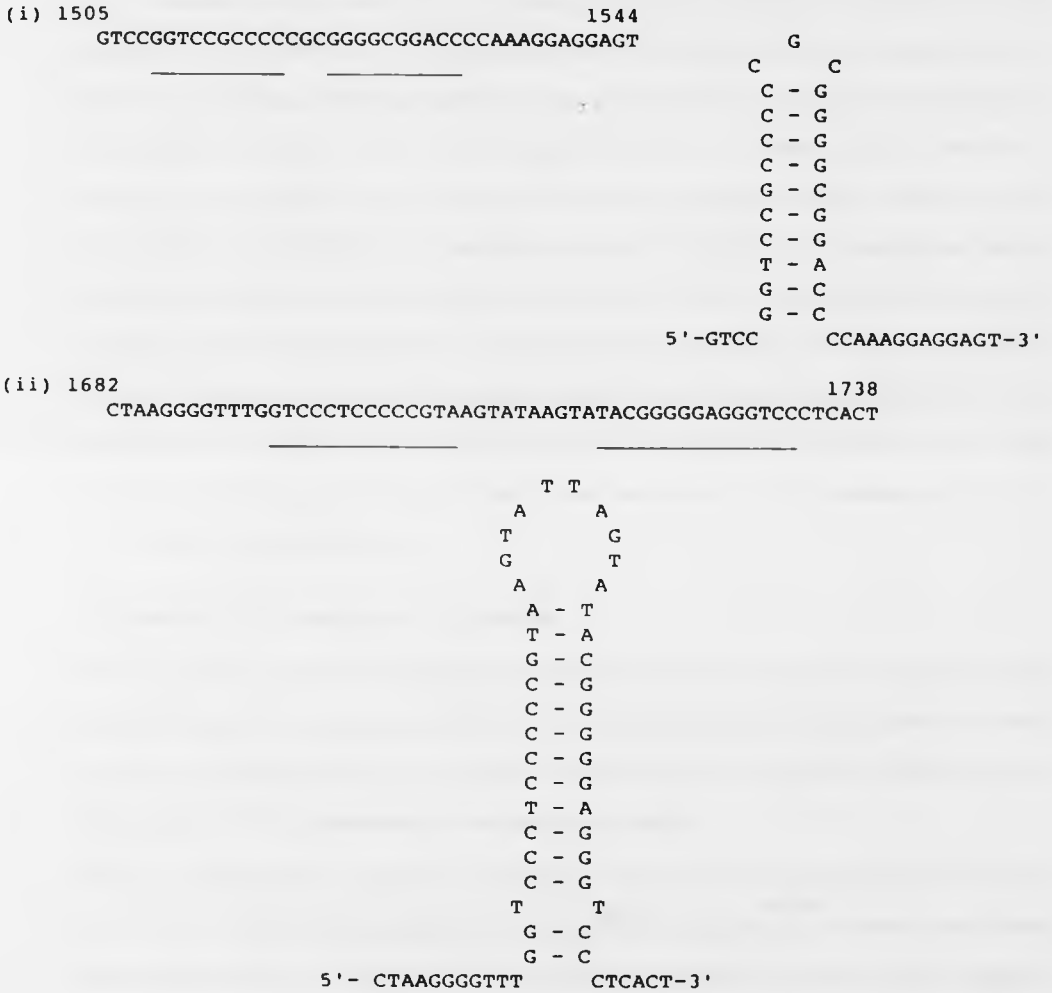
             3'-A T T C T T A T A-5'
(b)          5'-T A A G A A T A T- 309 bp upstream of the gene for subunit-8

                                3'-A T T C T A T A-5'
                                5'-T A A G A T A T ---50 bp upstream of the
                                                      gene for subunit-6

(c)                                       3'-A T A A A T A T T C-5'
                                          5'-T A T T T A T A A G - 27 bp up-
                                                                  stream of
                                                                  the gene
                                                                     for
                                                                  subunit-6

(d)                          3'-A T A A A T T C T A T-5'
                             5'-T A T T T A t G A T A- 11 bp upstream of the 2nd
                                                      ATG codon of the gene for
                                                      subunit-6
```

Figure 5.16. (G+C) rich clusters found in the sequence upstream of the gene for subunit-6 and their potential secondary structures. Sequences shown are from the nontranscribing strand of the DNA



clusters. In a stretch of 709 bp sequence, (G+C) constitutes only 86 residues or about 12.1% of the total. If two (G+C) rich cluster of about 30 bp each are deducted from the total, the (G+C) constitutes only 3.6%. The Two (G+C) rich clusters can potentially form characteristic stem and loop structures (Fig. 5.16) which may participate in the maturation of the mRNA by providing some sort of signal for RNA processing enzymes. Interestingly enough, a seven nucleotide sequence (ATTCTTA), which has been shown by Thalenfeld *et al* (1983) to be a possible candidate for the processing signal of the Oli-1 transcript, occurs about 6 bp upstream of the first hair-pin structure formed by the (G+C) rich cluster. Possibly both hair-pin structures formed by (G+C) clusters and other signal sequences (like that of the heptanucleotide ATTCTTA), are responsible for the processing of mRNA in mitochondria. Analogous to Shine and Dalgarno sequences (Shine & Dalgarno, 1975) or the putative ribosome binding sites, sequences complementary to the mitochondrial 15S rRNA occur twice upstream of the subunit-6 gene (Fig. 5.15c). The first one occurs 50 bp upstream and the second one occurs 27 bp upstream of the first ATG codon of the subunit-6 gene.

Localisation of the other *mit* mutations

Two other *mit* mutations (pho8 & mit-175) which have been localised genetically, have not been localised precisely as yet at the DNA sequence level. Because pho8 has been localised genetically upstream of the pho9, it is possibly due to mutation within the gene for subunit-8 or further upstream in the regulatory region.

Genetic localisation of the mit-175, downstream of the Oli-2 is very interesting. From the sequence data obtained from CD40, a strain of origin (D273-10B/A1), it has been found that sequence downstream of Oli-2 contains several open reading frames with a coding capability of 4.2 X 10^3 dalton to about 16 X 10^3 dalton. One reading frame which is a major candidate as a possible gene is 142 aminoacids long and can potentially give a 16.2 kilo dalton protein (Fig. 5.17). It is a highly charged protein (28% charged amino acids) with 26 basic and 14 acidic aminoacid residues. However, in this reading frame the initiation

Figure 5.17 Nucleotide and deduced aminoacid sequences of one of the URFs found downstream of the Oli2 gene.
The nucleotide sequence runs from the HpaII site of the 586 bp HpaII fragment of the ECO-7 band. The sequence beyond the EcoR1 site has been taken from the data of Macino & Tzagoloff (1980) for strain DS-14 to show the tentative termination site of transcription (See Osinga et al, 1984). The sequence shown is that of the non-transcribed strand and the aminoacids are in single letter code.

```
3851
CCGGGAACCC CACAAGGAGA TATTAAAAAT AAATTATTGA TTAGCTGGTT TTACAGCTGC AGATGGTTC
HpaII                                                          PstI

          3928   M   Y   N   P   K   D   T   L   L   F   K   D   M   R   P
T TTTTTATCATCT  ATA TAT AAT CCT AAA GAT ACA TTA TTA TTT AAA GAT ATA AGA CCT

 S   Y   V   I   S   Q   V   E   T   R   K   E   L   I   Y   L   I   Q   E
AGT TAT GTT ATT TCA CAA GTT GAA ACA CGT AAA GAA TTA ATC TAT TTA ATT CAA GAA

 S   F   D   L   S   I   S   N   V   K   K   V   G   N   R   K   L   K   D
TCT TTT GAT TTA TCT ATT TCT AAT GTT AAA AAA GTT GGT AAT AGA AAA TTA AAA GAT

 F   K   L   F   T   R   T   T   D   E   L   M   K   F   I   Y   Y   F   D
TTT AAA TTA TTT ACC AGA ACT CTT GAT GAA TTA ATA AAA TTT ATT TAT TAT TTT GAT

 K   F   L   P   L   H   D   N   K   Q   F   N   Y   I   K   F   R   F   N
AAA TTT TTA CCT TTA CAT GAT AAT AAA CAA TTT AAT TAT ATT AAA TTT AGA TTT AAT

 L   F   I   K   S   Y   N   W   N   N   R   V   F   G   L   V   L   S   E
CTA TTT ATT AAA TCA TAT AAT TGA AAT AAT AGA GTC TTT GGT TTA GTA TTA TCT GAA

 Y   I   N   N   I   K   I   D   N   Y   D   Y   Y   Y   Y   N   K   Y   I
TAT ATC AAT AAT ATT AAA ATT GAT AAT TAT GAT TAT TAT TAT TAT AAT AAA TAT ATT

 N   M   H   N   A   R   K   P   K   G   Y   I   K  Ochre 4361
AAT ATA CAT AAT GCA CGT AAA CCT AAA GGA TAC ATT AAA TAA   TTAATTATCC TTACTA
                                                                  4435
TTAT AATTATTCTA TATATTATAT ATAAAAATAA ATATATAAAA TTTTATAATA CAAAAA GAATTCTC
                                                                  EcoR1
                                                                 4501
ATATTCTTTT TTTTTAATA ATATTCTTTC ATAAATATAT CTATTTATTT ATATTATTAT
          Transcript. Termination (?)
```

codon is an ATA, rather than ATG. The codon usage by this protein (Table 5.3) is similar to other mitochondrial genes coding for the O.S.ATPase.

To establish whether or not this is another gene, the labelled mitochondrially translated products in the presence of cycloheximide (the drug which prevents protein translation of cytoribosomes) were analysed by SDS gel electrophoresis (Lamelli, 1970, Weber & Osborn, 1971). No extra protein band corresponding to 16-17 kilodalton or in this scale (data not shown) are observed.

Transcriptional analysis of the Oli-2 region

To study the nature of transcripts from the Oli-2 region, the total mt-RNAs from a grande strain (D273-10B/A1) was analysed using the Oli-2 specific petite (DS-14) mt-DNA as probe. Six major species of RNAs (9000, 5100, 4800, 3000, 800 and 600 nucleotides) hybridize to the DS-14 mt-DNA (Fig. 5.18). Of these the 5100, 4800 and 3000 nucleotide species are the most prominent. The 5100 and 4800 nucleotide species have been described by Cobon *et al* (1982) to represent the major transcripts of the Oli-2 region. However, in the present study the 3000 nucleotide species is the major one which is probably the matured mRNA for both the subunit-6 and subunit-8 genes. The 9000 nucleotide species possibly represents the cotranscript for both Oxi-3 and Oli-2 region. It has been shown by several workers (Hensgens *et al*, 1983; Osinga *et al*, 1984) that the Oxi-3 and Oli-2 regions are cotranscribed as a longer transcript which is processed into 2100 nucleotides long (18S) Oxi-3 gene specific mRNA and other transcripts for the Oli-2 region. Two minor bands of RNA (800 and 600 nucleotides long) which are not as prominent as the others also show week hybridization signal for the Oli-2 region. Cobon *et al* (1982) showed that these two transcripts are transcribed from downstream of the Oli-2 region which are not found in certain wild type (JM6) strain. The authors concluded that they are the optional transcripts of this region and are without significance. In view of the localisation of the *mit* mutation, mit-175, to this region of the mt-genome in the present study, they are likely to be functionally significant. This also indicates that the mit-175

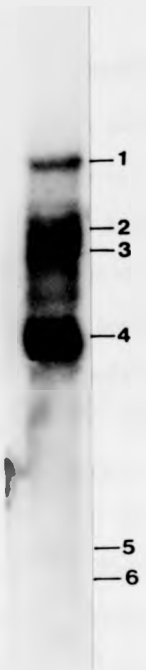


Figure 5.18 Transcripts of the Oli-2 region

The total mt-RNAs isolated from the grande ρ' strain D273-10B/A1 was transferred from a agarose gel onto a sheet of nitrocellulose filter according to the method of Thomas (1980) and then probed with nick translated ^{32}P labelled DS-14 mt-DNA. Six major bands have been marked on the autoradiogram. The molecular size of the RNAs are: (1) 9000, (2) 5100, (3) 4800, (4) 3000, (5) 800 and (6) 600 nucleotides.

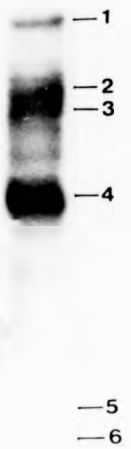


Figure 5.18 Transcripts of the Oli-2 region

The total mt-RNAs isolated from the grande ρ⁻ strain D273-10B/A1 was transferred from a agarose gel onto a sheet of nitrocellulose filter according to the method of Thomas (1980) and then probed with nick translated ^{32}P labelled DS-14 mt-DNA. Six major bands have been marked on the autoradiogram. The molecular size of the RNAs are: (1) 9000, (2) 5100, (3) 4800, (4) 3000, (5) 800 and (6) 600 nucleotides.

locus is expressed at the transcriptional level. As the 5' and 3' ends of these RNAs have not been mapped on the mt-DNA, it is difficult to relate the transcripts with the tentative reading frame suggested for the mit-175 locus.

DISCUSSION

I. Oligomycin and Ossamycin Resistivity of the O.S. ATPase

Oligomycin and Ossamycin resistant yeast strains (Oli2^R-76 and Oss1^R-92) used in this study have been shown to have both *in vivo* and *in vitro* resistivity towards oligomycin and ossamycin, respectively (Griffiths & Houghton, 1974). DNA sequence analyses in this study reveals that the former is due to the alteration of a highly conserved isoleucine residue by a phenylalanine at the 171st position of the subunit-6 gene. Macino & Tzagoloff (1980) have also demonstrated a similar change in an independently isolated resistant strain (Oli2^R-118) where the same isoleucine has been substituted by methionine. Novitski *et al* (1984) have also obtained a similar result in Oli2^R-23 strain where a conserved serine residue at the 175th position has been converted into a threonine. This region of the gene (Oli-2 block) is highly conserved (Fig. 5.19a) throughout evolution and is possibly involved in the energy conservation system of the O.S. ATPase.

The other mutation (*Oli*4^R) in the subunit-6 genes offering oligomycin resistivity has been demonstrated by Macino and Tzagoloff (1980) and this was due to the alteration of the 232nd residue, where a conserved leucine was changed to phenylalanine. This region (Oli4 block) of the subunit-6 also shows a substantial homology among diverse organisms (Fig. 5.19b). In an oligomycin resistant mouse cell line, Slott *et al* (1983) have also observed a change in the same region of the corresponding subunit where a conserved valine has been replaced by a glutamic acid (Fig. 5.19b). All these results point up the fact that subunit-6, along with the proteolipid subunit-9 which also can mutate independently to give rise to oligomycin resistant strains (Sebald *et al*, 1979), are involved in the O.S. ATPase-oligomycin interaction.

Both Oli2 and Oli4 loci associated changes have one characteristic in common. They centre around a charged residue, glutamic acid within a hydrophobic segment. Because the drug resistant strains have comparable ATP synthetase activity to that of wild type (Griffiths & Houghton, 1974), it is likely that the aminoacid changes observed here, associated with drug resistivity, are themselves not directly involved in proton translocation in the F_o sector of the O.S. ATPase. Probably, the conserved aminoacid residues are involved in the binding of oligomycin by hydrophobic interaction, which alters the proton channel, formed by relatively hydrophilic and charged residues in the F_0 sector. This inhibits proton pumping through the O.S.ATPase and consequently blocks oxidative phosphorylation. All these possibilities, in conjunction with the secondary and tertiary structures of this protein, based on statistical predictive analyses, will be discussed in the next chapter.

Ossamycin resistivity is due to a change of a conserved charged residue (Fig. 5.19c) in the carboxyl terminal end of the subunit-6 gene. The 254th residue, aspartic acid changes to asparagine due to change in a single nucleotide (G → A). As will be evidenced in the next chapter this region, based on predictive analyses, lies outside the membrane. Probably ossamycin, due to its comparatively larger structure, cannot enter into the membrane and interacts on the surface protruding segment of the subunit-6 and after binding, either alters the conformation of the segment, or masks the proton-channel at its entrance and thereby blocks oxidative phosphorylation.

II. *Mit* mutations

Transcriptional studies revealed that the nature of transcripts, in wild types and *mit* mutants of the Oli2 locus were very similar, though they produced aberrant translational products (Beilharz *et al*, 1982). Therefore, it was predicted that *mit* mutations are probably due to the occurrence of the termination codon within the functional reading frame, which can produce proteins of aberrant molecular weight (Roberts *et al*, 1979; Beilharz *et al*, 1982). Analysis of the *mit* mutation, pho9, indicates that this does not belong to this

Figure 5.19. Comparison of the drug resistant sites of the subunit-6 from various organisms. * indicates the altered aminoacid in drug resistant strain. Aminoacids are shown as single letter code (See Appendix)

		*	*	*	*
S. cerevisiae	P L L V L I E F I S Y L			L E F A I G I I Q S Y V W	
A. nidulans	P L L V I I E F I S Y L			L E L A I A F I Q A Q V F	
Bovine	P M L V I I E T I S L F			L E F A V A M I Q A Y V F	
Human	P M L V I I E T I S L L			L E I A V A L I Q A Y V F	
Mouse	P M L V I I E T I S L F			L E F A V A L I Q A V V F	
Rat	P M L I I I E T I S L F			L E F V V A L I Q A Y V F	
D. melanogaster	P F M V C I E T I S N I			L E S A V A M I Q S Y V F	
D. yakuba	P F M V C I E T I R N I			L E S A V T M I Q S Y V F	

(a) OLI2 Mutational site (b) OLI4 Mutational site

	*
S. cerevisiae	Y L - - - K D T L Y L H
A. nidulans	Y I - - - K D G L D L H
Bovine	Y L - - - H D N T
Human	Y L - - - H D N T
Mouse	Y L - - - H D N T
Rat	Y L - - - H D N T
D. melanogaster	Y S - - - S E V N
D. yakuba	Y S - - - S E V N
E. coli	Y L S M A S E - - E H

(c) Ossl Mutational site

category. Most likely it is due the mutation within the regulatory regions of the genes for subunit-6 and subunit-8.

Biochemically the pho9 mutation shows normal cytochrome spectra (Darlison, 1979) but with little or no mitochondrial membrane potential and little or no ATPase activity (unpublished result, this laboratory). From the DNA sequence analysis, it appears that the mutation is not due to any alteration within the reading frames for subunit-6 and subunit-8. Preliminary studies on biogenesis of the F_o from this mutant strain have shown that the anti-F_1 antibody fails to immunoprecipitate the subunit-6, subunit-8 and subunit-9 (Connerton, I.F., unpublished results). This indicates that probably the mutation is a regulatory one which controls the expression of the subunit-6 and subunit-8. This is very interesting for the fact that no controlling elements for mitochondrial gene expression, with the exceptions of the tRNA biosynthetic locus (Underbrink-Lyon *et al*, 1983) and the so called maturases (Grivell, 1983) are known to be located on yeast mitochondrial genome. Therefore future studies involving DNA sequence analysis and transcriptional analysis of this region of the mt-genome of the mutant strain (CD24) would be enlightening.

The absence of the subunit-9 in the O.S.ATPase from the pho9 mutant (CD24) is surprising. It is possible that the subunit-6 and subunit-8 are required for the assembly of the subunit-9. Marzuki *et al* (1983), however, have shown that the subunit-8 is needed for the assembly of subunit-6 but not for subunit-9 in the membrane. It has been noted that at certain concentrations of Triton-X, subunit-9 does not precipitate during immunoprecipitation (Dr. Velour, personal communication). Therefore, the possibility remains that under our experimental conditions subunit-9 is not precipitated and remains undetected.

III. Mit-175: another gene?

Localisation of mit-175 at downstream to the coding frame of subunit-6 gene has been confirmed on the following basis:

1. Petite deletion mapping shows (Chapter 2) that it is located downstream of the $Oss1^R$ marker which is located on the 254th aminoacid residue of the subunit-6 gene.

2. Because of the location of ossamycin resistivity in the 6th aminoacid from the C-terminal end of the subunit-6 structural gene, we sequenced the carboxyl terminal end of Oli-2 gene as well as further 200 bp downstream from mit-175, and we do not find any change in this region when compared to the wild type sequences. Therefore, we presume that it is located further downstream of the same region.

3. CDS-14, a cytoductant of DS-14 (Macino & Tzagoloff, 1980), which retains the major part of the Eco-6 and the whole of the Eco-7 (two adjacent *EcoR*1 restriction fragments containing the structual gene for subunit-6 and further downstream) can restore growth on glycerol upon crossing to the mit-175 strain, i.e., the mit-175 mutation is complemented by its normal counterpart on the Eco-7 fragment of mt-DNA (segment downstream of Oli-2) from CDS-14 (See chapter 2).

On the basis of the above results it can be argued that a functional gene is retained downstream of the subunit-6 gene which, when mutated loses the growth property on nonfermentable glycerol medium. The presence of a transcript from this region (Cobon *et al*, 1982), further strengthens this view. Two points however, do not support the view:

Firstly, biogenesis studies using [^{35}]S-methionine do not show any novel protein band which could be correlated with the open reading frame we have suggested. Secondly, in a wild type mitochondrial strain JM6, it has been observed that this region of mt-DNA is missing (Cobon *et al*, 1982). The first point can be counter-argued from the point of view that the protein synthesized from this locus, is a regulatory protein either present in trace amount which remains undetected in our gel system, or remains masked by other known protein of mitochondrial origin. The possibility of direct participation of RNA in catalytic activity also can not be ruled out, as the association of RNA with catalytic activity of certain proteins has been proved (e.g. RNase P, Guerrier-Takada *et al*, 1983; Alanine synthesizing enzyme, Huang *et al*, 1984). The second point can also be counter-argued by

the transpositional activity of the mt-DNA segment within the genome or between itself and the nuclear genome. It is possible that this part of the genome in JM6 has been translocated elsewhere in the mt-genome or to the nucleus.

IV. Identification problem of subunit-6 and the subunit-6 reading frame

Assignment of the Oli2 locus to the subunit-6 gene has always been a controversial topic due to the anomalous mobility of the subunit-6 polypeptide in SDS gel electrophoresis. Studies of cycloheximide resistant protein synthesis in wild type yeast have shown subunit-6 to have a molecular weight of 22.5 kilodalton (Tzagoloff & Meagher, 1971; Roberts *et al*, 1979). However, Somlo *et al* (1982) have shown that, the 22.5 kilodalton protein which is very prominent in Coomassie-blue stained gels of the O.S.ATPase complex is not subunit-6, and that a cycloheximide resistant 20.0 kilodalton protein which does not form a prominent band in Coomassie blue stained gels, is the real candidate.

Added to this fact, is the DNA sequence data of the Oli-2 reading frame which has a coding capability of 28.5 kilodalton protein. Lack of aminoacid sequence data for this particular protein has highlighted this controversy.

However, when the reading frame of subunit-6 gene is examined carefully, it is found that it has a second ATG codon, 81 aminoacid downstream of the first ATG codon. If this second ATG is taken as the initiation codon, the rest of the reading frame can potentially form a 19 kilodalton protein (Fig. 5.20). When the aminoacid sequences of this 2nd frame is compared with that of the experimentally obtained aminoacid composition data from *N. crassa* subunit-6 (Sebald, 1977), it is found that the values are similar (Table 5.4). Therefore it is possible that *in vivo*, the second ATG might act as an initiation codon for this protein. This is partly supported by the fact that C-terminal two-third of subunit-6 from various organisms are more conserved than the NH_2-terminal one third. To prove this alternative translational initiation we will have to wait for NH_2 terminal sequence data from purified subunit-6 or from cyanogen bromide cleavage data of the same.

Figure 5.20. Second reading frame of the gene for Subunit-6. Underline region is complementary to 3' end of the mitochondrial 15S rRNA. One letter and three letters aminoacid codon has been used to discriminate the two reading frames.

```
MetPheAsnLeuLeuAsnThrTyrIleThrSerProLeuAspGlnPheGluIleArgThrLeuPheGlyLeuGln
ATGTTTAATTTATTAAATACATATATTACATCACCATTAGATCAATTTGAGATTAGACTATTATTTGGTTTACAA

SerSerPheIleAspLeuSerCysLeuAsnLeuThrThrPheSerLeuTyrThrIleIleValLeuLeuValIle
TCATCATTTATTGATTTAAGTTGTTTAAATTTAACAACATTTTCATTATATACTATTATTGTATTATTAGTTATT

ThrSerLeuTyrThrLeuThrAsnAsnAsnAsnLysIleIleGlySerArgTrpLeuIleSerGlnGluAlaIle
ACAAGTTTATATCTATTAACTAATAATAATAATAAAATTATTGGTTCAAGATGATTAATTTCACAAGAAGCTATT
                                                                  |___
TyrAspThrIleMetAsn   M   T   K   G   Q   I   G   G   K   N   W   G   L   Y
TATGATACTATTATAAAT  ATG CTT AAA GGA CAA ATT GGA GGT AAA AAT TGA GGT TTA TAT
_____|
 F   P   M   I   F   T   L   F   M   F   I   F   I   A   N   L   I   S   M
TTC CCT ATG ATC TTT ACA TTA TTT ATG TTT ATT TTT ATT GCT AAT TTA ATT AGT ATG

 I   P   Y   S   F   A   L   S   A   H   L   V   F   I   I   S   L   S   I
ATT CCA TAT TCA TTT GCA TTA TCA GCT CAT TTA GTA TTT ATT ATC TCT TTA AGT ATT

 V   I   W   L   G   N   T   I   L   G   L   Y   K   H   G   W   V   F   F
GTT ATT TGA TTA GGT AAT ACT ATT TTA GGT TTA TAT AAA CAT GGT TGA GTA TTC TTC

 S   L   F   V   P   A   G   T   P   L   P   L   V   P   L   L   V   I   I
TCA TTA TTC GTA CCT GCT GGT ACA CCA TTA CCA TTA GTA CCT TTA TTA GTT ATT ATT

 E   T   L   S   Y   I   A   R   A   I   S   L   G   L   R   L   G   S   N
GAA ACT TTA TCT TAT ATT GCT AGA GCT ATT TCA TTA GGT TTA AGA TTA GGT TCT AAT

 I   L   A   G   H   L   L   M   V   I   L   A   G   L   T   F   N   F   M
ATC TTA GCT GGT CAT TTA TTA ATG GTT ATT TTA GCT GGT TTA CTA TTT AAT TTT ATG

 L   I   N   L   F   T   L   V   F   G   F   V   P   L   A   M   I   L   A
TTA ATT AAT TTA TTT ACT TTA GTA TTC GGT TTT GTA CCT TTA GCT ATG ATT TTA GCT

 I   M   I   L   E   F   A   I   G   I   I   Q   S   Y   V   W   T   I   L
ATT ATG ATT TTA GAA TTC GCT ATT GGT ATT ATC CAA TCT TAT GTT TGA CTT ATC TTA

 T   A   S   Y   L   K   D   T   L   Y   L   H  Ochre
ACA GCA TCA TAC TTA AAA GAT ACA TTA TAC TTA CAT TAA
```

Figure 5.20. Second reading frame of the gene for Subunit-6. Underline region is complementary to 3' end of the mitochondrial 15S rRNA. One letter and three letters aminoacid codon has been used to discriminate the two reading frames.

```
MetPheAsnLeuLeuAsnThrTyrIleThrSerProLeuAspGlnPheGluIleArgThrLeuPheGlyLeuGln
ATGTTTAATTTATTAAATACATATATTACATCACCATTAGATCAATTTGAGATTAGACTATTATTTGGTTTACAA

SerSerPheIleAspLeuSerCysLeuAsnLeuThrThrPheSerLeuTyrThrIleIleValLeuLeuValIle
TCATCATTTATTGATTTAAGTTGTTTAAATTTAACAACATTTTCATTATATACTATTATTGTATTATTAGTTATT

ThrSerLeuTyrThrLeuThrAsnAsnAsnAsnLysIleIleGlySerArgTrpLeuIleSerGlnGluAlaIle
ACAAGTTTATATCTATTAACTAATAATAATAATAAAATTATTGGTTCAAGATGATTAATTTCACAAGAAGCTATT
                                                                        |___

TyrAspThrIleMetAsn   M   T   K   G   Q   I   G   G   K   N   W   G   L   Y
TATGATACTATTATAAAT  ATG CTT AAA GGA CAA ATT GGA GGT AAA AAT TGA GGT TTA TAT
_______|

 F   P   M   I   F   T   L   F   M   F   I   F   I   A   N   L   I   S   M
TTC CCT ATG ATC TTT ACA TTA TTT ATG TTT ATT TTT ATT GCT AAT TTA ATT AGT ATG

 I   P   Y   S   F   A   L   S   A   H   L   V   F   I   I   S   L   S   I
ATT CCA TAT TCA TTT GCA TTA TCA GCT CAT TTA GTA TTT ATT ATC TCT TTA AGT ATT

 V   I   W   L   G   N   T   I   L   G   L   Y   K   H   G   W   V   F   F
GTT ATT TGA TTA GGT AAT ACT ATT TTA GGT TTA TAT AAA CAT GGT TGA GTA TTC TTC

 S   L   F   V   P   A   G   T   P   L   P   L   V   P   L   L   V   I   I
TCA TTA TTC GTA CCT GCT GGT ACA CCA TTA CCA TTA GTA CCT TTA TTA GTT ATT ATT

 E   T   L   S   Y   I   A   R   A   I   S   L   G   L   R   L   G   S   N
GAA ACT TTA TCT TAT ATT GCT AGA GCT ATT TCA TTA GGT TTA AGA TTA GGT TCT AAT

 I   L   A   G   H   L   L   M   V   I   L   A   G   L   T   F   N   F   M
ATC TTA GCT GGT CAT TTA TTA ATG GTT ATT TTA GCT GGT TTA CTA TTT AAT TTT ATG

 L   I   N   L   F   T   L   V   F   G   F   V   P   L   A   M   I   L   A
TTA ATT AAT TTA TTT ACT TTA GTA TTC GGT TTT GTA CCT TTA GCT ATG ATT TTA GCT

 I   M   I   L   E   F   A   I   G   I   I   Q   S   Y   V   W   T   I   L
ATT ATG ATT TTA GAA TTC GCT ATT GGT ATT ATC CAA TCT TAT GTT TGA CTT ATC TTA

 T   A   S   Y   L   K   D   T   L   Y   L   H  Ochre
ACA GCA TCA TAC TTA AAA GAT ACA TTA TAC TTA CAT TAA
```

TABLE 5.4 Comparison of aminoacid composition of the two reading frames of the Yeast Oli-2 gene with that of Subunit-6 of *N. crassa* ATPase

Amino acid	Yeast		*N. crassa*	
	1st Reading frame (M%)	2nd Reading frame (M%)	protein† analysis (M%)	DNA sequence‡ analysis (M%)
Ala	5.023	6.3	7.85	6.35
Arg	1.55	1.06	2.48	1.98
Asx	6.95	3.7	7.1	7.54
Cys	0.38	0.0	ND	0.39
Gly	6.18	7.4	8.0	6.75
Glx	3.48	2.11	6.05	4.76
His	1.55	2.11	2.55	2.38
Ile	14.36	13.23	11.9	13.88
Leu	18.15	16.9	16.6	16.67
Lys	1.93	2.1	1.41	0.79
Met	3.47	4.75	1.08	1.98
Phe	8.11	9.0	9.63	10.32
Pro	3.10	3.7	4.01	3.17
Ser	7.34	5.8	9.06	8.73
Thr	7.72	5.29	4.75	5.16
Trp	1.93	2.1	ND	0.39
Tyr	4.25	3.7	3.05	3.97
Val	4.63	5.2	4.56	4.76

† Data from Sebald (1977); ‡ Data deduced from Morelli & Macino (1984); M% = Molar percentage; ND = not determined.

While this chapter was written, Morelli & Macino (1984) published the gene structure and sequence of subunit-6 from *N. crassa*. In this fungus, the subunit-6 gene has two introns within the reading frame: the first one is 93 bp long and situated just after the first two aminoacids and the second one is 1370 bp long and contains an open reading frame continuous with the upstream reading frame. After excision of the intron, the coding frame for this gene has been shown to give rise to similar aminoacid sequences to that of *A. nidulans* and *S. cerevisiae*. It demonstrates that the longer reading frame is expressed at the transcriptional level but does not rule out the post-translational modification of the protein.

Comparison of subunit-6 from various organisms

The subunit-6 reading frame was studied from various organisms after their alignment to establish maximum homology, as shown in Fig. 5.21. It is quite obvious from such comparison that the primary sequence of this protein is highly conserved, at least in its C-terminal part. Interestingly, all the mutations associated with this gene have been ascribed to this region. As shown in the figure, Oli-2, Oli-4 and Oss-1 sites are highly conserved throughout evolution. This indicates their functional importance in oxidative phosphorylation. Homologous protein (subunit-a or uncB product) from *E.coli* (a prokaryote) also reveals similarity at the COOH end, with the exception of Oli-4 site which is mising in *E. coli* (Fig. 5.21; see also Fig. 6.12, Chapter-6). This could be the reason why *E.coli* ATPase is not inhibited by oligomycin. Enns & Criddle (1977) demonstrated, by affinity binding studies of oligomycin with yeast ATPase and its reducton by sodium [^{3}H]-borohydride that, major incorporation of radioactivity occurs at subunit-9. They claimed that proteolipid subunit-9 is itself enough to bind oligomycin. From the present result it appears that, subunit-6 is equally important in oligomycin binding. This is based on mainly two facts:

(i) a single aminoacid change in the subunit-6 gene can alter oligomycin binding affinity and consequently gives rise to a drug resistant mutant (Lancashire & Mattoon, 1979b).

Figure 5.21 Comparison of aminoacid sequences of the subunit-6 of the O.S.ATPase from various organisms. (1) E. coli, (2) Aspergillus nidulans (3) Saccharomyces cerevisiae, (4) Bovine, (5) Human (6) Mouse, (7) Drosophila melanogaster, (8) Drosophila yakuba, The aminoacid sequences have been arranged to establish maximum homology.
A "-" indicates nothing but a gap within the sequences to establish homology.

```
1.MASENMT----PQDYIGHHLNNLQLDLRTFSLVDPQNPQNPPATFWTINIDSMFFSVVLGLLFLVLFRSVAKK
2.MYQ--FNFILSPLDQFEIRDLFSLNANVLGNIHLSITNIGLYLSIG-------------LLL-TLGYHL-AHN
3.MFNLLNTYIFSPLDQFEIRTLFGLQSSFIDLSCLNLTTFSLYIIV--------------LLVITSLYTLTNNN
4.MN--------------EN--LFT---SFITPVILGLPLVTL--IVLF-PS---------LLFPTSNR-LV--S
5.MN--------------EN--LFT---SFIAPTILGLPAAVL--ILLF-PP---------LLIPTSKY-LI--N
6.MN--------------EN--LFA---SFITPTMMGFPIVVA--IIMF-PS---------ILFPSSKR-LI--N
7.MMTNLFSVF----DP-----LAIFNFSLNWLSTFLGLLMIPS-IYWLMPSRYNIMWNS-ILLTLHK-------
8.MMTNLFSVF----DP-----LAIFNLSLNWLSTFLGLLMIPS-IYWLMPSRYNIMWNS-ILLTLHK-------

ATSGVPGKFGTAIELVIGFVNGSVKDMYHGKSKLIAPLALTIFVWVFL-----------MMNLMDLLPYIAEHV
NKIPNN-WSISQEAIYATVHSIVINQLNPTKGQL-----------YFP-FIYALFIFILVNNLIGMVPYSFAST
NKIIGSRWLISQEAIYDTIMNM--------KGQIGG----KNWWGLYFP-MIFTLFMFIFIANLISMIPYSFALS
NRFVTLQWMLQLVS----------------K-QMMSIHNSKGQYWTL---MLMSLILFIGSTNLLGLLPHSFTPT
NRLITTQWLIKLTS----------------K-QMMTMHNTKGRTWSL---MLVSLIIFIATTNLLGLLPHSFTPT
NRLITTQWLIKLTS----------------K-QMMLIHTPKGRTWTL---MIVSLIMFIGSTNLLGLLPHSFTPT
------------EF----------------KTLLGPSGHHNGSTFIFISL--FSLILFNNFM---GLFPYIFTST
------------EF----------------KTLLGPSGHHNGSTFIFISL--FSLILFNNFM---GLFPYIFTRT

LGLPALRVVPSADVNVTLSMALFIGVLILFYSIKMKGIGFTKELTLGPFNHWAFIPVNLILEGVSLL----SKPV
SHFILTFSMSFTIVLGATF-LGLQRHGL-------K----FFSLFVPSGCPLGLLPLLVLIEFISYL----SRNV
AHL------VFIISLSIVIWLGNTILGLY------KHGWVFFSLFVPAGTPLPLVPLLVIIETLSYI----ARAI
TQL------SMNLGMAIPLWAGAVITGF------RNKTKASLAHFLPQGTPTPLIPMLVIIETISLFIQPMALA-
TQL------SMNLAMAIPLWAGTVIMGF------RSKIKNALAHFLPQGTPTPLIPMLVIIETISLLIQPMALA-
TQL------SMNLSMAIPLWAGAVITGF------RNKLKSSLAHFLPQGTPISLIPMLIIIETISLFIQPMALA-
SHLTLT------LSLALPLWLCFMLYGW------INHTQHMFAHLVPQGTPAILMPFMVCIETISNIIRPGTLA-
SHLTLT------LSLALPLWLCFMLYGW------INHTQHMFAHLVPQGTPAILMPFMVCIETIRNIIRPGTLA-

SLGLRLFGNMYAGELIFILIAGLLPWWSQWILNVPWAIFIFHILIITL----------QAFIFMVLTIVYLSMA
SLGLRLAANILSGHMLLSILSGFTYNIMTSGILFFFLGLIPLAFIIA-FSGLELAIAFIQAQVFVVLTCSYI---
SLGLRLGSNILAGHLLMVILAGLTFNFMLNLFTLVFGFFVPLAMILA-IMILEFAIGIIQSYVWTILTASYL---
---VRLTANITAGHLLI-HLIGGATLALMSISTTTALITFT---ILILLTILEFAVAMIQAYVFTLLVSLYL---
---VRLTANITAGHLLM-HLIGSATLAMSTINLPSTLIIFT---ILILLTILEIAVALIQAYVFTLLVSLYL---
---VRLTANITAGHLLM-HLIGGATLVLMNISPPTATITFT---ILLLLTILEFAVALIQAVVFTLLVSLYL---
---VRLTANMIAGHLLLTLL-GNTGSS-MSYM----LMTFLLMAQIALLV-LESAVAMIQSYVFAVLSTLYS---
---VRLTANMIAGHLLLTLL-GNTGPS-MSY-----LLVFLLVAQIALLV-LESAVTMIQSYVFAVLRTLYS---

SE---E-H
KD-GLDLH
KD-TLYLH
HDNT
HDNT
HDNT
SEVN
REVN
```

(ii) *E.coli* ATPase does not bind to oligomycin; it lacks the (Oli-4) site in its subunit-6 homologous protein, though it has a similar subunit-9 protein (Sebald & Hoppe, 1981).

The occurence of a conserved isoleucine at the 226th aminoacid residue position of subunit-6 is very interesting in the context of the strain D22. This unrelated wild type yeast strain of *S. cerevisiae* has undergone a nucleotide change T → C and thereby changing the codon from ATT to ATC (this has generated a new *Sau*3A within the reading frame, Fig. 5.11a & b; See also chapter-4). However the gene retains the same aminoacid coding capability as both ATT and ATC code for isoleucine. Because this isoleucine is highly conserved throughout the evolution, it is assumed that the isoleucine plays an important role in the function of this protein.

The NH_2 terminal one third of the subunit-6, though diverged among various organisms, is conserved within the same phylogenetic group (See Fig. 5.21). For example, mammalian group (bovine, human, rat and mouse), insect group (examplified by *D. melanogaster, D. yakuba*), fungal group (*S. cerevisiae, A. nidulans, S. pombe*) are similar among themselves.

The NH_2-terminal part of the *E. coli* subunit-a (homologous protein of subunit-6) contains more charged aminoacids and is radically different from its eukaryotic counterpart. It is possible that this part might have a homologous counterpart in a different unknown subunit of eukaryotic O.S. ATPase.

Comparison of subunit-8 from various organisms

Based on homology with the subunit-8 gene of *S. cerevisiae*, the unknown reading frames (URFs) found upstream of the subunit-6 gene of several organisms have been claimed to be the corresponding subunit-8 gene. Comparison of aminoacid sequences which have been deduced from DNA sequence data for this gene from various organisms, shows a conserved amino terminal end (Fig. 5.22). The Carboxyl terminal ends are similar within the same phylogenetic group but are different from distantly separated groups. However the frequent occurrence of basic aminoacid residues in the carboxyl terminal part appears

Figure 5.22 Comparison of Amino acid Sequences of Subunit-8 from Various Organisms. * indicates same aminoacid in more than two phylogenetic groups. A + indicates same aminoacid in three or more organisms. Names of organisms are: (1) Aspergillus amstelodemi, (2) Aspergillus nidulans, (3) Saccharomyces cerevisiae (4) Human, (5) Bovine, (6) Mouse, (7) Drosophila melanogaster (8) Drosophila yakuba. "-" has been introduced within the sequences to establish maximum homology. Homologous regions have been shown in colour. Pink, common to all or fungi only; yellow, mammals and insects or mammals only orange, insects only.

```
  ****                                  *       *             *
1.MPQLVPFFFVNQVV-YAFVNL-TVLIYAFTK---FIIPKLLRIFISRIVI----------NKL
2.MPQLVPFFFVNQVI-FAFIVL-TVLIYAFTK---YILPRLLRTYISRIYI----------NKL
3.MPQLVPFYFMNQLITYGFLLM-ITLLILFSQ---FLLPMILRLYVSRLFI----------SKL
4.MPQLNTTVWPTMITPMLLTL-FLITQLKMLNTNYHLPPSP-KPMKMKNYNK---PWEPKWTKICSLHSLPPQS
5.MPQLDTSTWLTMILSMFLTL-FLITQLKVSKHNFYHNPELTPTKMLKQNT----PWETKWTKIYLPLLLPL
6.MPQLDTSTWFITISSM-ITLFFLLTQLKVSSQTFPLAPSPKSLTTMKVKT----PWELKWTKIYLPHSLPQQ
7.IPQLAPISWLLLFIIFSTL--FLLTCSINYYSYMPNSPKSNEL---KNINLNSMNW--KW
8.IPQLAPIRWLLLFIVFSTL--FLLTCSINYYSYMPTSPKSNEL---KNINLNSMNW--KW
```

significant and could be functionally important.

A homologous counterpart of subunit-8 is not observed in prokaryotes. It is probable, therefore, that it has evolved in a later stage of evolution due to the complexity of the energy conservation system of eukaryotes.

Transcripts of the Oli-2 Region

The Oli-2 region in *S. cerevisiae* produces a co-transcript for subunit-6 and subunit-8 genes. Six RNA species, which hybridise to this region (Fig. 5.18) are observed in wild type. It is therefore probable that the common transcript is processed before being translated. It has been shown in Fig. 5.16 that the segment of DNA between subunit-6 and subunit-8 gene can potentially form two hair pin structurs. Upstream of the first hairpin structure, a so called transcript processing signal (ATTCTTA) (Thalenfeld *et al*, 1983) occurs. Downstream of the second hairpin, tentative ribosome binding sites are seen. It is therefore presumed that the initiation of translation of subunit-6 starts after being processed to a shorter transcript (probably the 3000 nucleotides species, which is predominant in Fig. 5.18). It can not however be ruled out that the translation of both subunit-6 and subunit-8 starts as a polycistronic message similar to that of prokaryotes. The tentative ribosome binding site is situated at such a long distance from the first ATG of subunit-8 gene that it is very difficult to speculate whether there is yet another mechanism (or other sequences) for ribosome binding during mitochondrial translation.

The occurrence of an 600-800 bp transcript from downstream of the Oli-2 region is very interesting. The sequence analyses from this region (Fig.5.23) shows that few 20 bp G+C rich clusters occur within the 1.7Kb *EcoR*1 fragment. The occurrence of similar sequences on two sides of the 250 bp and 447 bp *Hpa*II fragments is similar to the structure of the terminal repeats of transposable elements. If these sequences are transposable like elements, it is possible that they exert their regulatory effect on the neighbouring structural gene in the same way the transposable elements do after their integration into a new site. This might also explain why this particular region is missing in the homologous part of

Figure 5.23. DNA sequence of the 1.70 Kbp EcoR1 fragment.
The sequence starts from the EcoR1 site within the Oli2 gene. Dotted portion of the fragment has not been sequenced. The regions between the nucleotides 3680-3730 and 2920-3000 have been taken from the data of Macino & Tzagoloff (1980). The sequence shown is that of the nontranscribed strand.
*** indiates the termination codon, Ochre. Four URFs (URF I, URF II, the tentative URF for mit-175 and URF III) have been indicated. Note that URF I and URF II donot have the initiation codon (ATG) within the sequence shown. Terminal repeats have been coloured.

```
OLI 2 |

GluPheAlaIleGlyIleIleGlnSerTyrValTrpThrIleLeuThrAlaSerTyrLeuLysAspThrLeuTyr
GAATTCGCTATTGGTATTATCCAATCTTATGTTTGACTTATCTTAACAGCATCATACTTAAAAGATACATTATAC
 2570       2580       2590       2600       2610       2620       2630       2640
EcoR1

LeuHis***
TTACATTAAATTATAAAATAAAAATTATATAAAAAAAAAATATAATAATAATAATAATATAAAATAAAAAAATAAA
      2650       2660       2670       2680       2690       2700       2710

AAATAAAACAATGAAAAAACAAAATTTAAATTCTATTTTATTAATTGTATATTAATTATATTATTAATTATTTAA
 2720       2730       2740       2750       2760       2770       2780       2790

TAATATTCATAAAATCAATTAAAAAAGACTAGATTATAGATATGATATATATATAATTTTAATAAATAATATACTA
      2800       2810       2820       2830       2840       2850       2860

TGTTTTATTAAATGAGATAATAATAAAATTTTATTATTATTAGATATATATTATAATGTATTATATAACTATCAT
 2870       2880       2890       2900       2910       2920       2930       2940

AAACAACGTACACCTATATCTAATAAAAGATTAATAAATTCAAAAAATATTATAGTT----------
      2950       2960       2970       2980       2990       3000       3010
(88 + aminoacid) URFI
  GlyThrProGlnGlyAspTyrLysLeuLeuTyrThrTyrPheTyrIleLeuAsnLysMetLysMetGluMetAsp
CCGGAACCCCGCAAGGAGATTATAAATTATTATATCTTTATTTTTATATTTTAAATAAAATAAAAATAGAAATAG

 3020       3030       3040       3050       3060       3070       3080       3090
HpaII  Terminal repeat
  AsnTyrAsnAsnAsnAsnAsnIleSerLeuLysTyrAsnGluLeuLeuLysAsnIleMetAsnAsnLeuAsn
ATAATTATAATAATAATAATAATATTTCATTAAAATATAATGAATTATTAAAAAATATTATAAATAATTTAA
      3100       3110       3120       3130       3140       3150       3160

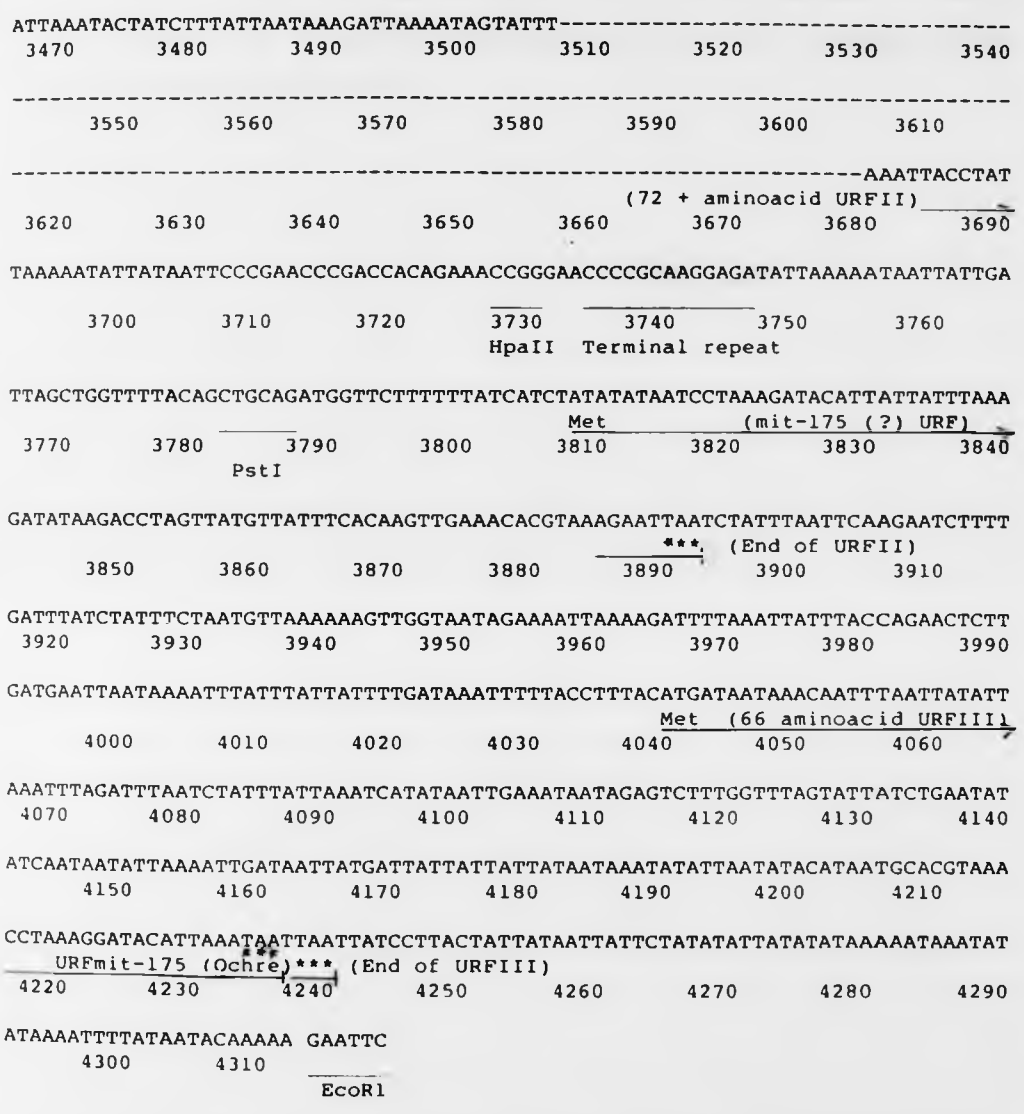
  TyrLysLeuSerAsnIleGluThrAsnLeuSerAsnAsnPheTyrLeuMetAspLysTyrLeuIleAsnLysTyr
ATTATAAACTATCTAATATTGAACTTAATTTATCTAATAATTTTTATTTAATAGATAAATATTTAATTAATAAAT
 3170       3180       3190       3200       3210       3220       3230       3240

  MetLysTyrLeuValProGlyProAlaArgArgMetTyr***
ATATAAAATATTTAGTCCCGGGACCCGCAAGGAGAATATATTAAATATAATTCCTAATAATTATATATTTAATAA

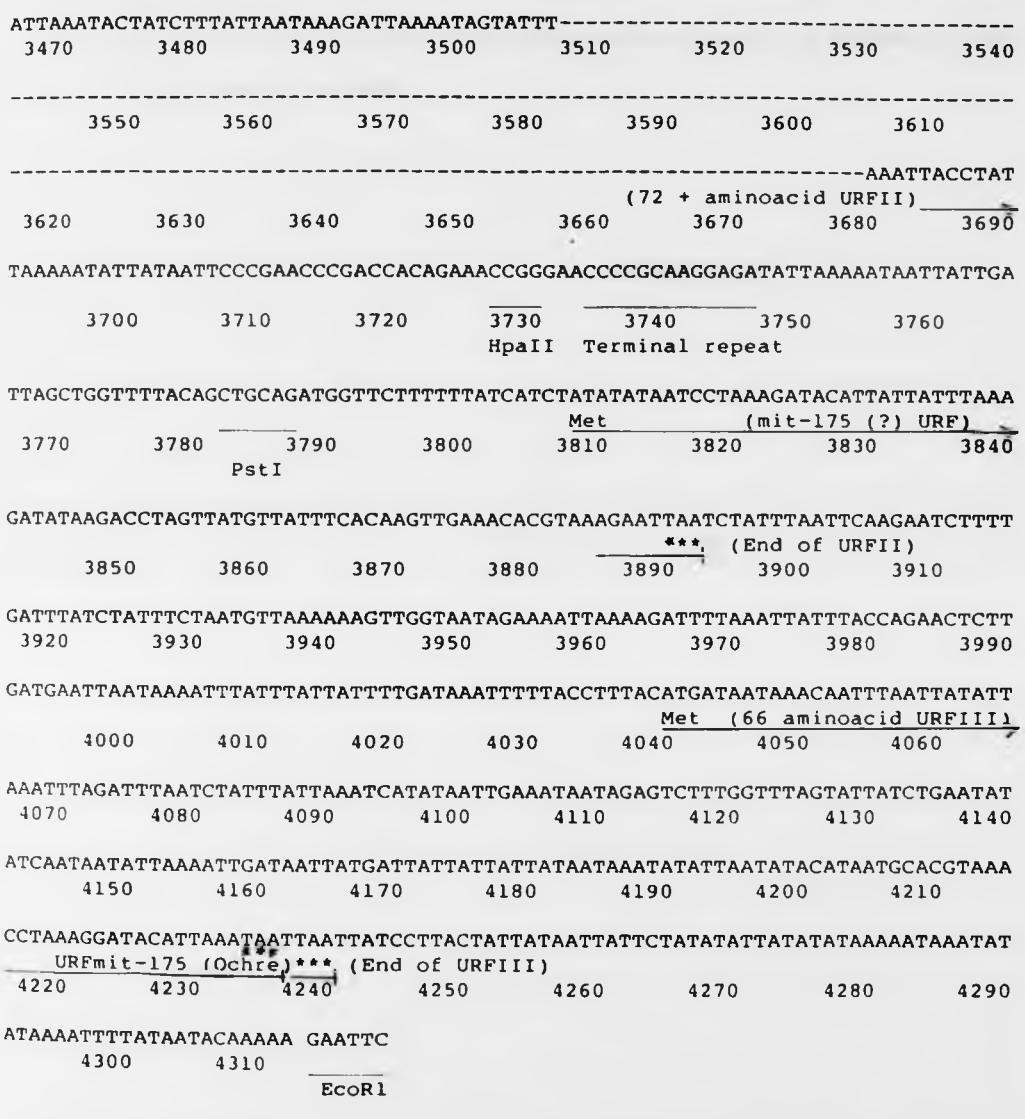
      3250       3260       3270       3280       3290       3300       3310
                  HpaII Terminal Repeat

TATTAATTATAAAGCGTAAATTAAATATTAAAACAGTATTAGATTTAAATAATAATGAATTTTATGATTATTTAT
 3320       3330       3340       3350       3360       3370       3380       3390

CAGGGCTTAATTGAAGCCTGATGGCTTATATTGGTCCTAAAGGCTATTACAATTCTAAATCATGCCTAATGATGT
      3400       3410       3420       3430       3440       3450       3460
```



Note that the numbers below the sequence are arbitory as the number of nucleotides in the nonsequenced regions of the DNA is not correctly known.



Note that the numbers below the sequence are arbitory as the number of nucleotides in the nonsequenced regions of the DNA is not correctly known.

the mt-DNA in JM6. Nonetheless, they might well be the processing signal for transcription. In the absence of any direct experimental evidence, it would be premature to support one hypothesis or the other.

Chapter-6

Secondary and tertiary structure of the proton conducting F_o part of mitochondrial ATP synthase

INTRODUCTION

Combined genetic and molecular analysis of various components of the proton translocating ATPase in the mitochondria have provided mainly three kinds of information. First, the primary structure (vis a vis aminoacid sequences) of different subunits of the ATP synthase have been revealed. Secondly, the sites of mutations involving both drug resistant sites and sites involving the kinetics of enzyme reaction are known. Thirdly, the evolutionarily conserved sites in the protein (and consequently functionally important) have been defined. All this information is quite helpful in its own right, in order to understand some of the basic characteristics of such a complex energy conserving system. But such details as to how different subunits of the complex interact with each other and how they are spatially oriented in a three dimensional structure within the membrane and also their interaction with different drugs during oxidative phosphorylation are not known. This is mostly due to the lack of data on individual subunits or the complex as a whole, or their three dimensional structures by x-ray crystallography or electron density mapping or by other physical methods. Other factors, e.g. controversy regarding the number of components constituting the complex, the state of their structures at different physiological conditions, their *in vivo* dynamics and the interaction of all these components in the membrane have not been settled.

The purpose of the present chapter however, is to discuss the secondary and tertiary structure of the different components of the F_o complex in the mitochondrial membrane solely on the basis of theoretical prediction methods, aided by some experimental evidence obtained from homologous subunits from other organisms. This may help to interpret the probable mechanism of drug interaction (e.g. oligomycin and ossamycin) and proton translocation across the F_o sector of the O.S. ATPase complex.

Membrane proteins involved in proton translocation

It has already been mentioned that the F_o part of the mitochondrial ATP synthase consists of mainly three subunits: subunit-6, subunit-8 and subunit-9. Participation of other subunits could not be ruled out, at least in physiological states of the organelle. A bias towards three subunits has probably stemmed from the work on bacteria, where it has been found to have only three: subunit-a, subunit-b, and subunit-c. Subunit-a is homologous to subunit-6; subunit-c is homologous to subunit-9. Subunit-b and subunit-8 are structurally so different that they appear to serve different functions. However, as will be discussed later in this chapter, they have some strong analogous features.

It is natural that the prokaryotic counterpart of ATP synthase could be much simpler in comparison to a eukaryotic one - as experienced in other biochemical processes. So the claim that other subunits like coupling Factor-B (Sanadi, 1982), F_6 (Kanner *et al*, 1976; Racker, 1979) participate in $F_1.F_o$ complex activity can not be ruled out. Recently the F_6 from bovine heart mitochondria has been sequenced (Fang *et al*, 1984) and the study indicates that the protein has two strong hydrophobic regions with the scattered charged polar amino acids, a characteristic feature of the membrane associated protein. There might be even more factors, as experienced in our mutation mit-175, where yeast cells cannot grow on non-fermentative glycerol medium, but have all three subunits, mentioned above in $F_o.F_1$ complex. However, in this chapter I shall consider only three subunits (subunit-6, 8 & 9) for which extensive sequence data are available, to predict their membrane conformations and their probable drug binding sites.

MATERIAL AND METHODS

Sources of amino acid sequence data

Primary aminoacid sequence data for subunit-6 and subunit-8 of the yeast *S. cerevisiae* have been deduced from DNA sequence analyses (See chapter-5). Aminoacid sequences for subunit-9 have been taken from Macino and Tzagoloff (1979), Sebald *et al* (1979) and

Sebald and Hoppe (1981). Amino acid sequences for subunit-6 and subunit-8 (or corresponding homologous ORFs) have been taken from following references:

Human (Anderson *et al*, 1981), Bovine (Anderson *et al*. 1982), Mouse (Bibb *et al*, 1981), Rat (Grosskopf & Feldman, 1981), *Drosophila melanogaster* (de Bruijin, 1983), *D. yakuba* (Clary & Wolstenholme, 1983), *Aspergillus nidulans* (Grisi *et al*, 1982; Netzker *et al*, 1982), *A. amstadium* (Lazarus & Kuntzel, 1981), *Neurospora crassa* (Morelli & Macino, 1984). Aminoacid sequences for subunit-a, subunit-b, and subunit-c of *E. coli* has been taken from Walker *et al* (1984).

Prediction basis of secondary and tertiary structure

Chou & Fasman's method of prediction of secondary structure of a protein has been used extensively for a large number of proteins and has been shown to be successful (Chou & Fassman, 1978). Their prediction method is based on some data derived from statistical analyses of x-ray crystallographic studies of 29 globular proteins. From a set of values obtained for a particular aminoacid in their property of α-helix, β-sheet, β-turn and random coil formation, they have proposed some empirical rules which can be used to predict the secondary structure of a protein. However, it has been pointed out (Wootton, 1974; Green & Flanagan, 1976; Furthmeyer *et al*, 1978) that this method can not be applied to all proteins, especially the hydrophobic membrane proteins (Sebald & Hoppe, 1981; Mao *et al*, 1982). As the F_0 part of the O.S. ATPase complex is made up of mainly hydrophobic proteins, Chou & Fasman's method could not be employed directly to these proteins. Therefore I have used the hydrophobicity profile of the proteins, complemented by a slightly modified form of Chou & Fasman's method as a predictive basis of the secondary and tertiary structure of the F_o components.

Hydrophobicity Profile

The hydrophobicity profile of a protein is generally generated by calculating an average "hydropathy" value of a short chain of aminoacids along a string of polypeptides. The

TABLE 6.1 Conformational parameters (P_α and P_β) of 20 aminoacids based on the residues in the α-helix and β-sheet regions of 15 proteins (Chou & Fasman, 1974). Symbols to assign aminoacids as Formers, Breakers, and Indifferent for helical and β-Sheet conformation are as in text †

Helical residues	P_α	Helical assignment	β-Sheet residues	P_β	β-Sheet assignment
Glu	1.53	H_α	Met	1.67	H_β
Ala	1.45	H_α	Val	1.65	H_β
Leu	1.34	H_α	Ile	1.60	H_β
His	1.24	h_α	Cys	1.30	h_β
Met	1.20	h_α	Tyr	1.29	h_β
Gln	1.17	h_α	Phe	1.28	h_β
Trp	1.14	h_α	Gln	1.23	h_β
Val	1.14	h_α	Leu	1.22	h_β
Phe	1.12	h_α	Thr	1.20	h_β
Lys	1.07	I_α	Trp	1.14	h_β
Ile	1.00	I_α	Ala	0.97	I_β
Asp	0.98	i_α	*Arg*	0.90	i_β
Thr	0.82	i_α	Gly	0.81	i_β
Ser	0.79	i_α	*Asp*	0.80	i_β
Arg	0.79	i_α	*Lys*	0.74	b_β
Cys	0.77	i_α	Ser	0.72	b_β
Asn	0.73	b_α	*His*	0.71	b_β
Tyr	0.61	b_α	Asn	0.65	b_β
Pro	0.59	B_α	Pro	0.62	b_β
Gly	0.53	B_α	*Glu*	0.26	B_β

† Conformational parameters given in this table have later been corrected by Chou & Fasman (1978) on the basis of more data from 32 proteins.

Table 6.2 Conformational parameters (P_α and P_β) of 20 aminoacids based on the residues in the α-helix and β-sheet regions of 29 proteins (Chou & Fasman, 1978). Symbols to assign aminoacids as Formers, Breakers, and Indifferent for helical and β-sheet conformation are as in text.

α-Residues	P_α	Helical assignment	β-Residues	P_β	β-Sheet assignment
Glu	1.51	H_α	Val	1.70	H_β
Met	1.45	H_α	Ile	1.60	H_β
Ala	1.42	H_α	Tyr	1.47	H_β
Leu	1.21	H_α	Phe	1.38	h_β
Lys	1.16	h_α	Trp	1.37	h_β
Phe	1.13	h_α	Leu	1.30	h_β
Gln	1.11	h_α	Cys	1.19	h_β
Trp	1.08	h_α	Thr	1.19	h_β
Ile	1.08	h_α	Gln	1.10	h_β
Val	1.06	h_α	Met	1.05	h_β
Asp	1.01	I_α	*Arg*	0.93	i_β
His	1.00	I_α	Asn	0.89	i_β
Arg	0.98	i_α	*His*	0.87	i_β
Thr	0.83	i_α	Ala	0.83	i_β
Ser	0.77	i_α	Ser	0.75	b_β
Cys	0.70	i_α	Gly	0.75	b_β
Tyr	0.69	b_α	*Lys*	0.74	b_β
Asn	0.67	b_α	Pro	0.55	B_β
Pro	0.57	B_α	*Asp*	0.54	B_β
Gly	0.57	B_α	*Glu*	0.37	B_β

Table 6.3 Conformational parameters (P_α and P_β) of 20 aminoacids based on the modifications suggested in the present study. See text for the groundof modification. Symbols to assign aminoacids as Formers, Breakers, and Indifferent for helical and β-sheet conformation are as in text.

α-Residues	P_α	Helical assignment	β-Residues	P_β	β-Sheet assignment
Glu	1.6	H_α	Val	1.6	H_β
Met	1.6	H_α	Ile	1.6	H_β
Ala	1.6	H_α	Tyr	1.6	H_β
Leu	1.6	H_α	Phe	1.2	h_β
Lys	1.2	h_α	Trp	1.2	h_β
Phe	1.2	h_α	Leu	1.2	h_β
Gln	1.2	h_α	Cys	1.2	h_β
Trp	1.2	h_α	Thr	1.2	h_β
Ile	1.2	h_α	Gln	1.2	h_β
Val	1.2	h_α	Met	1.2	h_β
Asp	1.0	I_α	*Arg*	0.8	i_β
His	1.0	I_α	Asn	0.8	i_β
Arg	1.0	i_α	*His*	0.8	i_β
Thr	0.8	i_α	Ala	0.8	i_β
Ser	0.8	i_α	Ser	0.7	b_β
Cys	0.8	i_α	Gly	0.7	b_β
Tyr	0.7	b_α	*Lys*	0.7	b_β
Asn	0.7	b_α	Pro	0.5	B_β
Pro	0.5	B_α	*Asp*	0.5	B_β
Gly	0.5	B_α	*Glu*	0.5	B_β

"hydropathy" value for a given aminoacid can be calculated from its transfer of free energy (ΔG°) on the basis of its partition of coefficient between water and a non-interacting, isotropic phase (Nozaki & Tanford, 1971; Hine & Mookerjee, 1975). Ethanol has been used as the choice of a solvent, as it has been assumed to be a neutral and non-interacting agent and is a phase resembling the interior of a protein (Nozaki & Tanford, 1971). Von Heijne (1981) has used such estimated values (free energies of transfer of residues originally in a helix in water to a helix in a non-polar phase lacking hydrogen bonding capacity) to generate a computer program which could plot hydrophilicity and hydrophobicity of a protein along its aminoacid sequences. However, it has been suggested (Kyte & Doolittle, 1982) that ethanol might not fulfil the criteria of an ideal isotropic non-interacting phase and retains many of the unpredictable peculiarities of water itself. The latter workers, therefore, have used water-vapour transfer free energies (Hine & Mookerjee, 1975) as an alternative index of hydropathy of aminoacids which calculates transfer free energies of aminoacid side-chains between an aqueous solution and the condensed vapour. The hydrophobic plot of proteins used in this study is based on this later data, using a computer program HYDROPLOT, based on the algorithm of Kyte & Doolittle (1982).

Prediction of α-helix and β - sheet

A computer program has been devised for the prediction of α-helix and β-sheet formation probability of a protein along its aminoacid sequences. The program has the option of using any one of three tables of α-helix and β-sheet conformation potential (P_{α} and P_{β} respectively) of 20 aminoacids (Table 6.1, Table 6.2 and Table 6.3 respectively). Table 6.1 describes the original values of P_{α} and P_{β} of 20 aminoacids developed by Chou & Fasman (1974). Table 6.2 is the revised one produced by the same authors based on 29 proteins. Table 6.3 is a modified table proposed by the present author. The reason for this modification will be described in the results section of this chapter. The algorithm of the computer program is given in the appendix of this thesis. The program uses a moving

segment approach that continuously determines the average P_α and P_β within a segment of predetermined length as it advances through the sequences. The consecutive scores are plotted from the amino to the carboxy terminus. At the same time, a mid point line is printed, that corresponds to the probability value of 1.0 depicting the fact that values below this line break the continuity of α-helix or β-sheet. Secondary structure predicition will utilise both this program and other empirical rules proposed by Chou & Fasman (1978). Assignment of α-helix and β-sheet potential of a given amino acid is similar to that of Chou & Fasman (1978):

Helical assignment: H_α, strong α former; h_α, α former; I_α, weak α former; i_α, α indifferent; b_α, α breaker; B_α, strong α breaker.

β-sheet assignment: H_β, strong B former; h_β, β former; I_β, weak β former; i_β, β indifferent; b_β, β breaker; B_β, strong β breaker.

Representation of three dimensional structures of helices

Three dimensional structures of helices can be represented by two dimensional projections which are called "helical wheels" (Schiffer & Edmundson, 1967). In this model, the successive C_α- atoms in a helical peptide would, in projection down the helical axis, lie on a circle with successive positions rotated by 100^0. Lines or spokes emanating from the centre of the circle and passing through the C_α positions would show the projected direction of the aminoacid side chain, giving the appearance of a helical "wheel". As on average, only 3.6 residues per turn of an helix can exist, there would be only 18 unique C points separated by 20°. On the basis of neutron-scattering experiments and electron density map of bacteriorhodopsin, Engelman and Zacci (1980) indicated which helical faces of this protein remain in contact with the lipid bi-layer. Because most membrane proteins like bacteriorhodopsin are an "inside-out" protein in contrast to the structural organisation of soluble protein, they found out that charged and polar residues were distributed on the interior faces exposed to the protein while the strongly non-polar groups were on the exterior faces associated with the lipid bi-layer of the membrane. On the basis of this

Table 6.4 The membrane-buried preference parameters for the twenty aminoacids calculated from a 1125-residue data base (After Argos et al. 1982)

Amino acid	Normalized preference value
Met (M)	2.96
Leu (L)	2.93
Phe (F)	2.03
Ile (I)	1.67
Ala (A)	1.56
Cys (C)	1.23
Val (V)	1.14
Trp (W)	1.08
Thr (T)	0.91
Ser (S)	0.81
Pro (P)	0.76
Tyr (Y)	0.68
Gly (G)	0.62
Gln (Q)	0.51
Arg (R)	0.45
His (H)	0.92
Asn (N)	0.27
Glu (E)	0.23
Lys (K)	0.15
Asp (D)	0.14

result and other results for membrane proteins, Argos *et al* (1982) deduced the membrane buried preference parameter for all 20 aminoacids (based on 1125 - residue data base). The same authors also proposed that aminoacids with membrane-buried preference parameters less than 1.0 (Table 6.4) would tend to cluster on one side of a helical wheel and, on the basis of this, the lipid facing and protein facing helical sides can be inferred.

To examine the three dimensional structure of the helices of the F_o components within the hydrophobic membrane environment and to apply them in interpretation of proton channel conformation and drug interaction, the predictive method of Argos *et al* (1982) has been used in the present study.

RESULTS

Secondary structure of bacteriorhodopsin

Bacteriorhodopsin, a light-driven proton translocating membrane protein of halophilic bacteria, is a relatively well understood protein for which different physical data (based on x-ray crystallography, electron microscopy and CD spectral studies) and biochemical data (involving primary structure, proteolytic degradation products) are known (Henderson, 1977; Long *et al*, 1977; Ovchinnikov *et al*,1979; Engelman *et al*, 1980). When this protein was analysed for its secondary structure based on Chou & Fasman's method, using table 6.2, it is found to overpredict β-structure (Fig. 6.1) and a similar result is obtained with the transmembrane segment of human erythrocyte glycophorin (Fig. 6.3). X-ray crystallography and CD spectral analyses show 75-80% α-helix content for bacteriorhodopsin (Engelman *et al*, 1980) and about 96-100% of helicity for the transmembrane segment of glycophorin (Marvin & Wachtel, 1975; Furthmayr *et al*, 1978). From table 6.8 and Fig. 6.1 and 6.3, it is evident that Chou & Fasman's prediction rule predicts only 52% and 47% of α-helix for the above two proteins respectively which undoubtedly overpredicts β-structure. For this reason an empirical approach of modifying the values for P_α and P_β of Table 6.2 (Table V of Chou & Fasman, 1978) has been adopted. From

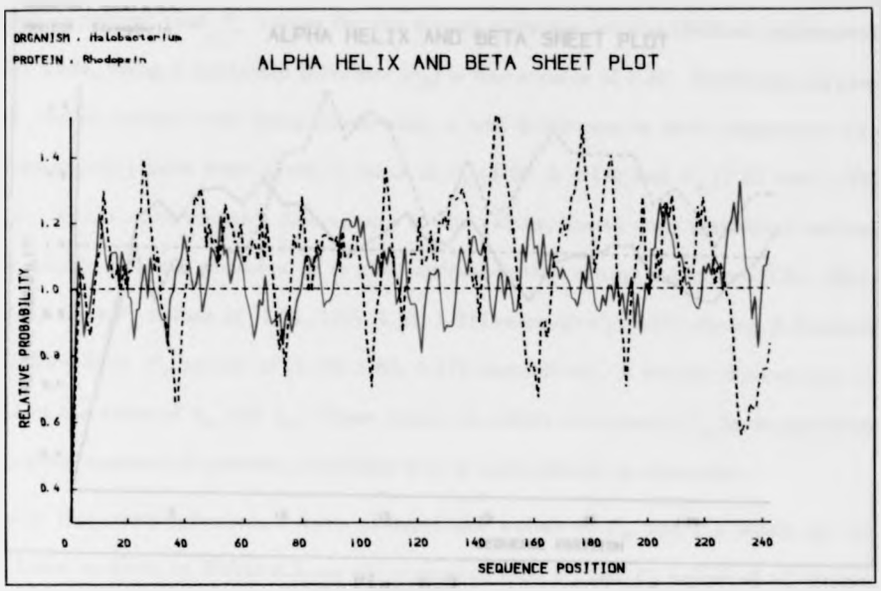


Fig. 6.1
red line (α-helix)
blue line (β-sheet)

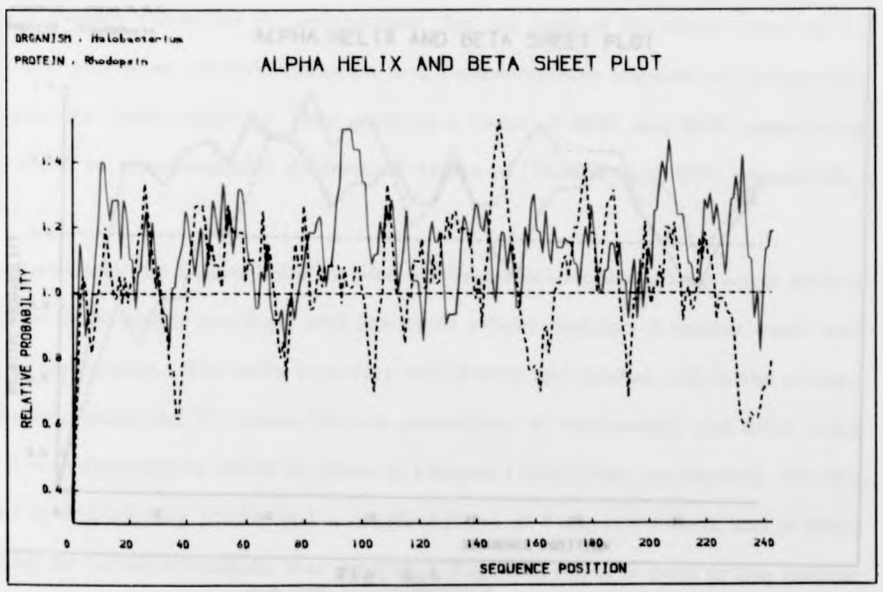


Fig. 6.2
red line (α-helix)
blue line (β-sheet)

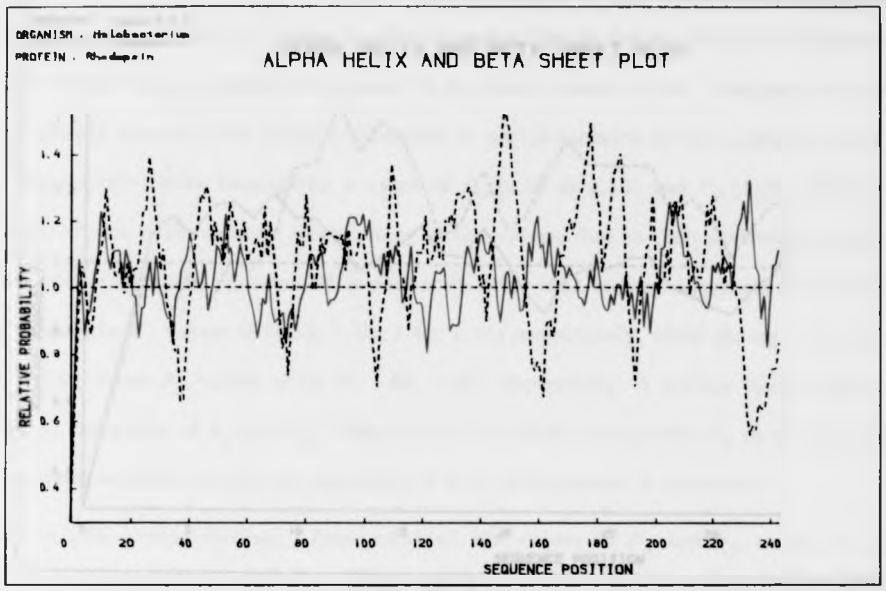


Fig. 6.1
red line (α-helix)
blue line (β-sheet)

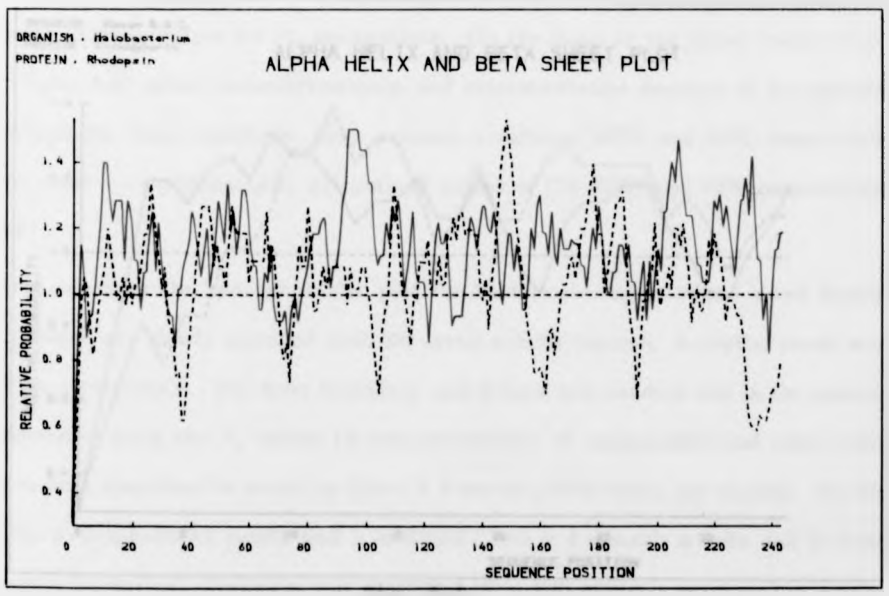


Fig. 6.2
red line (α-helix)
blue line (β-sheet)

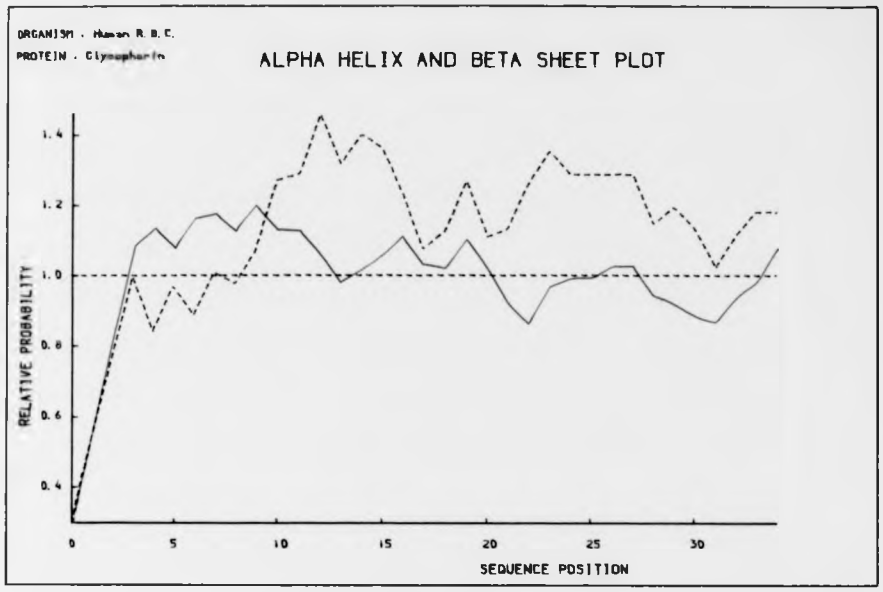


Fig. 6.3
red line (α-helix)
blue line (β-sheet)

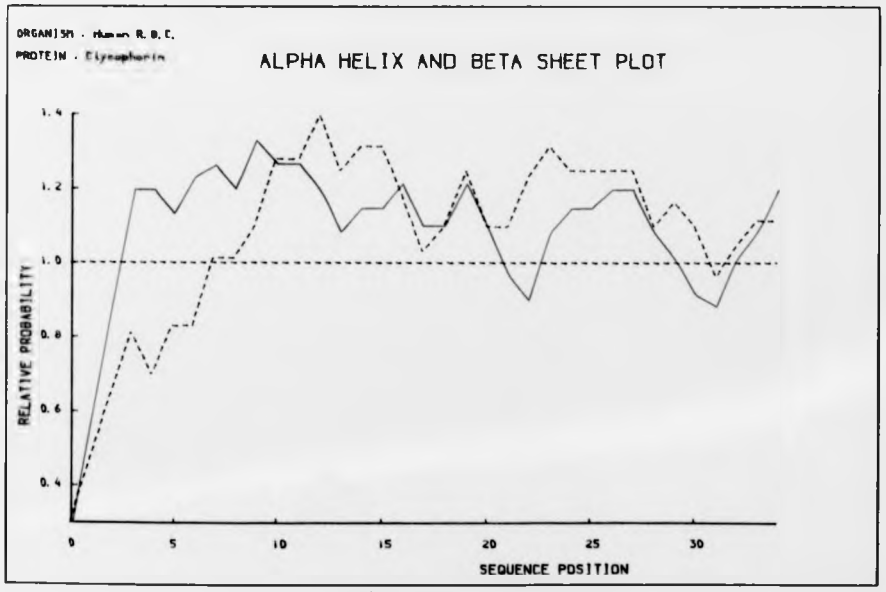


Fig. 6.4
red line (α-helix)
blue line (β-sheet)

Table 6.2 it is evident that P_α values for the strong α-former leucine (Helical assignment H_α) is 1.21 while, being a moderate β-former (h_β) it has a value of 1.30. Similarly, tryptophan and phenyl alanine both being moderately α and β-formers in their assignment (h_α and h_β, respectively) have been given a value of P_α (1.08 & 1.13) and P_β (1.37 and 1.38) respectively, which again shows a discrepancy in the values, due to their statistical derivation from soluble globular proteins. It is also noticeable that strong α-formers (Glu, Met, Ala and Leu) have P_α values of (1.51, 1.45, 1.42, 1.21) respectively, while strong β-formers (Val, Ileu, Tyr) have P_β values of (1.70, 1.60, 1.47) respectively. A similar discrepancy is also found in the value of h_α and h_β. These values therefore overpredict P_β in comparison to P_α in a given segment of protein, especially if it is hydrophobic in character.

To minimise this overprediction, I have changed the values of P_α and P_β, solely on an empirical basis, as given in Table 6.3, on the argument that P_α and P_β values of all strong α-formers (H_α) and strong β-former (H_β) should be the same. Similarly, all moderate α-formers (h_α) and β-former (h_β) should have the same values and so on. The values for H_α or H_β, h_α or h_β, I_α or I_β, i_α or $i\beta$, b_α or b_β, B_α or B_β were determined empirically from the approximate average values for P_β assignments. On the basis of the above values of P_α and P_β (Table 6.3) when bacteriorhodopsin and intermembrane segment of glycophorin are calculated for their α-helicity, they produce a value of 82% and 90% respectively which are closer to experimentally determined values of (75-80%) and 96% respectively, (Table 6.8).

Fig.6.5 demonstrates the hydrophobicity plot for bacteriorhodopsin whose seven hydrophobic segments are clearly matched with the seven α-helix regions. A similar result was obtained for glycophorin. The helix boundary and β-turn and random coil in the protein were determined using the P_t values (β-turn probability of aminoacids) and other rules which have been described in detail by Chau & Fasman (1978) (data not shown). On the basis of the hydrophobicity profile and a modified Chou & Fasman's α-helix and β-sheet plot, a model for bacteriorhodopsin was generated (Fig. 6.6) which is more or less similar to the one described by Engelman *et al* (1980), based on physical and biochemical data.

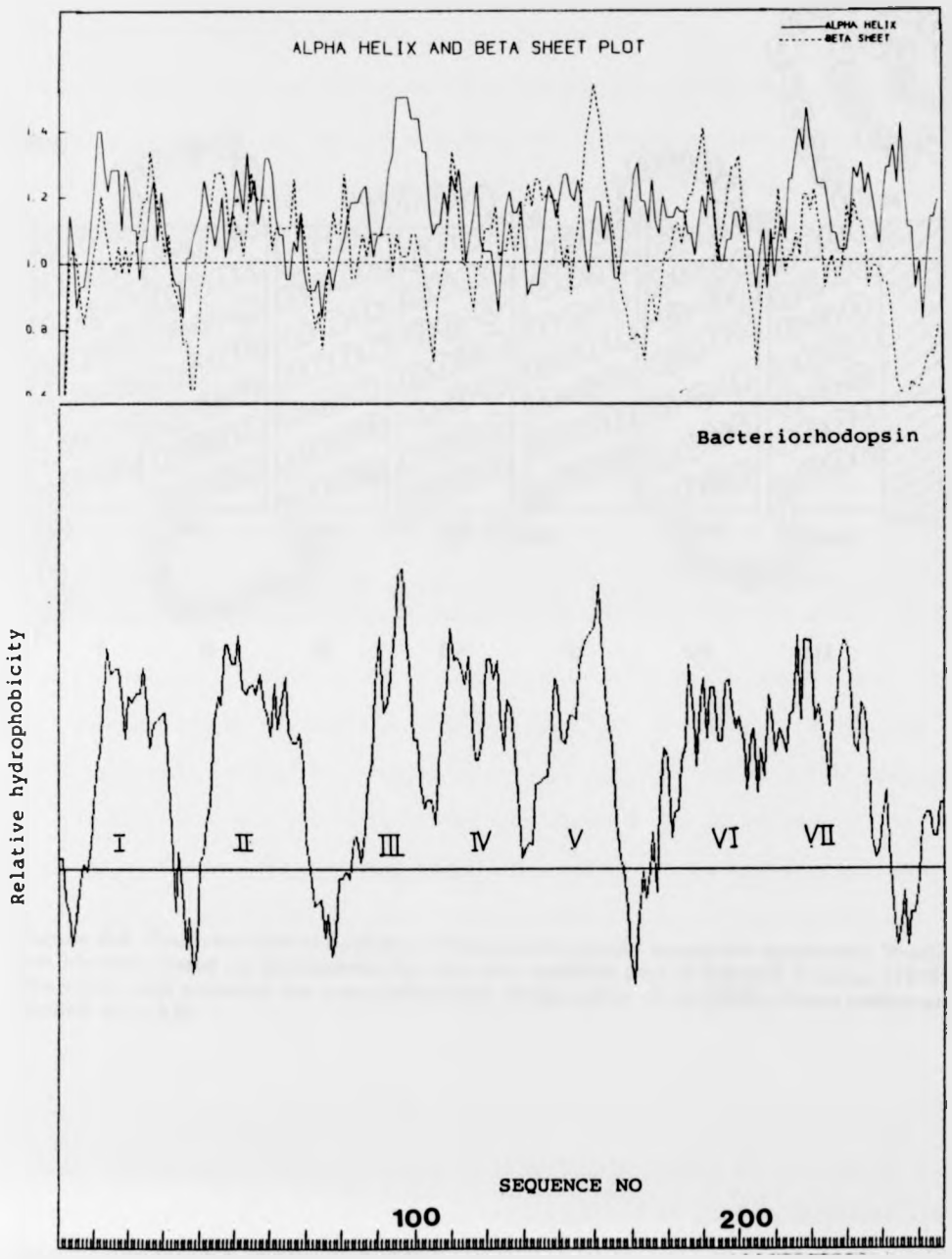


Figure 6.5 Superimposition of the secondary structure plot (α-helix and β-sheet plot) on the hydrophobicity plot of bacteriorhodopsin.

The analysis shows that seven helical regions correspond to the prominent seven hydrophobic regions of this membrane protein. Electron density mapping of the X-ray crystallographic studies have proved these regions to be membrane buried helical regions (Engelman et al, 1980)

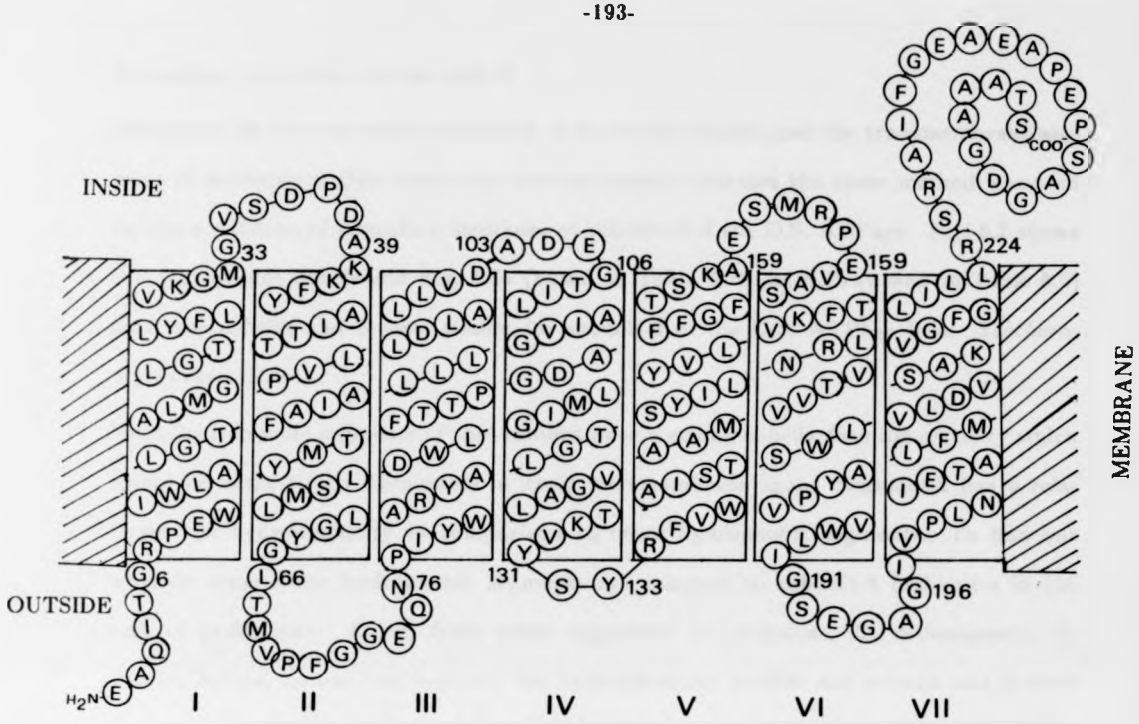


Figure 6.6 Predicted helical pathway of bacteriorhodopsin across the membrane. Prediction has been based on hydrophobicity plot and modified plot of Chou & Fasman (1978). The amino acid sequence has been taken from Ovchinnikov *et al* (1979). Seven helices are labelled as I - VII.

Secondary structure of subunit-6

Stimulated by the successful prediction of bacteriorhodopsin and the transmembrane segment of erythrocyte glycophorin for their secondary structure the same method was used for the prediction of secondary structure of subunit-6 of the O.S. ATPase. Fig. 6.7 shows the hydrophobic profile and Fig. 6.8a (based on Table 6.2) and 6.8b (based on Table 6.3) show the α-helix and β-sheet profile for subunit-6 of the O.S. ATPase from *Saccharomyces cerevisiae.*

Seven hydrophobic segments of this subunit have a strong α-helix forming capacity which would be energetically favourable in their intramembrane span. Segment-2 has a relatively low hydrophobicity in comparison to other hydrophobic segments. To find out whether these seven hydrophobic segments are common to subunit-6 (subunit-a in the case of prokaryotic *E.coli*) from other organisms (*A. nidulans, D. melanogaster, D. yakuba*, bovine, mouse, rat, human), the hydrophobicity profiles and α-helix and β-sheet plots were compared. Fig. 6.9 and Fig. 6.10 depict the comparable hydrophobic profile and their corresponding α-helix and β-sheet plot, respectively. In all cases a string of six to seven aminoacids was used to calculate the average values of their secondary structure conformations. It is quite clear from such comparisons that the protein subunit-6 of the yeast O.S. ATPase and equivalent subunits in other species are very similar in their secondary structure. The probable boundary of different hydrophobic segments with the α-helix forming capacity of each segment from various organisms is shown in Table 6.5. On the basis of this, a model for the subunit-6 of *Saccharomyces cerevisiae* is proposed in Fig. 6.11.

In the present model the N-terminal end of subunit-6 has been shown to lie on the matrix side and C-terminal end has been shown to be on the periplasmic side by analogy with studies on subunit-a of *E. coli* ATPase and assuming that both subunits are homologous (based on the homologies in their primary and secondary structures). Hoppe & Sebald (1984) have also reported that about 20 aminoacid residues of subunit-a of *E.coli* (homologous to subunit-6 of eukaryotic O.S. ATPase) could be removed proteolytically from the

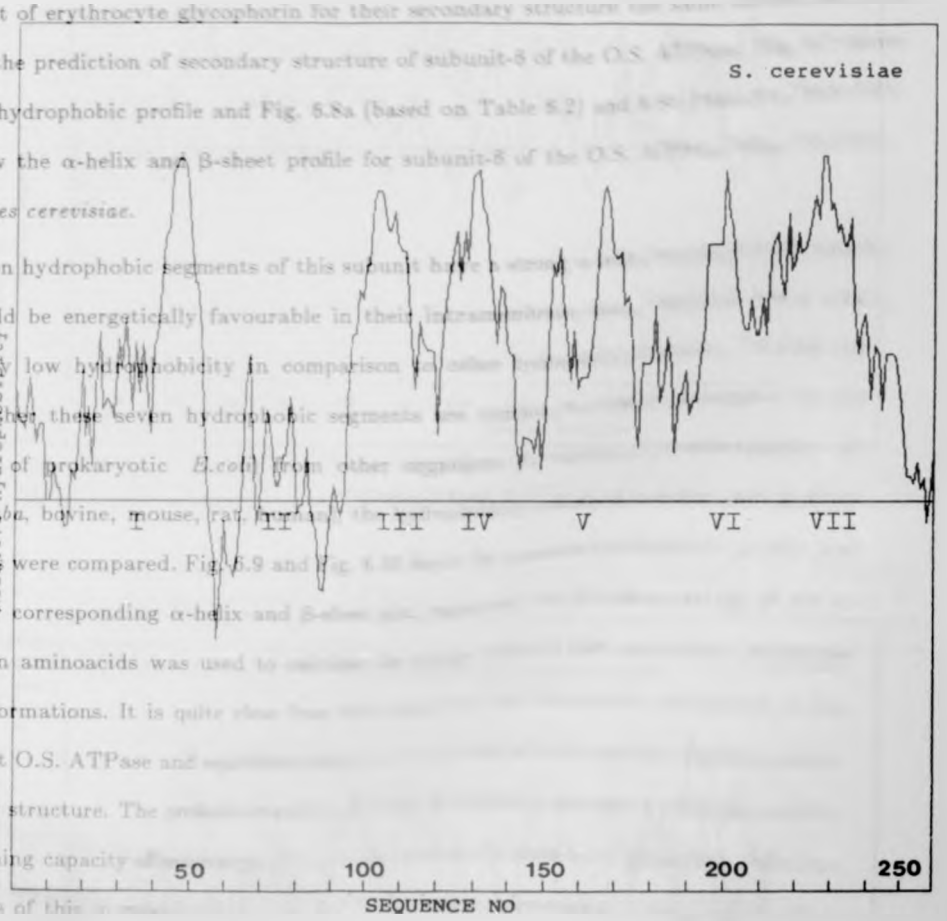


Figure 6.7 Hydrophobicity profile of subunit-6 of yeast O.S.ATPase. A string of nine amino acids were taken to calculate the average values for this plot. Seven hydrophobic regions (I-VII) of the protein are very clear, which are proposed to be membrane buried.

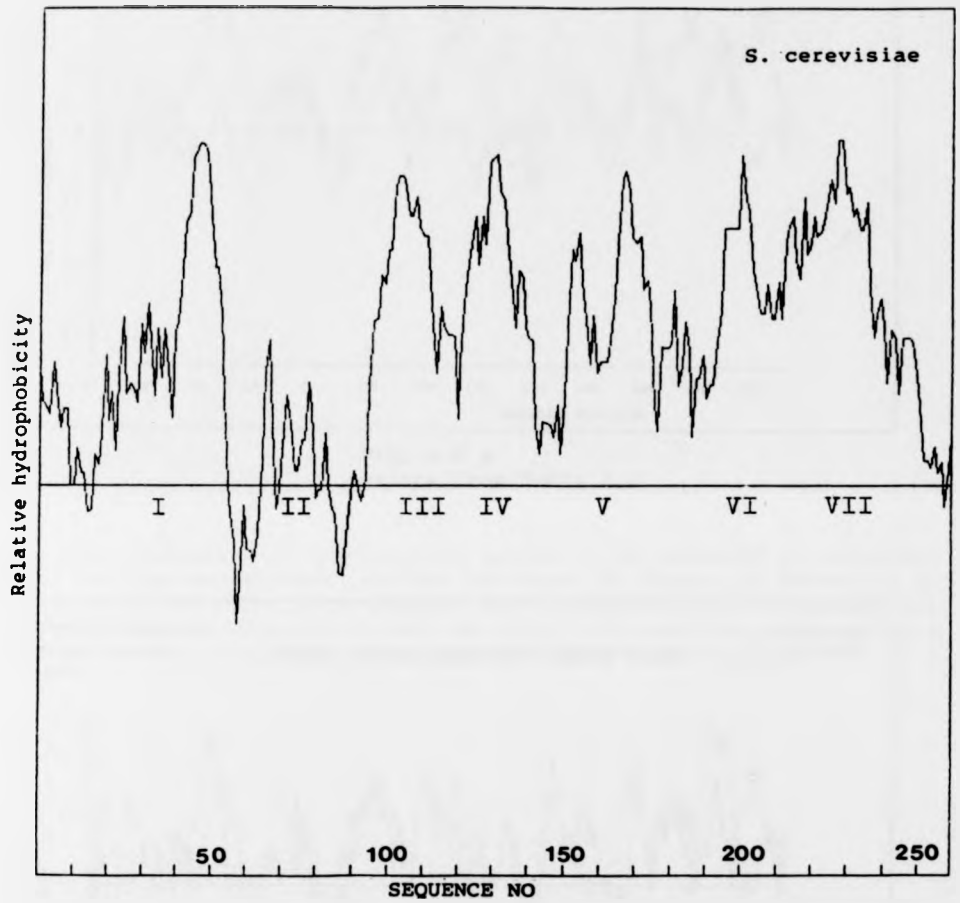


Figure 6.7 Hydrophobicity profile of subunit-6 of yeast O.S.ATPase. A string of nine amino acids were taken to calculate the average values for this plot. Seven hydrophobic regions (I-VII) of the protein are very clear, which are proposed to be membrane buried.

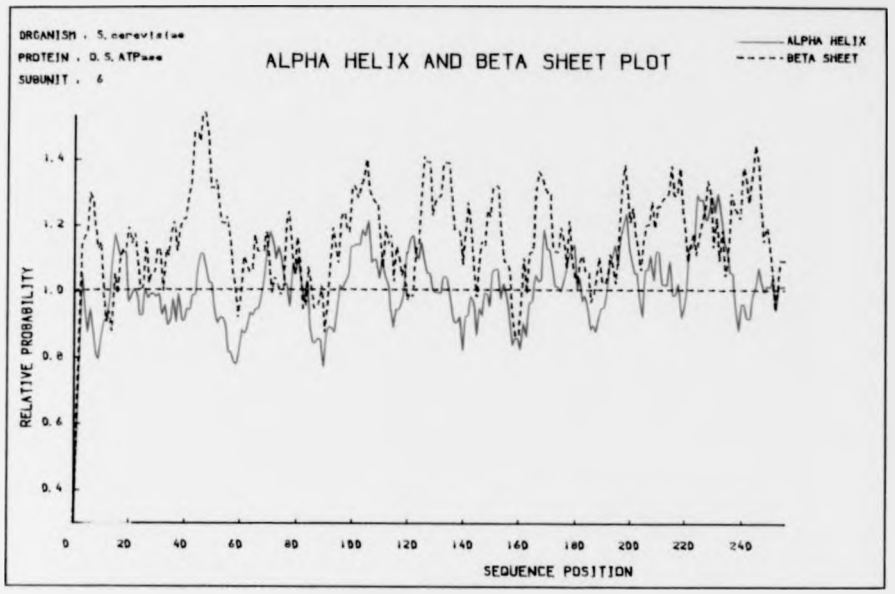


Fig. 6.8 a
values from Table 6.2

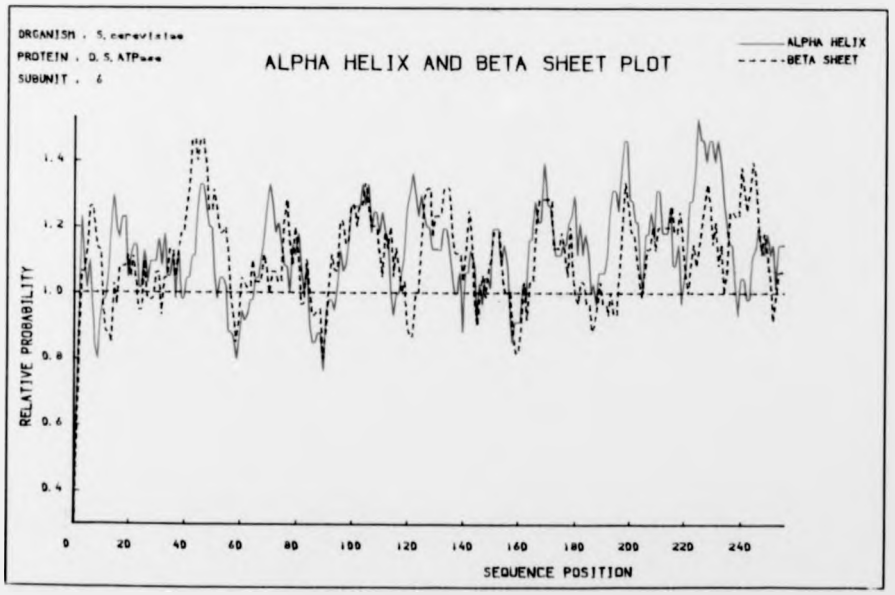
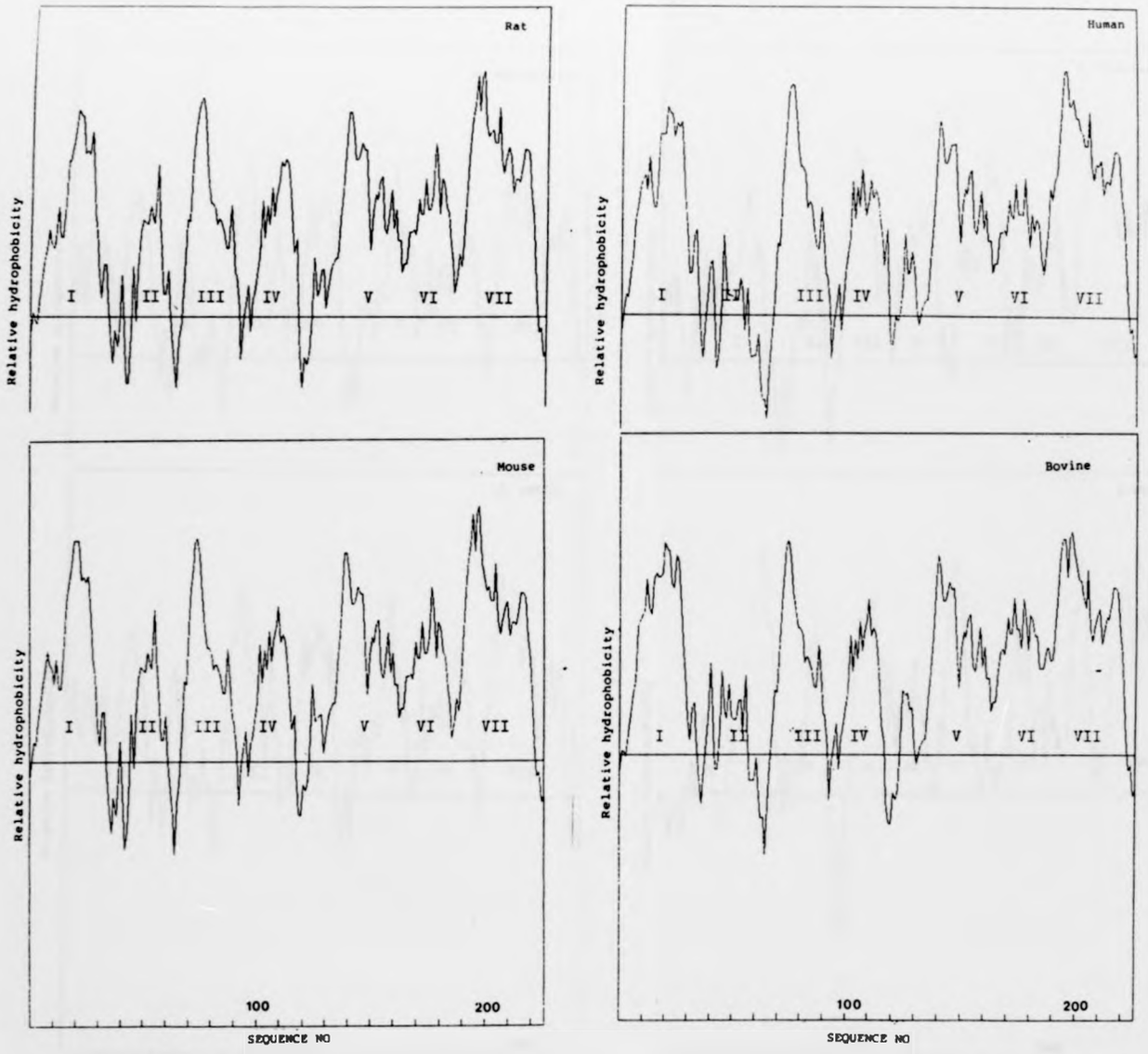


Fig. 6.8 b
values from Table 6.3

Figure 6.9 Comparison of hydrophobicity profiles of the subunit-6 (or equivalent subunit) from various organisms. (a) Rat, (b) Mouse, (c) Human, (d) Bovine, (e) D. melanogaster, (f) D. yakuba, (g) A. nidulans, (h) E. coli (subunit-a). The sequence has been plotted along the horizontal axis and calculating a string of nine amino acids at a time. I-VII represent the regions to be hydrophobic membrane buried regions.


Rat
Human
Mouse
Bovine
Relative hydrophobicity
I
II
III
IV
V
VI
VII
100
200
SEQUENCE NO

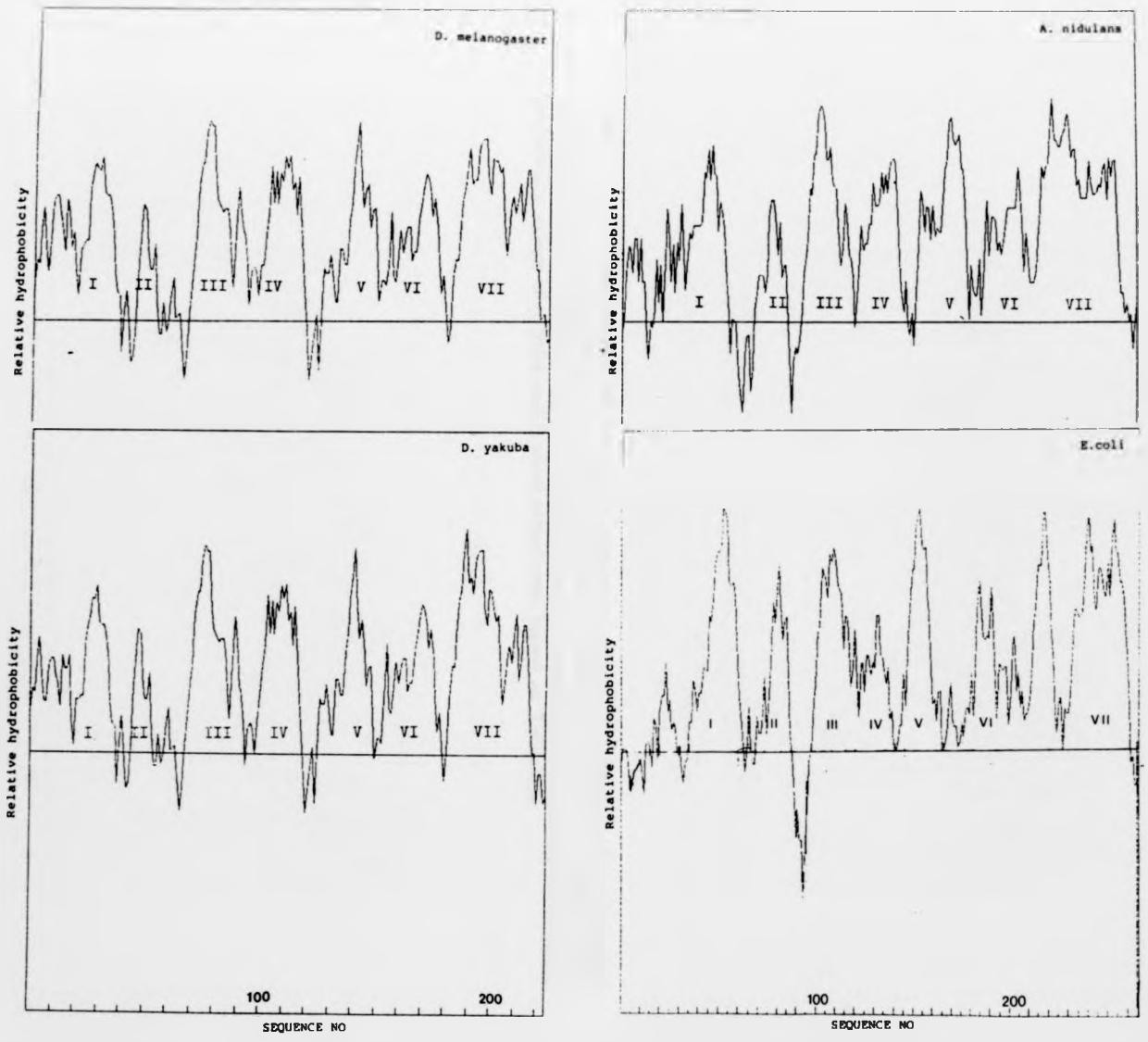

D. melanogaster
A. nidulans
D. yakuba
E.coli
Relative hydrophobicity
I
II
III
IV
V
VI
VII
100
200
SEQUENCE NO

Figure 6.10 Comparison of secondary structure plots of subunit-6 (or equivalent subunit). (a) *A. nidulans*, (b) *E. coli*, (c) Human, (d) Bovine, (e) Mouse, (f) Rat, (g) *D. melanogaster* (h) *D. yakuba*. A string of 6 amino acids were taken to calculate the average probability values of α-helix and β-sheet structures, based on Table 6.3

Figure 6.10 Comparison of secondary structure plots of subunit-6 (or equivalent subunit). (a) *A. nidulans*, (b) *E. coli*, (c) Human, (d) Bovine, (e) Mouse, (f) Rat, (g) *D. melanogaster* (h) *D. yakuba*. A string of 6 amino acids were taken to calculate the average probability values of α-helix and β-sheet structures, based on Table 6.3

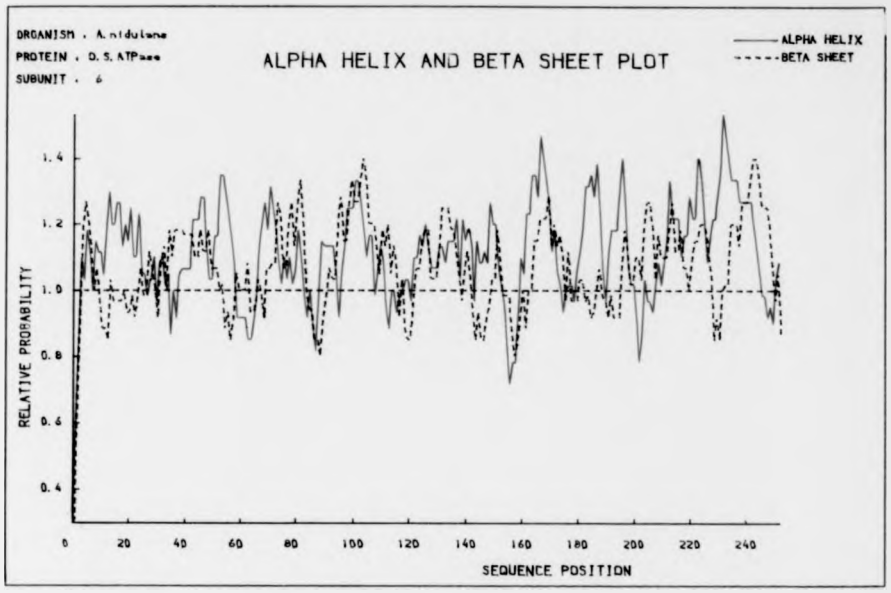


Fig. 6.10 a

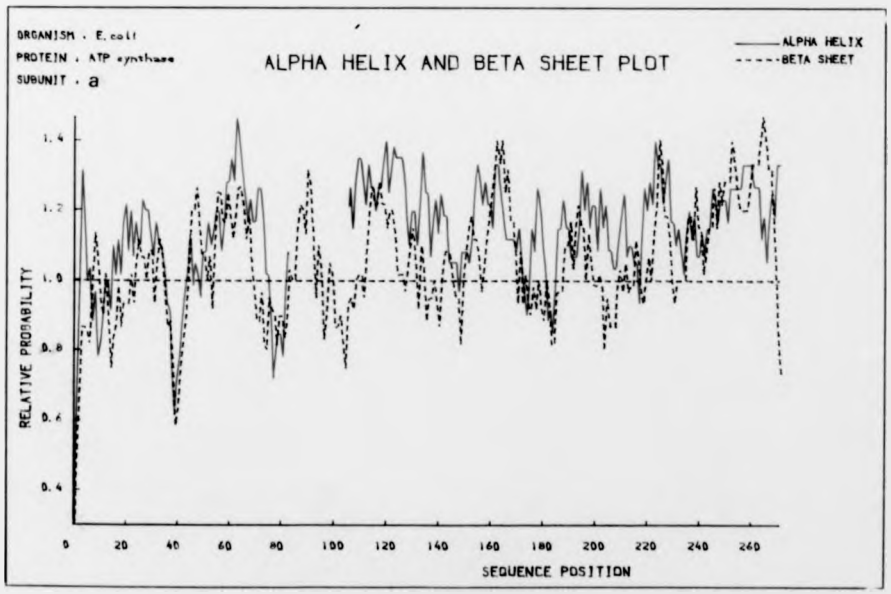


Fig. 6.10 b

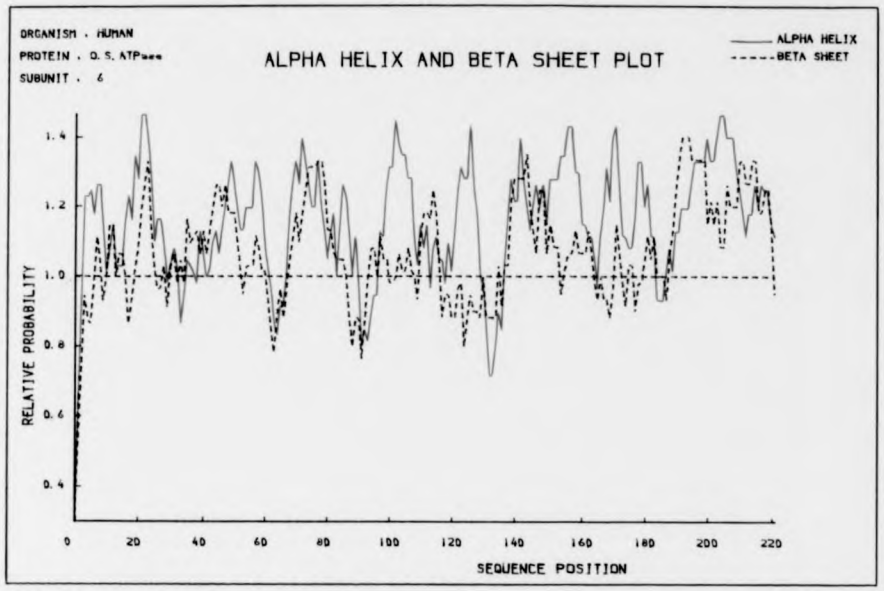


Fig. 6.10 c

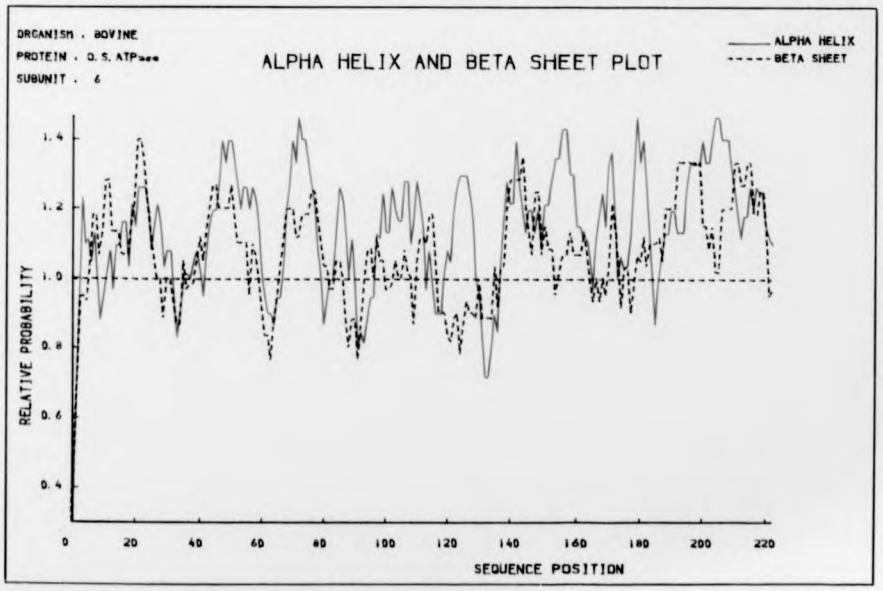


Fig. 6.10 d

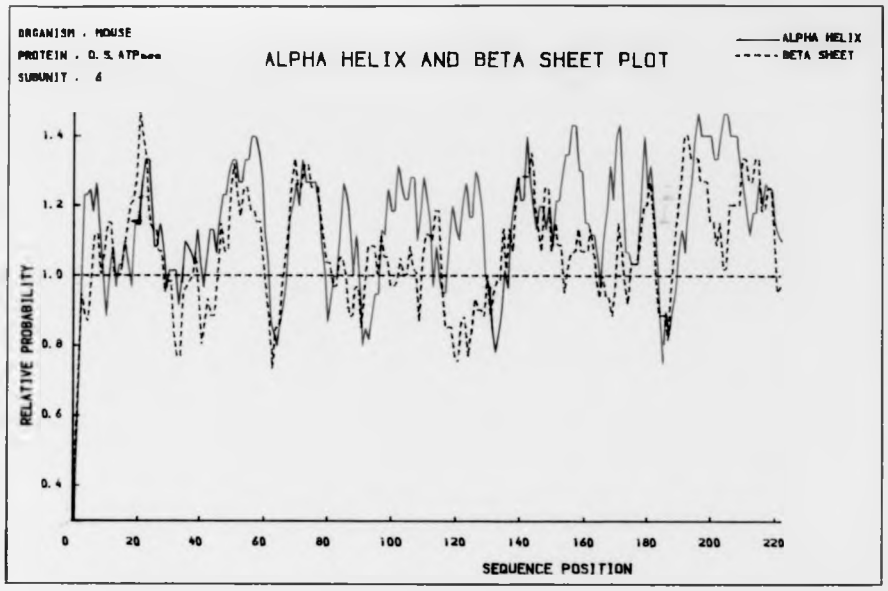


Fig. 6.10 e

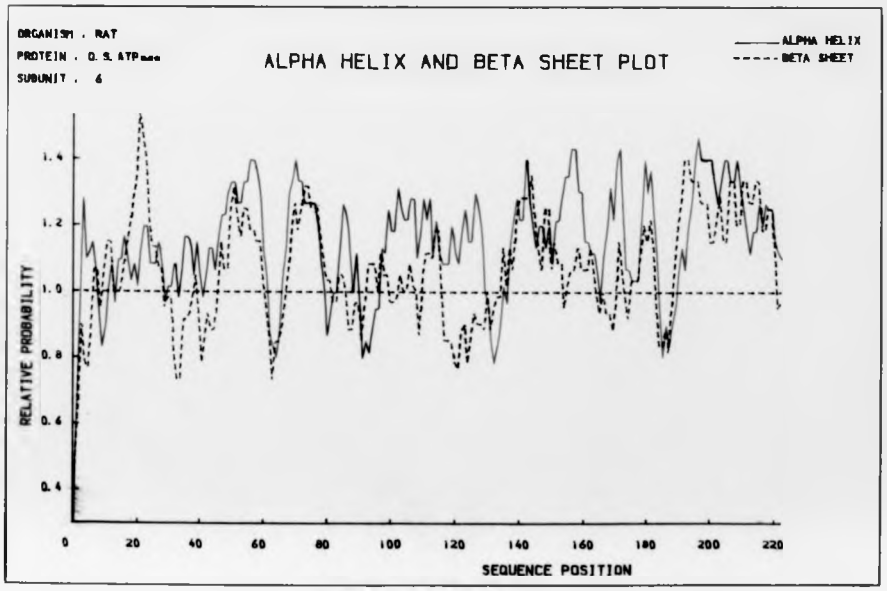


Fig. 6.10 f

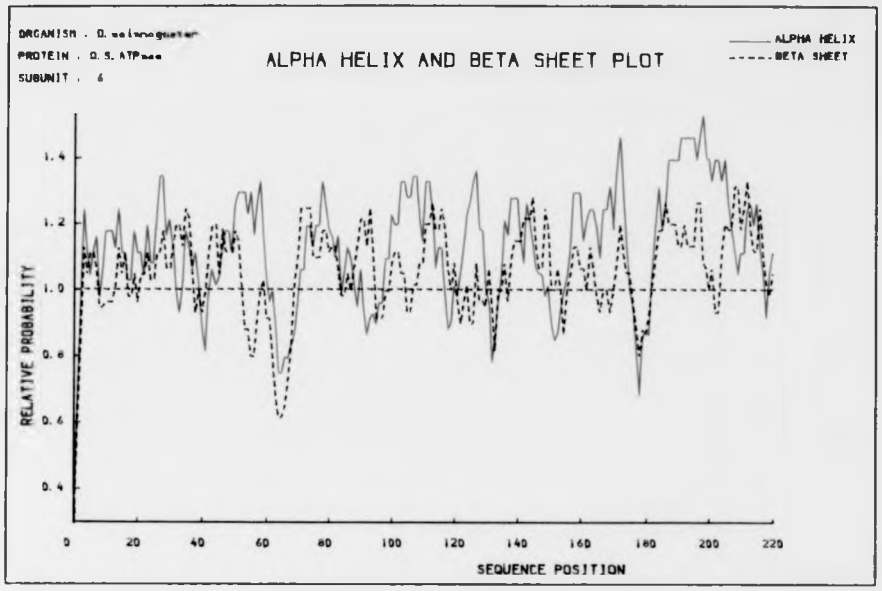


Fig. 6.10 g

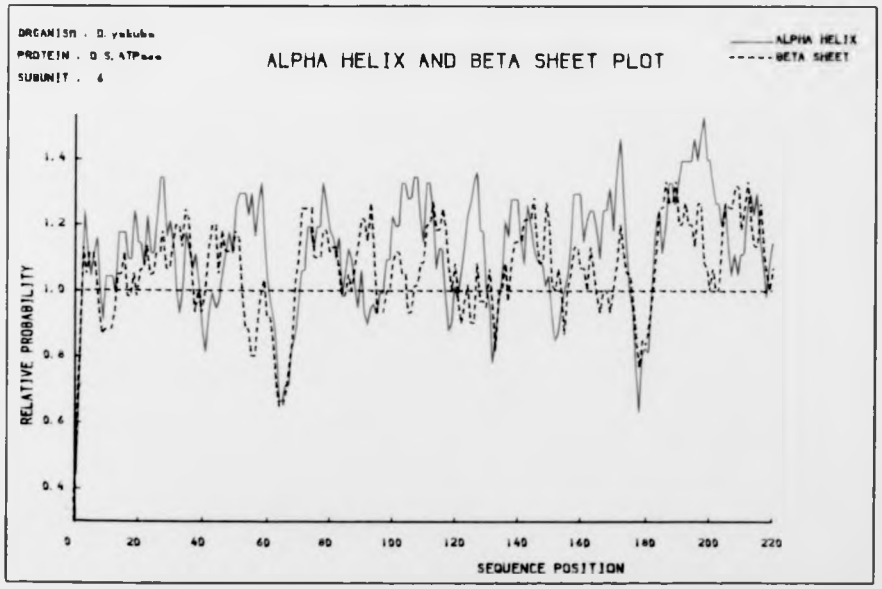


Fig. 6.10 h

Table 6.5 Predicted span of intramembrane segments of the Subunit-6 from various organisms. The predictions are based on both hydrophobicity plots and alpha-helix and beta-sheet plots. The percentage of alpha helicity of each segment is given within the brackets.

Organism	Hdrophobic Segment I	Hydrophobic Segment II	Hydrophobic Segment III	Hydrophobic Segment IV	Hydrophobic Segment V
S.cerevisiae	25-50 (92%)	61-86 (80%)	93-118 (92%)	120-145 (100%)	164-189 (100%)
A.nidulans	25-50 (90%)	59-84 (65%)	90-115 (90%)	118-142 (96%)	160-185 (95%)
D.melanogaster	6-31 (96%)	40-65 (80%)	69-94 (80%)	96-120 (90%)	123-148 (90%)
Bovine	5-30 (92%)	35-60 (96%)	66-91 (85%)	95-120 (80%)	137-162 (100%)
E. coli	35-60 (61%)	67-92 (70%)	100-125 (92%)	135-160 (96%)	178-203 (84%)

Hydrophobic Segment VI	Hydrophobic Segment VII	Total number of aminoacids	Total percentage of alpha-helicity
193-218 (100%)	225-250 (92%)	259	83%
188-214 (80%)	223-248 (90%)	256	82%
150-175 (90%)	194-219 (100%)	224	80%
167-192 (96%)	196-221 (100%)	226	86%
208-233 (96%)	243-268 (100%)	275	82%

cytoplasmic side, with subtilisin. Since the C-terminus of all the homologous subunits is very hydrophobic, it has been argued by the same authors that the N-terminal end is the protease susceptible end and therefore, would be located at the cytoplasmic side. The model also shows that the subunit can form seven helical transmembrane segments which can potentially form a proton pump similar to bacteriorhodopsin (Engelman *et al*, 1980). Another charecteristic feature of this model is that all acidic charged residues of the hydrophobic segments remain within the matrix half of the inner membrane while most of the basic charge residues remain within the other half of the membrane (i.e. towards the periplasmic side). One lysine residue (L_{84}) of the 2nd hydrophobic segment can possibly lie close to the 233rd glutamic residue (E_{233}), the neighbouring residue of the Oli4 mutational site (L_{232}) of the 7th hydrophobic segment. This might have significance in the formation of a Schiff base like structure during the binding of oligomycin, as has been proposed by Enns and Criddle (1977). Most of the conserved regions of the protein (see Fig. 5.21, Chapter-5) in this model (Fig.6.11) remain buried in the membrane, except one (P_{157}AGTP_{161} in yeast; PQGTP in mammals and insects) which might have some importance in interaction with the F_1 components.

Structural conservation of Subunit-6 and it's probable drug binding sites

From the hydrophobic plots and their corresponding α-helix & β-sheet plot, it is clear that subunit-6 from evolutionary diverged organisms (such as *E.coli, Aspergillus, Saccharomyces cerevisiae, Neurospora*, human, bovine, mouse, rat, *Drosophila melanogaster, D. yakuba*), have seven characteristic hydrophobic segments with potential α-helical structures. To find out whether these hydrophobic segments are conserved or not, the homologous hydrophobic segments from representatives of each evolutionary diverged group (for example bovine from the mammalian group, *D. melanogaster* from the insect group, *A. nidulans* and *Saccharomyces cerevisiae* from the fungal group, *E.coli* from the prokaryotes) were compared (Fig. 6.12). From such a comparison it is evident that there are few similarities in their aminoacid composition, but nonetheless, boundary sequences

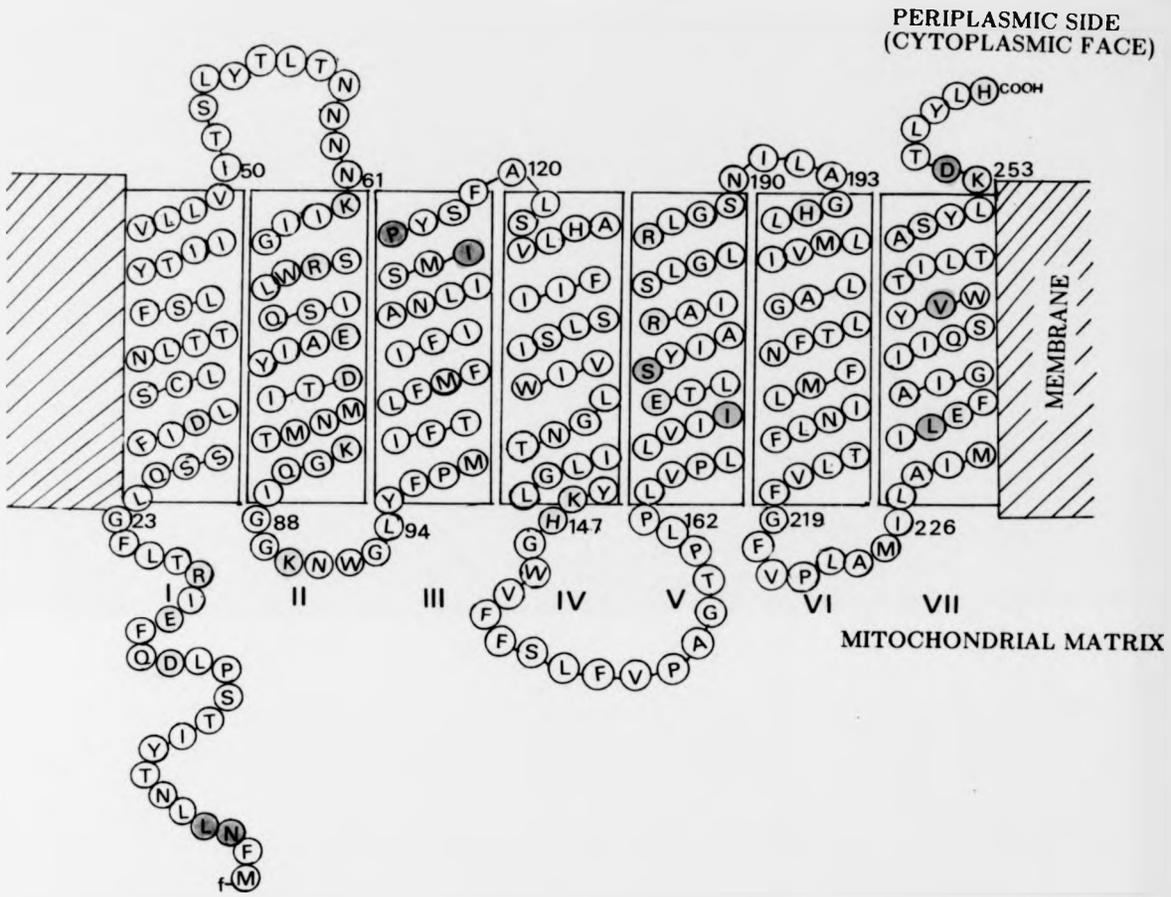
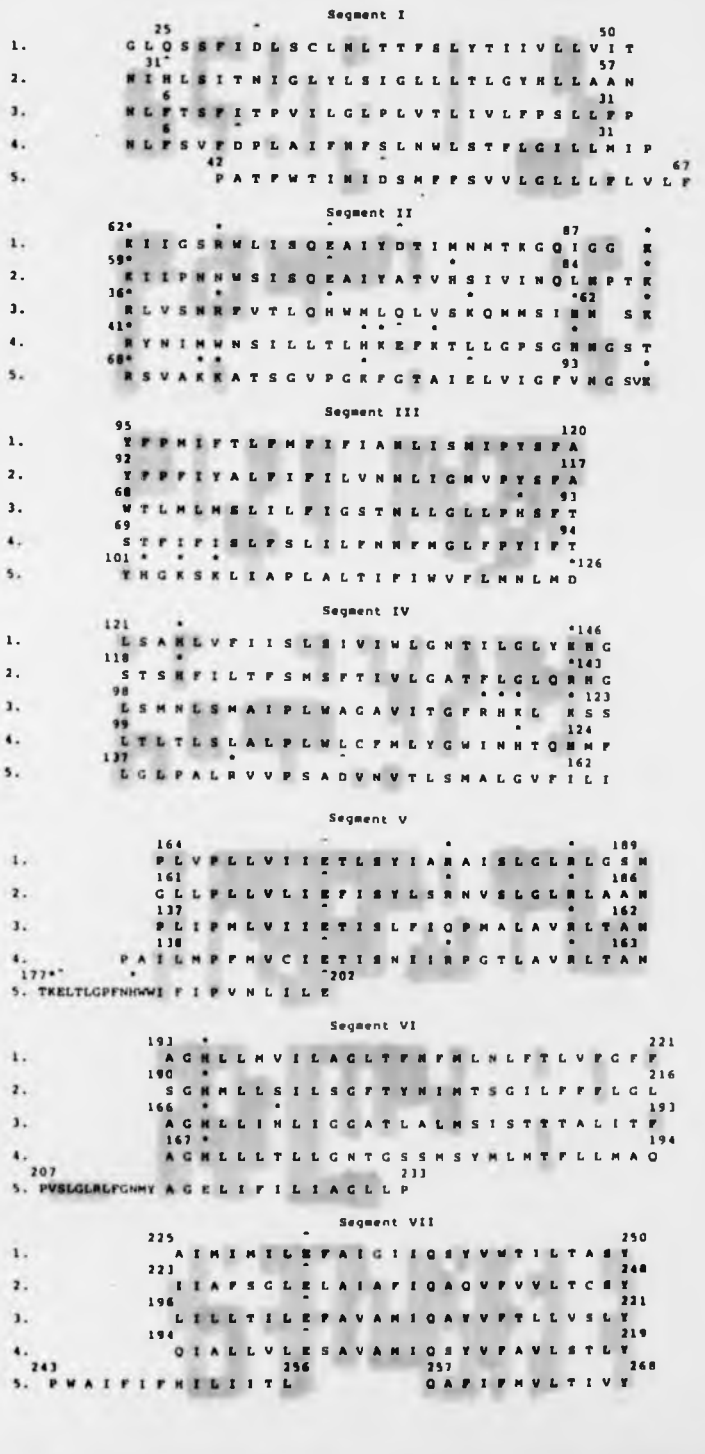


Figure 6.11 Predicted helical pathway of the subunit-6 of yeast O.S.ATPase. Seven helices are labelled as I - VII. Oli2 and Oli4 associated loci have been coloured in red while Oss1 associated locus has been coloured in green. Charged amino acids (acidic and basic) have been coloured yellow. Blue colour shows the position of intron found in *Neurospora* subunit-6 gene. The number indicates the position of aminoacid in the protein chain.

Figure 6.12. Comparison of aminoacid sequences of predicted seven transmembrane segments of the subunit-6 from various organisms.
(1) S. cerevisiae (2) A. nidulans (3) Bovine (4) D. melanogaster (5) E. coli
- indicates acidic amino acid residue and * indicates basic amino acid residue



of each segment tend to be similar. Similar characteristics have been noticed by Chou & Fasman (1978) in other proteins. Exceptions to this are the hydrophobic segments V, VI and VII, which are much more conserved, especially in relation to charged aminoacid distribution and their neighbouring aminoacids.

Interestingly, all oligomycin resistant mutations have been shown to lie within the hydrophobic segments V and VII (Oli-2 and Oli-4 block, respectively; See Chapter-5). Comparison of these segments from *E.coli* to the others is very interesting. The Oli-4 locus, which is due to a change in the neighbouring leucine of a conserved glutamic acid of the segment VII is absent in *E.coli*. In fact, a region of 10 aminoacids of segment VII are missing from the corresponding *E.coli* hydrophobic segment. This could be a reason why oligomycin cannot inhibit oxidative phosphorylation in *E.coli*. Added to this observation, is the presence of an isoleucine in place of a conserved valine residue (10 residue downstream of glutamic acid of hydrophobic segment VII) in the subunit-b of *E. coli*. Molecular analysis from a mouse cell line (Slott *et al*, 1983) has revealed that when this valine is replaced by a glutamic acid, the cells become oligomycin resistant. Therefore both these factors may have some bearing on why E.coli ATP synthase does not bind oligomycin.

Three other interesting aspects which emerge from comparison of these hydrophobic segments are:

i) The Oli-2 mutation site which is on segment V is present in *E.coli*, but on the whole, half of this segment is very similar to the eukaryotic counterpart, while the other half is not.

ii) Half of the hydrophobic segment VI from *E.coli* is similar to the eukaryotic counterpart.

iii) The left half of the hydrophobic segment VI from *E.coli* has some similarities (SLGLRL) to the right half of segment V from eukaryotes.

These discrepancies reflect a different organisation of the ATP synthetase in *E. coli* in comparison to the eukaryotic system, despite having similar function of ATP synthesis.

Other points which emerge from the subunit-6 model are:

a) The hydrophobic segments II and IV are comparatively more polar and are basic in charge.

b) The hydrophobic segments I, III, V, VI and VII contain less charged aminoacids.

c) The ossamycin resistant locus assigned to a carboxyl terminal conserved charged acidic aminoacid (aspartic acid in fungus and mammals, glutamic acid in *E.coli* and insect) lie outside the membrane in all cases.

The last observation is very interesting in the sense that ossamycin possibly reacts directly with this charged residue in contrast to oligomycin which appears to interact with non-polar aminoacids.

Secondary structure prediction of subunit-8

The hydrophobicity profiles of subunit-8 from various organisms is shown in Fig. 6.13. Characteristically, fungal subunit-8 (e.g. *Saccharomyces* and *Aspergillus*) shows a strong hydrophobic segment in the middle, in comparison to those from insects (*D. melanogaster* and *D. yakuba*) and mammals (bovine, human, mouse, rat) where it is the amino terminal end which is very hydrophobic. Secondary structure analysis (in terms of α-helix and β-sheet plot) reveals that the hydrophobic segment has a strong α-helix forming potentiality, with the exception of *Drosophila*, where the last few residues of this hydrophobic segment shows a tendency to form β-sheet. The boundaries of hydrophobic segments of this subunit from various organisms and their α-helix forming potentiality is shown in Table 6.6.

α-helix and β-sheet plot analysis of yeast subunit 8 using table 6.3 shows a strong α-helix potentiality (Fig. 6.13a) of about 85-90% and this conclusion is supported by CD spectral analysis of isolated subunit-8 reconstituted in lipid vesicles by Dr. Velours. The CD spectral analysis indicates a α-helix potentiality of more than 85% which is exactly similar to our predictive value (Dr. Velour, personal communication). On the basis of the above

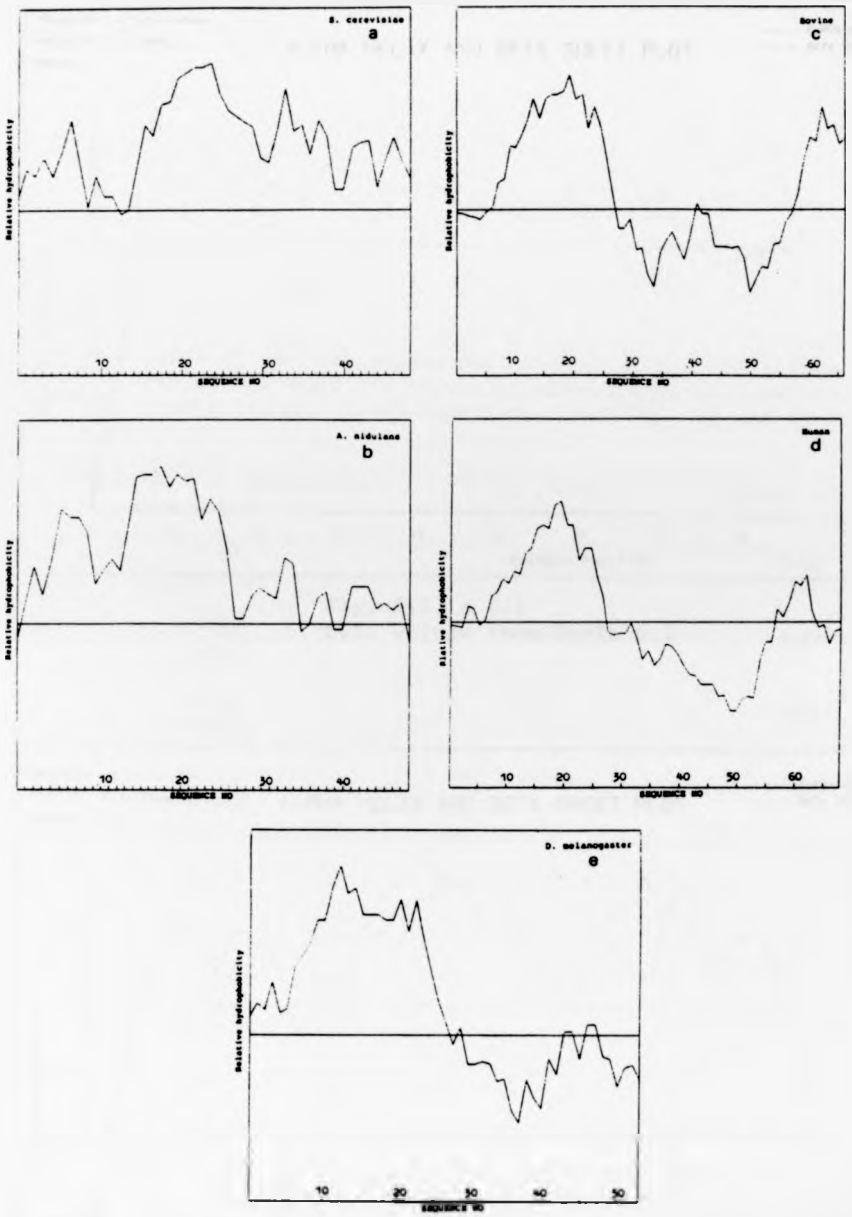


Figure 6.13 Comparison of the hydrophobicity profiles of subunit-8 from various organisms. (a) *S. cerevisiae*, (b) *A. nidulans*, (c) Bovine, (d) Human and (e) *D. melanogaster*. The sequence has been plotted along the horizontal axis and by calculating a string of 9 aminoacids at a time, a hydrophobicity profile can obtained.

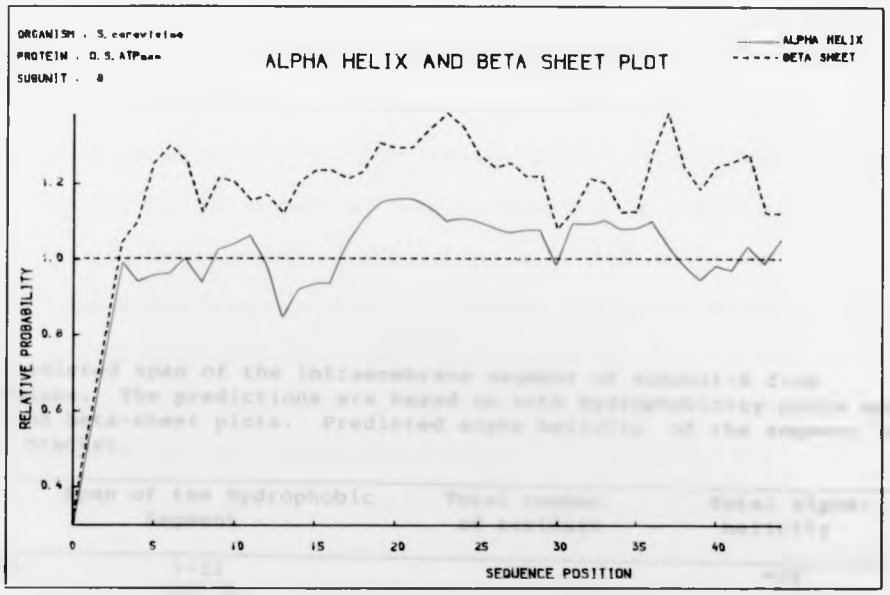


Fig. 6.13 a (1)
ref. values from Table 6.2

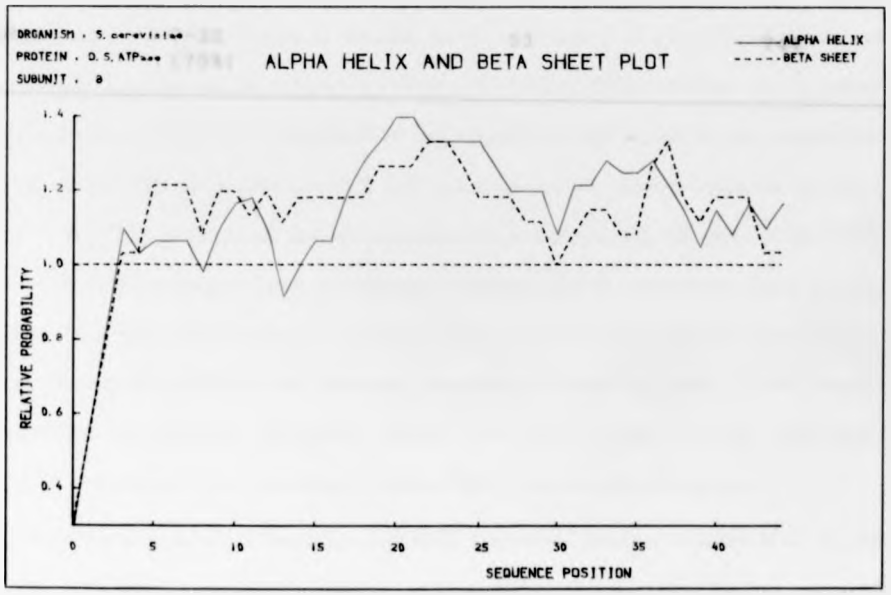


Fig. 6.13 a (2)
ref. values from Table 6.3

Table 6.6 Predicted span of the intramembrane segment of subunit-8 from various organisms. The predictions are based on both hydrophobicity plots and alpha-helix and beta-sheet plots. Predicted alpha helicity of the segment is given in the bracket.

Organism	Span of the Hydrophobic Segment	Total number of residues	Total alpha-helicity
S. cerevisiae	7-32 (93.3%)	48	87%
A. nidulans	7-32 (100%)	48	90%
Bovine	6-31 (100%)	66	85%
D. melanogaster	7-32 (70%)	53	74%

hydrophobicity and secondary structure plots, a tentative model is proposed in Fig. 6.14 for subunit-8 of *Saccharomyces cerevisiae*.

In this model, the amino terminal end (7th-32nd aminoacid residue) remains buried in the membrane and the basic charged tail remains exposed to the exterior but there is at present no independent supporting evidence due to the lack of protease cleavage and chemical modification data. Also there is no evidence whether the exposed basic tail of this subunit lies on the periplasmic side or in the matrix side of mitochondria. However, biogenesis studies have revealed that in the lack of this subunit in the yeast *Saccharomyces cerevisiae*, the other subunits fail to assemble the F_0 complex and consequently, the F_1 part of the O.S. ATPase does not bind to the membrane (Marzuki *et al*, 1983). It is therefore probable that this polar domain binds F_1 on the mitochondrial matrix side of the membrane.

Is subunit-8 homologous to prokaryotic subunit-b

It is interesting to note that subunit-b of *E.coli* ATP sythetase has a similar predicted secondary structure (See Hoppe & Sebald, 1984) to the one proposed here for subunit-8. The secondary structure of subunit-b (Hoppe & Sebald, 1984; Walker *et al*, 1984) has been predicted to have the N-terminal 26 aminoacid domain buried in the membrane and the rest of the 125 aminoacids, which are polar in nature, remain exposed on the cytoplasmic side. The C-terminal end can be removed proteolytically (Hoppe *et al*, 1983) and it has also been suggested from mutational analysis and F_1 protection from proteolytic cleavage, that this polar domain is involved in the binding of F_1 (Friedl *et al*, 1983). The primary structures of these two subunits, however are very different. These could be a reflection of evolutionary divergence (which we have noticed between mitochondrial subunit-6 and prokaryotic subunit-a), rather than a functional divergence.

The longer domain at the C-terminal end (125 aminoacid long) of subunit-b of *E.coli* has been shown to have a repeat of a similar segment (52-82 and 85-105) (Walker *et al*, 1982). On the basis of this it is facile to speculate that a monomer of subunit-b has two similar

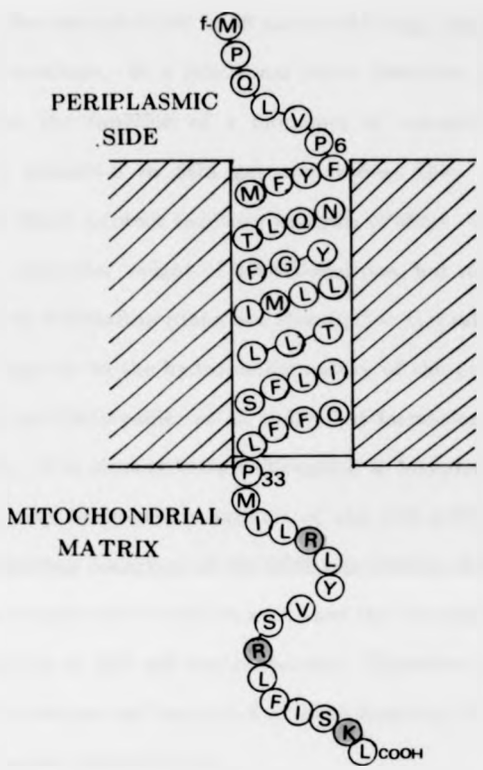


Figure 6.14 Predicted structure of yeast subunit-8 in the membrane. The protein has one membrane spanning helix towards the the N- terminal end. The extended C- terminal end with positively charged aminoacids (red colour) has been placed in the matrix side of mitochondria.

functional domains and that this is why subunit-b is longer (151 aminoacid long) than the structurally similar subunit-8 (48 to 66 aminoacid long, depending on the species) of mitochondrial ATP synthase. In a functional sense therefore, a dimer of subunit-8 may be required to serve the function of a monomer of subunit-b. The observed anomalous mobility of the subunit-8 in SDS gel may reflect this. The DNA sequence analysis (Chapter-5) and direct protein sequence analysis (Velour *et al*, 1984) indicate that yeast subunit-8 has a molecular weight of 5.8 Kilodaltons, but it moves to a region of gel calibrated to be 10-12 Kilodalton (data not shown; See Marzuki *et al*, 1983). This has been explained as being due to the hydrophobic nature of the protein which causes anomalous mobility in SDS gel (Macreadie *et al*, 1983), but formation of a stable dimeric species is also a possibility. It is already known (Tzagoloff & Meagher, 1971) that subunit-9, which is also an extremely hydrophobic subunit of the O.S.ATPase, can remain in hexameric form in the denaturing condition of the SDS gels (Weber & Osborn, 1969; Lamelli, 1970). The interaction of subunit-8 could be such that the dimeric form is not dissociated under the usual conditions of SDS gel electrophoresis. Therefore it is suggested that subunit-b of *E. coli* ATP synthase and subunit-8 of mitochondrial O.S.ATPase may be analogous, despite their apparent dissimilarities.

Structural conservation of Subunit-8

Although there is no marked homology between yeast subunit-8 and prokaryote subunit-b, subunit-8 from eukaryotes could be compared with respect to their evolutionary conservation. From the primary sequence data it is evident that this particular subunit has a N-terminal nonpolar domain and a C-terminal basic charged polar domain (See Fig. 5.22, Chapter-5). Comparison also demonstrates that the primary sequence is more conserved within the same phylogenetic group but is different from one group to another. Therefore it is possible that this particular subunit has undergone divergent selection pressure while retaining its secondary structure constant throughout evolution. Apparent dissimilarity of *E.coli* subunit-b with eukaryotic subunit-8 could readily be explained in terms of the

Figure 6.15 Comparison of the aminoacid sequences of the predicted intramembrane segment of the subunit-8 from various organisms. Predicted intramembrane segment of the E. coli subunit-b has also been included in the comparison. * indicates the charged (basic) aminoacid.

		7																							32
Yeast	P	F	Y	F	M	N	Q	L	T	Y	G	F	L	L	M	I	T	L	L	I	L	F	S	Q	F	L	L	P
		7																						*			32	
Aspergillus	P	F	F	F	V	N	Q	V	I	F	A	F	I	V	L	T	V	L	I	Y	A	F	T	K	Y	I	L	P
		7																				*		*			32	
Bovine	T	S	T	W	L	T	M	I	L	S	M	F	L	T	L	F	I	I	F	Q	L	K	V	S	K	H	N	F
		7																									32	
Drosophila	P	I	S	W	L	L	L	F	I	I	F	S	I	T	F	I	L	F	C	S	I	N	Y	Y	S	Y	M	P
		1																						*			26	
E. coli		M	N	L	N	A	T	I	L	G	Q	A	I	A	F	V	L	F	V	L	F	C	M	K	Y	V	W	P

strong divergent selective pressure.

When the comparison of primary sequence is taken into account for the interpretation of the boundaries of hydrophobic domains, it is observed that the 6th aminoacid residue in most cases, is proline, a known α-helix breaker (threonine, i_α or α-indifferent, in case of mammals) and at the 33rd amino acid residue, another proline or asparagine (also α-breaker). Taking these as the boundary aminoacids of the intramembrane domain, the structure of subunit-8 from various organisms would be fairly constant in respect to α-helicity. However in terms of hydrophobicity, the domain of the 13th to 38th aminoacids in *Saccharomyces cerevisiae* would be favourable as the intramembrane domain (See Fig. 6.13). If it is true that boundaries of helical domains are very conserved, then the tentative model for subunit-8 of yeast would be very similar to other organisms and some aminoacid residues would be fairly constant in their position. To determine experimentally the boundary aminoacids for the intramembrane segment of this subunit, we must wait for proteolytic cleavage and photoreactive surface labelling data. Fig. 6.15 demonstrates the conserved aminoacids in their comparable hydrophobic segments from various orgamisms. It is noticeable that the sequences are very divergent in different groups.

Secondary structure prediction of subunit-9

Prediction of secondary structure of the subunit-9 or DCCD binding protein (subunit-c in the case of *E. coli*) was undertaken in association with subunit-6 and 8 in the hope of better understanding of oligomycin and ossamycin interaction with the F_o complex.

Fig. 6.16 and Fig. 6.17 depict the hydrophobicity profile and α-helix and β-sheet plot from yeast which are comparable to those obtained from *E. coli* (Fig. 6.18 and Fig. 6.19, respectively). Evidently, both have two strong hydrophobic segments divided by a polar hydrophilic segment. Both hydrophobic segments have the ability to span a lipid bilayer of $35A^\circ$ and can form a hairpin structure (Fig. 6.20). Different drug resistant sites and other important mutational sites were also marked in the figure. The polarity of this subunit in the membrane has not been confirmed due to lack of data. It is possible that

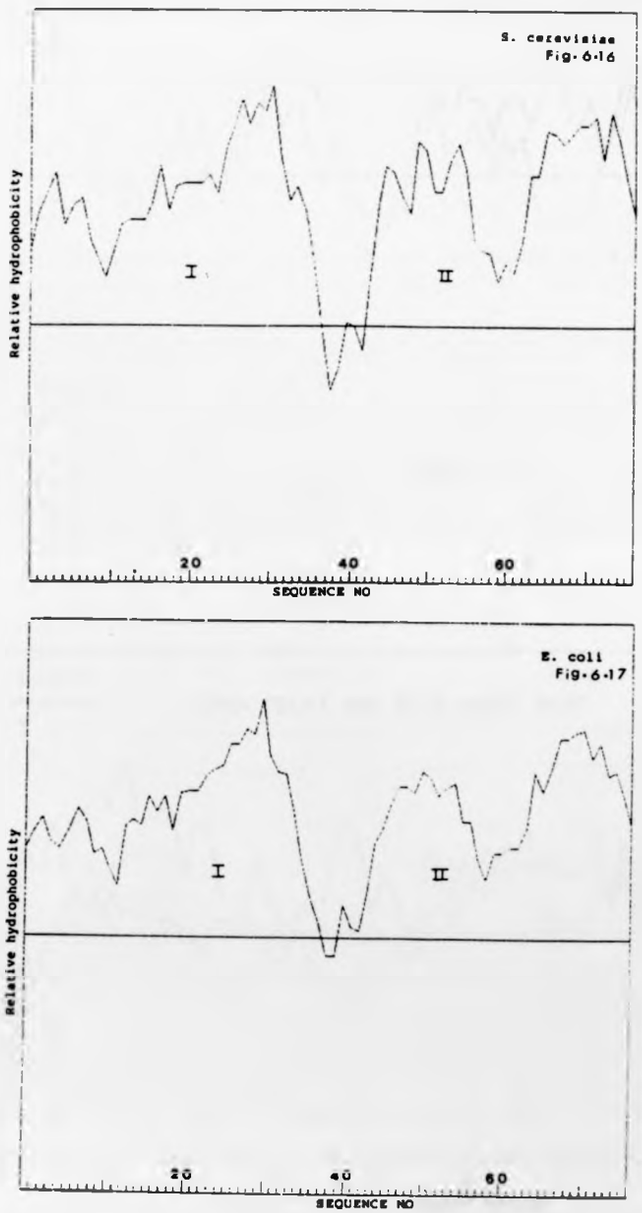


Figure 6.16 Hydrophobicity profile of the yeast subunit-9.

Figure 6.17 Hydrophobicity profile of the subunit-c of *E. coli*. The calculations were based on a string of 9 aminoacids at a time. I and II represents two strong hydrophobic segments which are predicted to be membrane buried.

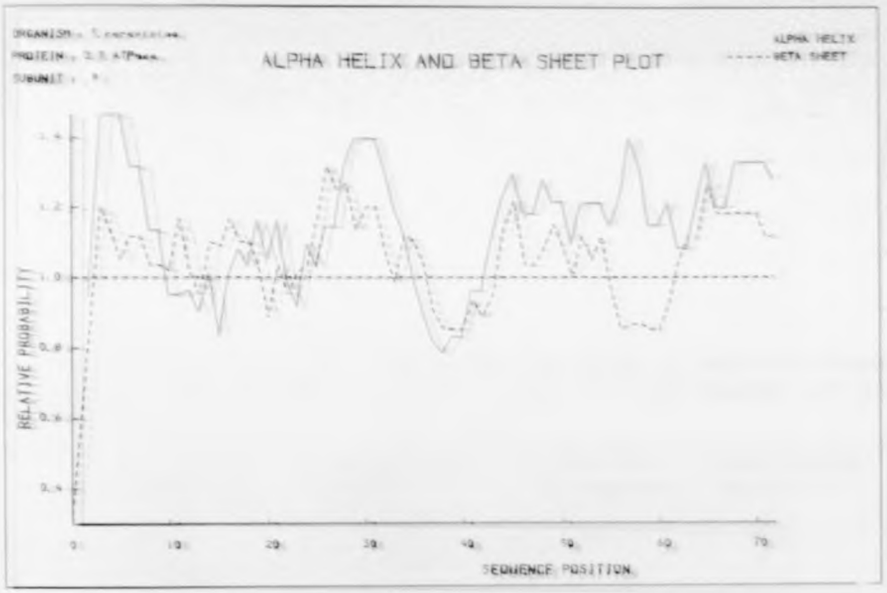


Fig. 6.18

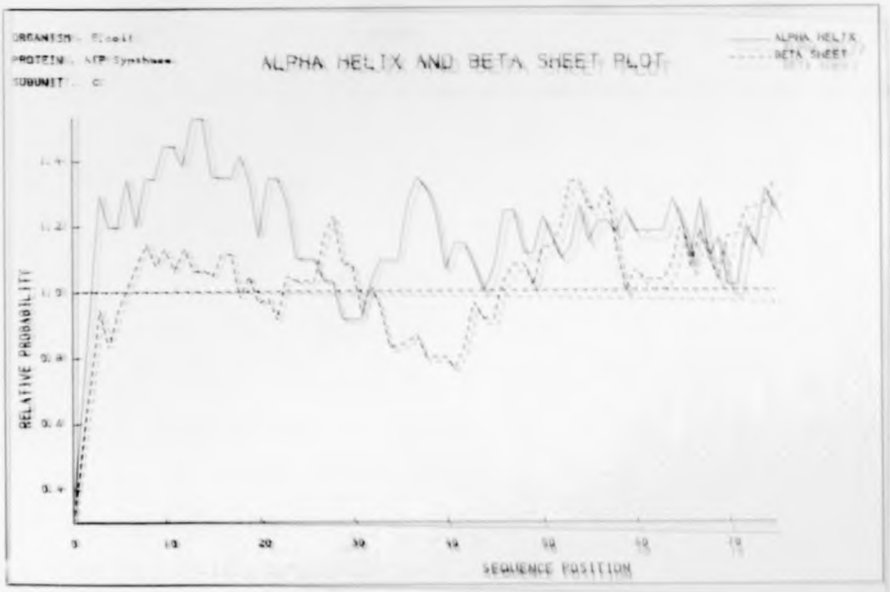


Fig. 6.19

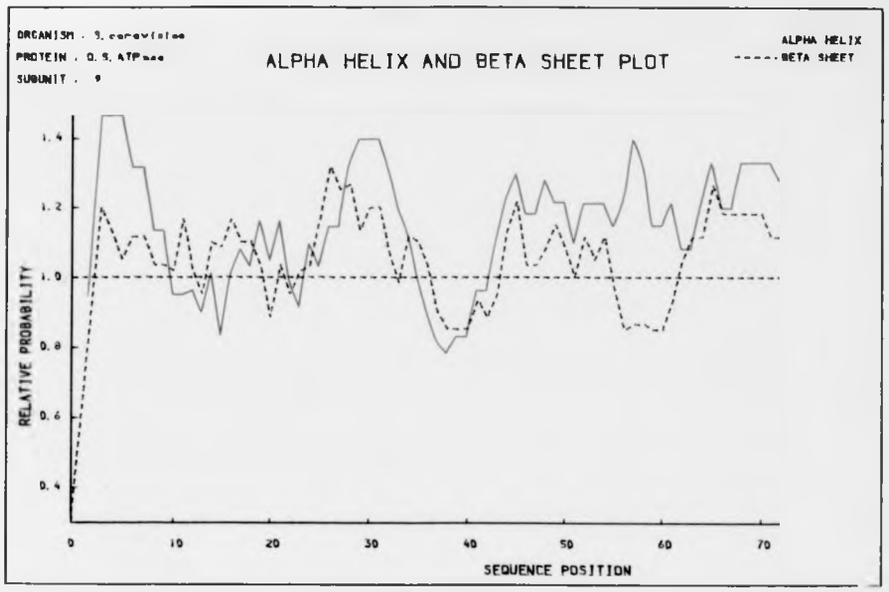


Fig. 6.18

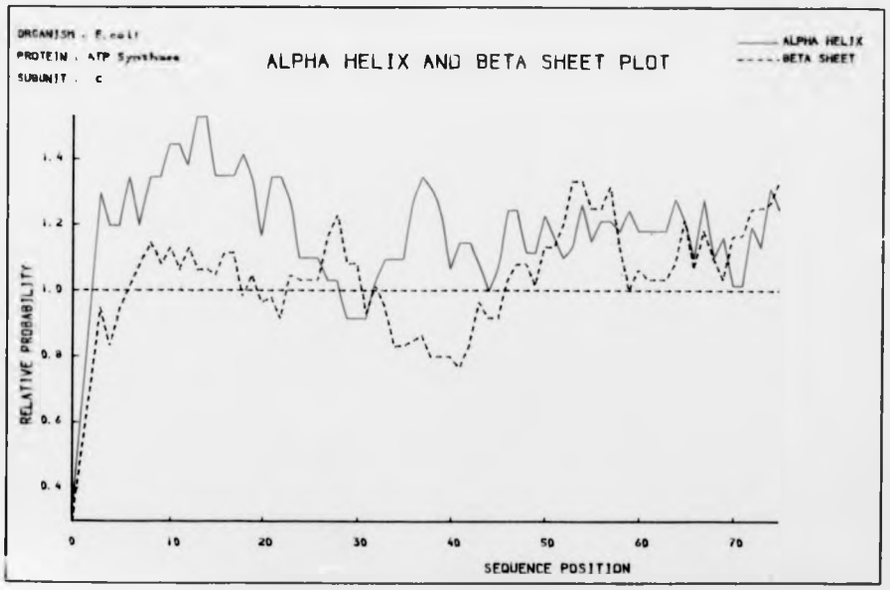


Fig. 6.19

Table 6.7. Predicted span of the intramembrane segments of subunit-9 from various organisms. Predicted alpha helicity values for each segment are given within the brackets.

Organism	Hydrophobic Segment I	Hydrophobic Segment II	Total No. of residue	Total alpha-helicity
S.cerevisiae	9-34 (77%)	46-71 (100%)	76	81%
Neurospora	15-40 (77%)	54-79 (100%)	81	79%
Bovine	8-33 (58%)	45-70 (100%)	75	78%
E. coli	11-36 (88%)	48-73 (100%)	79	87%

the middle hydrophilic domain, which is a strong antigenic determinant on the basis of predictive analysis, remains directed towards the cytoplasmic side where F_1 binds. In *E. coli*, it has been demonstrated that an antibody to the DCCD binding protein can prevent binding of F_1 to the F_o, as well as the leakage of protons across through the membrane, in an inverted but not in a right side out "vesicle" (Loo & Bragg, 1982), which supports the assumption.

α-helicity in the subunit-9 of yeast is predicted, using the modified method (table 6.3), to be 79% which fits well with the experimentally determined value of 75%-83% (Mao *et al*, 1982). This is in contrast to the method using Chou & Fasman's original data (Table 6.2), which predicts only 53% α-helicity. Subunit-c of *E. coli* gives a predictive value of 87% α-helicity using the modified values listed in Table 6.3.

Structural conservation of subunit-9 and drug resistant sites

When subunit-9 from four diverse groups of organisms (Yeast, *Neurospora*, Bovine and *E. coli*) are compared, it is found that their hydrophobicity profile and secondary structure are similar (data not shown). Conservation of amino acid sequences in two hydrophobic segments are found to be moderate (Fig. 6.21). From such comparison of homology and hydrophobicity profile, the boundary of the hydrophobic segments were determined from four various organisms, which are given in Table 6.7. The boundary shown for *E. coli* (11-36 and 48-73) is little different from one described by Senior (1982) (15-40 and 51-76). However, the DCCD reacting residue, glutamic acid (aspartic acid in the case of *E. coli*) and the associated alanine residue in the hydrophobic segment-II are highly conserved throughout evolution. All drug resistant sites, especially oligomycin resistance sites, are located surrounding the acidic charged residue. This indicates that oligomycin does not react with the charged residue in the membrane, rather it binds probably to a non-polar aminoacid by hydrophobic interaction and blocks the proton channel, either masking it directly or by its steric modification.

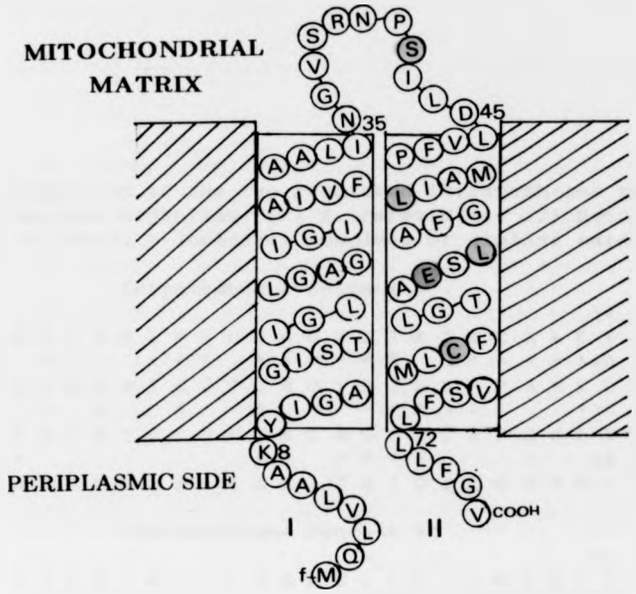


Figure 6.20 Predicted membrane spanning structure of yeast subunit-9. Two memrane spanning helices (I & II) have been shown with the Oligomycin binding sites (red colour) and the DCCD reacting glutamic acid (green). The region between the two segments has been shown to protrude into the matrix side of mitochondria.

Figure 6.21. Comparison of the two predicted intramembrane segments of subunit-9 from various organisms. (1) S. cerevisiae, (2) Neurospora (3) Bovine, (4) E. coli. * indicates homology or similar amino acids.

Intramembrane Segment I

```
          9                                                  34
1.    K Y I G A G I S T I G L L G A G I G I A I V F A A L I N G
      *15   *   *       * * *   * * * * * * *     * * * * *40 * *
2.    K N L G M G S A A I G L T G A G I G I G L V F A A L L N G
      * 8   *   *   *     *     *   *   * * *   * *     *33   *
3.    K F I G A G A A T V G V A G S G A G I G T V F G S L I I G
       11     *               * *   * * * *         *    36   *
4.    Y M A A A V M M G L A A I G A A I G I G I L G G K F L E G
```

Intramembrane Segment II

```
         46                                                  71
1.    D L V F P M A I L G F A L S E A T G L F C L M V S F L L L
       54   *     * * * * * *       *   * * *   * * *    79   *
2.    G Q L F S Y A I L G F A F V D A I G L F D L M V A L M K L
       54 * * *   * * * * * * * * * *   * * *   * * * * 79   *
3.    Q Q L F S T A I L G F A L S D A M G L F C L M V A F L I L
       48 *                     * * * *   *
4.    P L L R T Q F F I V M G L V D A I P M I A V G L G L Y V M
```

DISCUSSION

Prediction of secondary structure

The basis of prediction of the secondary structure described in this chapter has revealed that the use of both hydrophobicity profile (Kyte & Doolittle, 1982) in conjunction with the α-helicity plot using a modified table (Table 6.3) from Chou and Fasman (1978) is a useful way to look at the tertiary structure of membrane proteins. Modified data used in Table 6.3 has been assigned completely empirically, but using the argument that comparable α-former and β-former or comparable α-breaker and β-breaker should have a comparable value. On this basis the prediction of α-helicity correlates well with independent experimentally determined values of α-helicity, as shown in the case of bacteriorhodopsin, intramembrane segment of erythrocyte glycophorin, subunit-9 and subunit-8 of the O.S.ATPase (Table 6.8). The α-helix plots derived from modified values can be superimposed on the hydrophobicity plot of the respective protein with better correlation. This modified data used in Table 6.3 should be used for membrane proteins only. But for soluble proteins, Chou & Fasman's original table (Table 6.2) would probably be a better one. In all cases however, one should take the empirical rules depicted by Chou & Fasman (1978) into account for better prediction.

Model for oligomycin induced blockage of proton translocation in F_o

The inhibition of proton translocation and oxidative phosphorylation by oligomycin was established long ago (Lardy, 1958; Lardy *et al*,1964). But until recently it was not known which components of the O.S. ATPase complex are involved in the binding of oligomycin. By direct enzyme inhibition studies, it was inferred that it was the F_0 part of the complex, not the F_1, which is involved in oligomycin binding (MacLennan & Tzagoloff, 1968). The fact that the membrane sector of the O.S. ATPase is involved in oligomycin binding can be derived from the results where it has been demonstrated that the oligomycin sensitivity of beef heart SMP (sub mitochondrial particles) ATPase decreases by treatment

TABLE 6.8. Comparison of predicted alpha-helicity with those of experimentally determined values.

Name of the Pritein	Predicted helicity		Experimentally Observed helicity
	Using table 6.2 (Chou & Fasman)	Using table 6.3 (This thesis)	
Bacteriorhodopsin	52 %	82 %	75-80 %
Intermembrane segment of Glycophorin	47 %	>90 %	~ 100 %
Subunit 9 of the O.S.ATPase (Neurospora)	59 %	79 %	75-83 %
Subunit 8 of the O.S.ATPase (S.cerevisiae)	53 %	87 %	~ 85 %

with organic solvents (Broughall *et al*, 1972; Lenaz *et al*, 1975). Mitchell demonstrated that the functional effect of oligomycin is due to a decrease in the proton permeability of membranes (Mitchell, 1967).

Affinity labelling of mitochondrial ATPase by [^{3}H]-borohydride in the presence of oligomycin offered the suggestion that the low molecular weight proteolipid (assumed to be the subunit 9 or DCCD binding protein) is the site of oligomycin binding (Enns & Criddle, 1977). In the light of present DNA sequence data from oligomycin resistant mutants (this thesis, Macino & Tzagoloff, 1979, 1980; Novitski *et al*, 1984) it is well illustrated that at least two subunits, subunit-6 and subunit-9 of the O.S. ATPase can bind oligomycin. So far, no oligomycin resistance locus has been found in the subunit-8 structural gene. The participation of subunit-8 in oligomycin binding is therefore doubtful. However, participation of component(s), other than subunit-6 and subunit-9 can not be ruled out as some nuclear inherited mutations for oligomycin resistivity which show cross resistance to a range of other inhibitors of oxidative phosphorylation (e.g. Triethyl tin (TET), venturicidin (VEN), Rhodamine 6G (Rh6G), See Griffiths *et al*, 1972) have been reported. Although these mutations can be better explained in terms of a plasma membrane or cytoplasmic protein factor, in the absence of direct data, it is better to keep this question open.

However, with the present available data, it appears that at least two hydrophobic segments of the subunit-6 (segment V and segment VII) and one hydrophobic segment of the subunit-9 (Segment II) are involved in oligomycin binding. All the aminoacids so far, which have been shown to have undergone mutation in oligomycin resistant strains, are crowded around a single acid charged residue, glutamic acid, within a hydrophobic environment (Fig. 6.11 & 6.20). The only exception to this is the 42nd residue (serine) in subunit-9 from *Saccharomyces cerevisiae* (Sebald *et al*, 1979), which according to the present secondary prediction model, lies outside the membrane. Interestingly, this serine is also two aminoacids away from another acidic residue aspartic acid (Asp_{45}). The oligomycin resistivity in this strain however, was an acquired effect observed following a

reversion from an ATPase deficient pho-2 (*mit*) mutation.

The residues responsible for oligomycin resistivity (see Fig. 6.11 & 6.20), do not cause any change in the energy conservation system in yeast. The growth rates and growth yields of the mutant strains are the same as the parental strains (Griffiths & Houghton, 1974). In addition no differences in the respiratory rates of mitochondria and mitochondrial particles from parental and mutant strains have been observed. It was therefore interesting to examine whether the supposed oligomycin binding residues (i.e. mutational residue conferring oligomycin resistivity) of subunit-6 and subunit-9 are directed towards "assumed" proton channels or towards the lipid environment. For this, the "helical wheels" model (Schiffer & Edmundson, 1967) has been used to represent the structure of the hydrophobic segments of the subunits. Two dimensional projections or the "helical wheels" of each hydrophobic segment of subunit-6 and subunit-9 are given in Fig. 6.22 & 6.23.

As oligomycin mutations have been ascribed only to the carboxyl terminal segment of the subunit-9 (segment II of subunit-9) and the segments V and VII of the subunit-6, I will consider these three segments for interpretation of oligomycin interaction. Fig. 6.24 represents diagramatically, the helices of the above three segments surrounding a channel like structure. The mutated sites causing oligomycin resistance have also been illustrated in the figure. It is evident from the diagram that most oligomycin binding sites are in fact directed towards the lipid environment, while functionally important glutamic acid residues are directed towards the channel forming sides. Oligomycin, being hydrophobic in nature, probably binds from inside the lipid environment to the reacting aminoacids I_{171}, S_{175}, L_{231}, V_{242} of subunit-6 and L_{57}, L_{53} of subunit-9) and then probably sterically modifies the conformation of the proton channel which leads to the blockage of proton conduction and oxidative phosphorylation. The alteration of those above mentioned aminoacids prevent the binding of oligomycin, without affecting the conformation of the proton conducting channel. This is probably the reason why both mutant resistant strains and sensitive parental strains are equally efficient in oxidative phosphorylation. The other conserved residues, marked in the diagram, may also be involved in oligomycin binding.

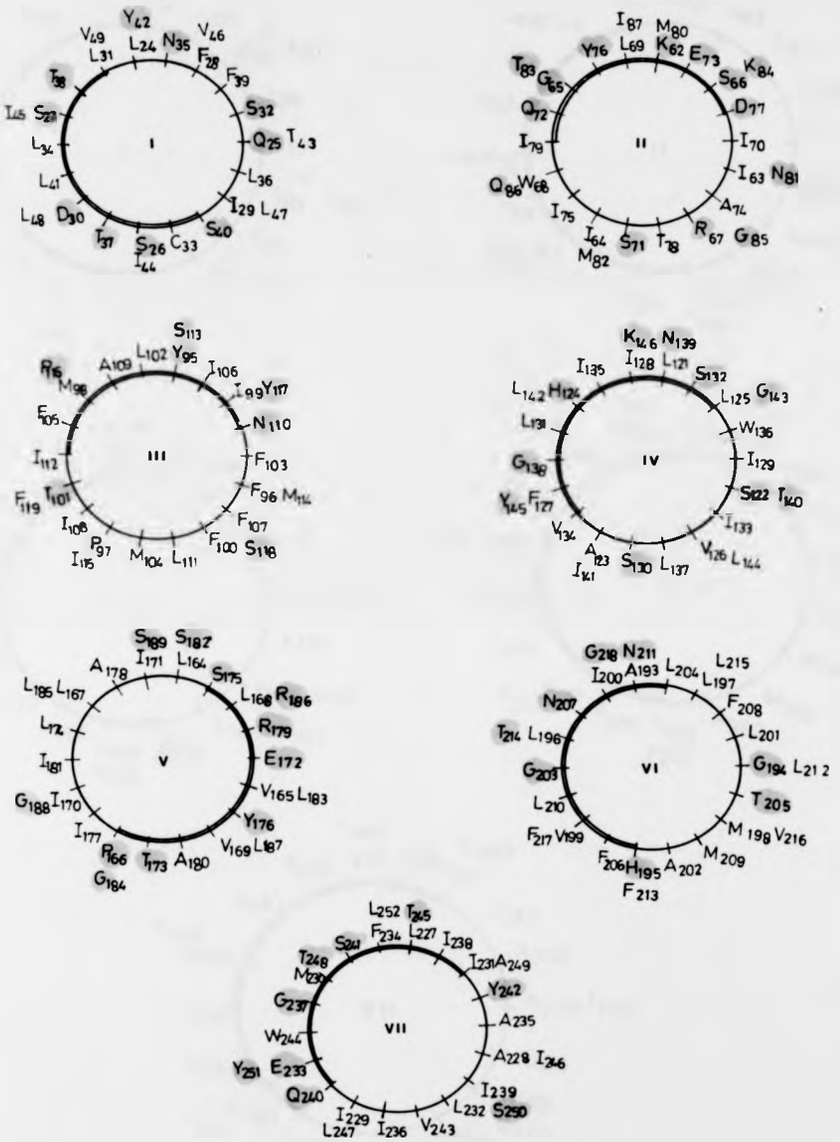


Figure 8.22 Helical wheel model for the seven hydrophobic segments (I-VII) of the subunit-6 of yeast. Aminoacids have been shown in single letter code, with the subscript numbers indicating the residue number in the protein chain. Aminoacids with membrane buried preference less than 1.0 have been shown in orange colour. The double line region of the wheels is predicted to face proton channel, while the single line region is predicted to face lipid bilayer.

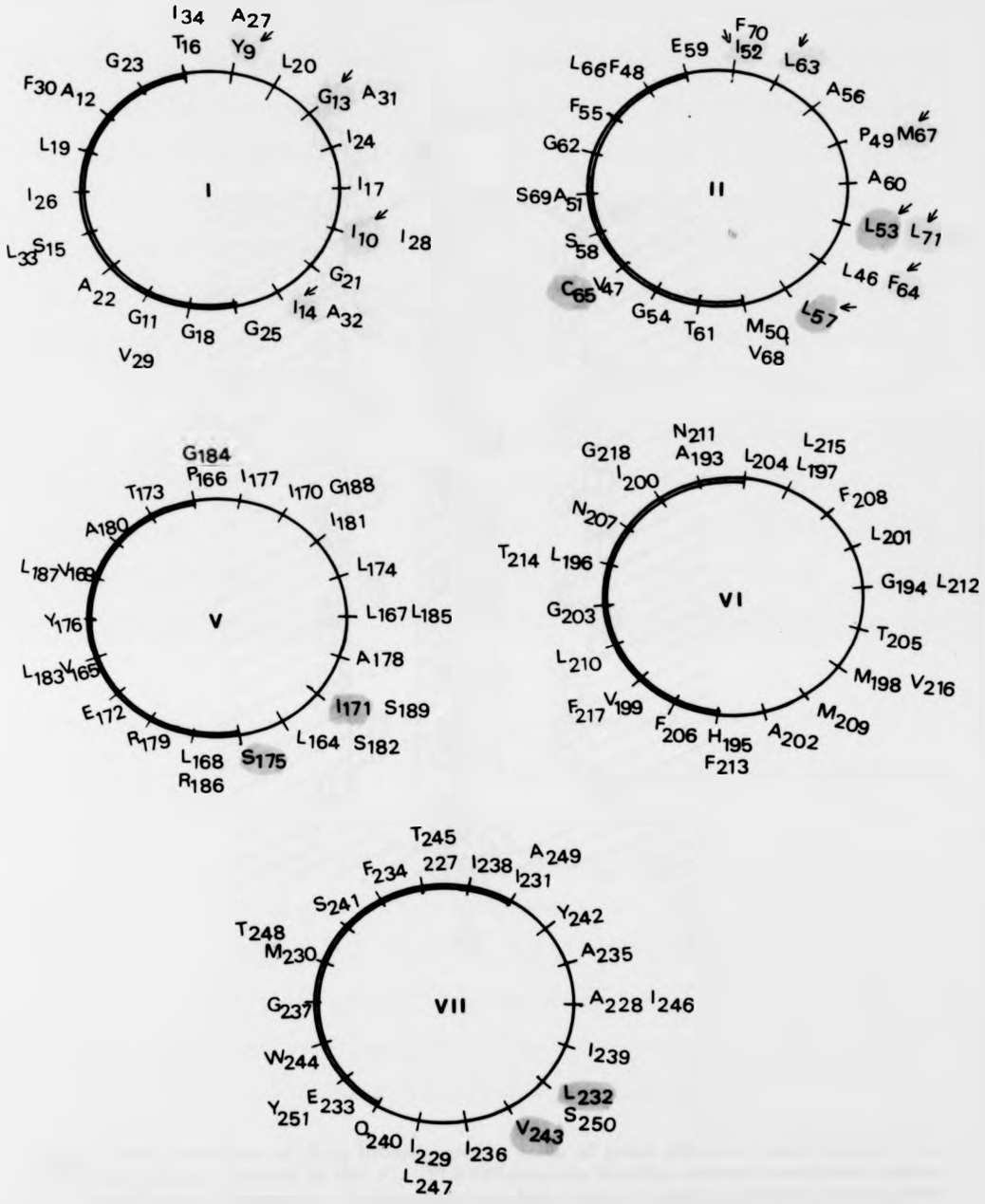


Figure 6.23 Helical wheel model for the two hydrophobic segments (I & II) of yeast subunit-9 and last three conserved hydrophobic segments (V, VI, & VII) of yeast subunit-6. Amino acids have been shown in single letter codes with the subscript numbers indicating the residue number on the protein chain. The double line regions are predicted to face the proton channel while single line regions are predicted to face lipid bilayer. The supposed oligomycin binding sites have been coloured in red and [^{125}I]-TID labelled aminoacids (Hoppe & Sebald, 1984), which are expected to face the lipid bilayer have been coloured in yellow.

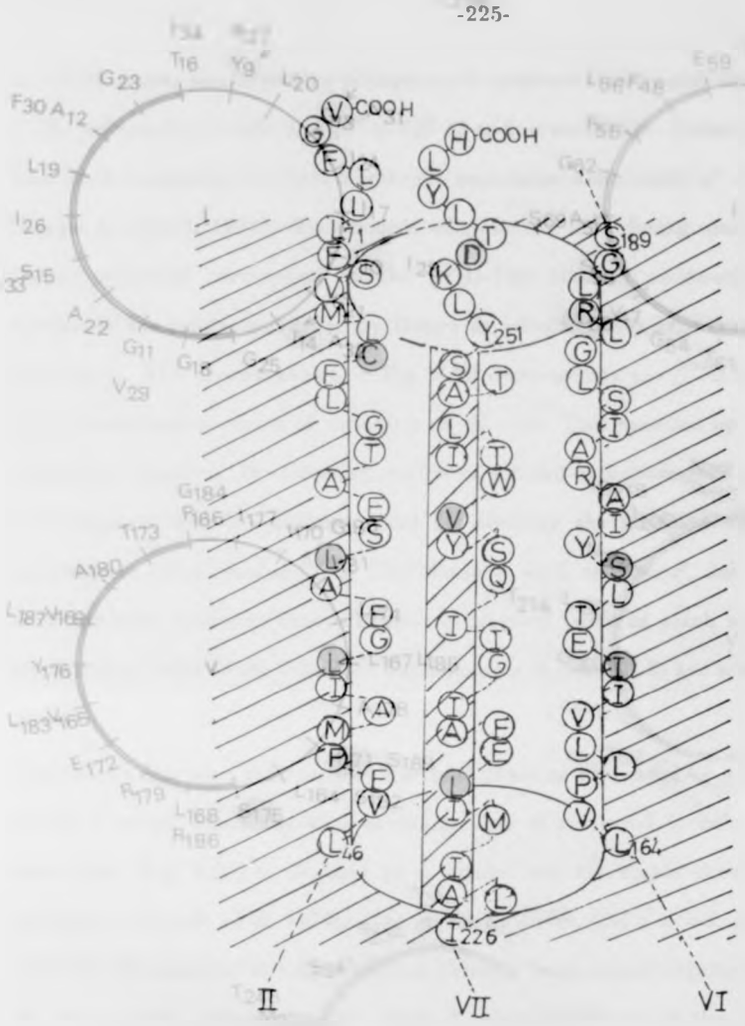


Figure 6.24 Location of drug binding amino acids of yeast subunit-6 and subunit-9 in relation to proton channel in the F_o. The Oligomycin binding residues have been shown in red while the Ossamycin binding residue has been shown in yellow. Only three intramembrane segments have been shown surrounding the proton channel for illustration: the second hydrophobic segment of the subunit-9 (II), the Vth (Oli2 site) and VIIth (Oli4 site) hydrophobic segments of subunit-6.

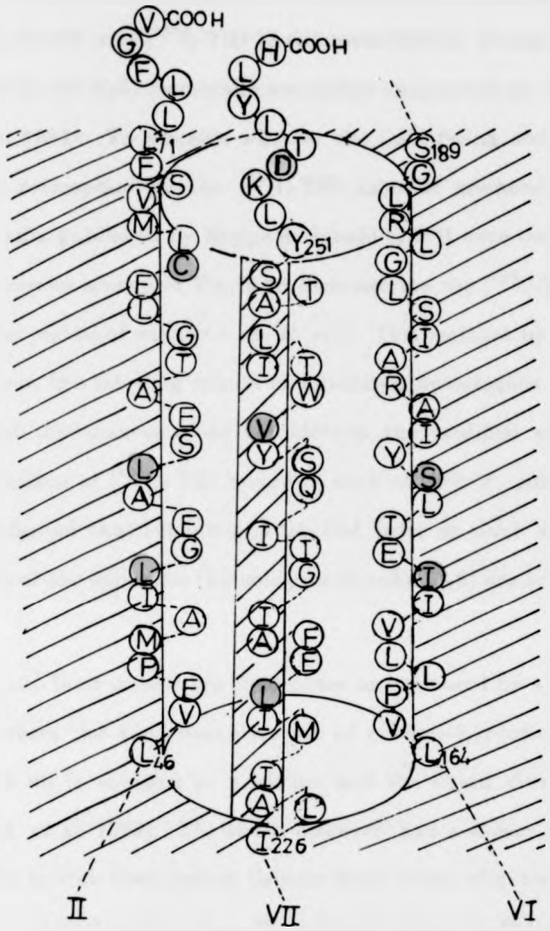


Figure 6.24 Location of drug binding amino acids of yeast subunit-6 and subunit-9 in relation to proton channel in the F_0. The Oligomycin binding residues have been shown in red while the Ossamycin binding residue has been shown in yellow. Only three intramembrane segments have been shown surrounding the proton channel for illustration: the second hydrophobic segment of the subunit-9 (II), the Vth (Oli2 site) and VIIth (Oli4 site) hydrophobic segments of subunit-6.

In recent years, photoreactive phospholipid analogues and small lipophilic reagents, e.g. (^{125}I)- iodonaphthylazide and (^{125}I)-TID (3-trifluormethyl-m- (iodophynyl)-diazirine) have been used to identify the lipid membrane associated aminoacids of *E. coli* F_o components (Hoppe & Sebald, 1984). To examine whether the lipid facing aminoacids of the hydrophobic segments correspond to the (^{125}I)-TID labelled aminoacids of subunit-6 and subunit-9, the results published by Hoppe & Sebald (1984) were compared to the present prediction. The arrows shown in Fig. 6.23 demonstrate the [^{125}I]-TID labelling aminoacids of homologous region of subunit-c of *E. coli*. This matches up well with the present prediction. However, the labelling results of subunit-a (homologous to subunit-6 of yeast) is ambiguous, and the authors could not identify the modified aminoacid residues. A quantitative estimation of [^{125}I]- TID bound to each of three F_o subunits reported by the same authors, indicated that subunit-a is labelled twice as much as subunit-b. In other words, large parts of the subunit-a (homologous to subunit-6) are accessible from the lipid phase.

Oligomycin may also bind outside the lipid phase as evidenced by a *mit* (pho2) revertant strain of yeast, where the 42nd serine residue of subunit-9 (predicted to be outside the membrane, Fig. 6.20) is changed to a leucine and the strain shows a weak oligomycin resistivity (Sebald *et al*, 1979). The strain however, had a slower growth rate. Presumably the alteration in this 42nd residue (leucine from serine) slightly affects the binding of F_1 (it has been proposed earlier that F_1 probably binds to the protruding intermembranous polar domain of subunit-9) and consequently proton conducting channel is somehow affected. The weak oligomycin resistivity observed therefore, could be due to the steric modification of the F_o as a consequence of a slightly altered binding of F_1 to F_o.

DCCD-binding sites and Oligomycin binding sites

Averer & Griffiths (1970) observed that oligomycin resistant mutants (Oli-1) had an increased resistance to DCCD. Quantitative estimation was not possible due to technical problems of absorption and hydrolysis of DCCD on drug-agar plate. Sebald *et al* (1979)

also demonstrated in *Neurospora* that alteration in the small proteolipid or subunit-9 result in a concommitant hypersensititity towards DCCD. It has been found that in DCCD resistant mutations of *E.coli* the mutated residues are clustered around the DCCD reacting aspartic acid (glutamic acid residue in case of mitochondria) as we see for the oligomycin resistivity. On the basis of all these evidences, it is likely that DCCD binding sites partly overlap with oligomycin binding sites of subunit-9.

Ossamycin inhibition of proton conduction in the F_0

Analyses of DNA sequences from ossamycin (Oss-1) resistant strains (CD40, D27/92) of yeast have established that a conserved aspartic acid residue (at the 254th position) of subunit-6 is involved in ossamycin binding. Comparison of this position from other organisms has revealed that in *E.coli* and in *Drosophila* the position is occupied by a glutamic acid instead of aspartic acid (Fig. 5.19c). In *Saccharomyces cerevisiae* when aspartic acid residue changes to asparagine, the strain becomes ossamycin resistant. This indicates that the conserved charged acidic residue of subunit-6 in the F_o is involved in ossamycin binding and/or inhibition.

Like the Oli2 linked Oss1 marker, mentioned above, the Oli1 linked Oss2 marker has been shown to affect ossamycin resistivity in *Saccharomyces cerevisiae* (Lancashire and Mattoon, 1979b). Although no sequence information is available from Oli1 linked Oss2 marker, it could be inferred that the subunit-9 (Oli1 locus) is also involved in ossamycin binding. No ossamycin resistance locus has been described for the subunit-8 gene. Therefore ossamycin, like oligomycin, probably does not bind to the subunit-8. Biochemically and genetically, it has been demonstrated that some oligomycin strains possess ossamycin resistivity (Griffiths & Houghton, 1974; Ray & Connerton, unpublished results). This is probably due to the overlapping of the oligomycin and ossamycin binding sites. Consequently, those aminoacids shown to bind oligomycin might be involved in ossamycin binding as well. This is supported by the fact that petite DS14, a *Oli*2^R strain (Macino & Tzagoloff, 1980), can grow on Ossamycin containing media after crossing to a sensitive ρ^+

strain (data not shown), despite the fact that it has retained an aspartic acid residue at the 254th position like the ossamycin sensitive parental strain.

Aspartic acid residue (D_{254}) of subunit-6 which seems to bind ossamycin, lies outside the lipid bilayer in the present predicted model of subunit-6 (Fig. 6.11) and directed towards periplasmic space of mitochondria. The structure of ossamycin is not known but possess amino sugar which is absent in oligomycin (See Appendix). It is assumed that ossamycin interacts both inside and outside the membrane, and in doing so it alters the steric conformation of the proton channel and blocks oxidative phosphorylation. Like oligomycin, ossamycin does not alter the proton conductive pathway directly, as evidenced by the fact that ossamycin resistant strain has got a similar growth rate and respiration to those of the wild type.

Proton conductive path in the F_o and mechanism of proton conduction

On the basis of models proposed for different subunits and other experimental data obtained, indication for a proton conductive path in F_o is favourable. But how the components of F_o assemble together to form the proton channel and how many such channels they form and how the proton conduction through the channel is linked to ATP synthesis in F_1 are not well understood. In a reconstitution experiment of subunit-9 into lipid vesicles, Schindler & Nelson (1982) have demonstrated that subunit-9 can itself self assemble to form selective proton channels. Loo & Bragg (1982) also showed that anti subunit-9 antibody can block proton translocation across the membrane. These experiments indicate that subunit-9 probably constitute the main proton channel in the F_o. However, these results are contradictory to the results obtained from a *E. coli* mutant (Friedl *et al*, 1983), where it has been demonstrated that the F_o containing only subunit-c fails to translocate protons. Hoppe & Sebald (1984) suggested that subunit-c might form the proton channel which are stabilised by the other two subunits (subunit a & b in *E. coli*, and subunit 6 & 8 in yeast or other eukaryotes). Genetic data in yeast however, indicate the participation of both subunit-6 and subunit-9 in the formation of proton channel.

From reconstitution experiments Schindler & Nelson (1982) proposed the possibility of three proton channels formed by a hexameric aggregate of the proteolipid or subunit-9. These proposals were based on the following arguments:

(i) The amount of protons conducted through the membrane could be best explained on the basis of a dimer constituting a single proton channel.

(ii) stoichiometry of three channels (formed by three dimers) per hexamer matches the occurrance of three α and three β subunits of ATPase complex (Todd *et al*, 1979).

(iii) Three protons are required to cross the membrane for the formation of each ATP molecule (McCarty, 1978).

Inhibitory data for oxidative phosphorylation from this laboratory however, favours the existence of a minimum of two channels, as it has been shown that in a certain concentration of Ve2383 (an organotin compound, See appendix for the structure), the ATPase reacton or ATP driven proton pump can be stopped completely, while ATP synthesis still continues (Emanuel *et al*, 1984, Griffiths & Connerton, unpublished results). Genetic data also favours existence of two possible channels. In Oli-1 resistant mutants, a 2 to 3 fold higher concentration of oligomycin is needed for inhibition of oxidative phosphorylation in comparison to Oli-2 resistant mutants both in *vitro* and *in vivo* (Griffiths & Houghton, 1974). Taking the stoichiometry of subunit-6 to subunit-9 as 4:3 (see Enns & Criddle, 1977; and Todd *et al*, 1980) and considering that, at least two hydrophobic segments of subunit-6 and one hydrophobic segment of subunit-9 bind oligomycin, the effective ratio of oligomycin reacting sites of subunit-6 to subunit-9 becomes 8:3. Therefore in Oli-2 resistant mutants, oligomycin inhibitory concentration, in the absence of subunit-6 binding sites, should be 2.6 fold as low as that of Oli-1 resistant mutants. This is what has been obtained by Griffiths & Houghton (1974). In other words, two binding sites of two subunits are working independently on two different proton channels. This result of course, does not disprove the possibility that both these subunits contribute towards forming a single proton channel with two different binding sites for oligomycin.

To prove conclusively that there are two related proton channels, one for directing protons from outside to inside mitochondria (as happens during oxidative phosphorylation) and the other in the reverse direction (observed during ATP driven proton translocation), one will have to wait for discrete mutations which can demonstrate one of the two phenomena. Interestingly, Fimmel *et al* (1983) reported two *E. coli* mutants (*unc* E408 and *unc* E463) which did not have ATP dependent atebrin-fluorescence-quenching activity (i.e., no membrane potential), but were capable of carrying out oxidative phosphorylation. These two strains, however, can generate NAD dependent atebrin-fluorescence-quenching suggesting that, despite having an apparent proton impermeable membrane, they can carry out oxidative phosphorylation.

Due to the lack of data, the contribution of subunit-8 in the formation of the proton channel can not be established at present. On the whole however, it is most likely that all three subunits are selectively arranged in the membrane at a particular physiological state. Loss of proton conduction in the presence of organic solvents point to the ease with which these arrangements can be disturbed.

The mechanism of proton translocation appears to be as complex as that of proton conductive paths. The invariant DCCD reacting glutamic residue (aspartic acid in *E. coli*) of subunit-9 is undoubtedly involved in proton translocation. Chemical modification of this residue and mutational analyses have proved that it is directly involved in proton translocation (Senior & Wise, 1983; Sebald & Hoppe, 1981). However, how this glutamic acid residue and other conserved glutamic residues found in the hydrophobic protein domain of subunit-6 are involved in proton translocation, remains unresolved.

Two types of mechanism have been proposed for proton translocation. The first type of mechanism (Nagle & Morowitz, 1978) have suggested that a continuous chain of hydrogen bonds formed from the protein side group can form a "proton wire" like structure which can drive protons across the membrane. In this scheme a hydrogen-bonded chain in a protein environment (Fig. 6.25) is a long lived and more like a solid structure than like liquid water. In such a structure two different processes are involved in proton conduction (Fig.

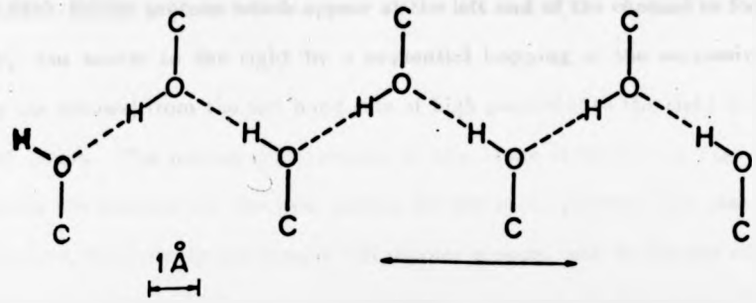


Figure 6.25 "Proton wire" or hydrogen bonded chains as carrier of protons (after Nagle & Morowitz, 1978).

A proton of a hydrogen bonded chain formed by the hydroxyl groups attached to serine side groups of a polypeptide chain (not shown). The chain is assumed to be symmetrical and perpendicular to the membrane (shown by arrow).

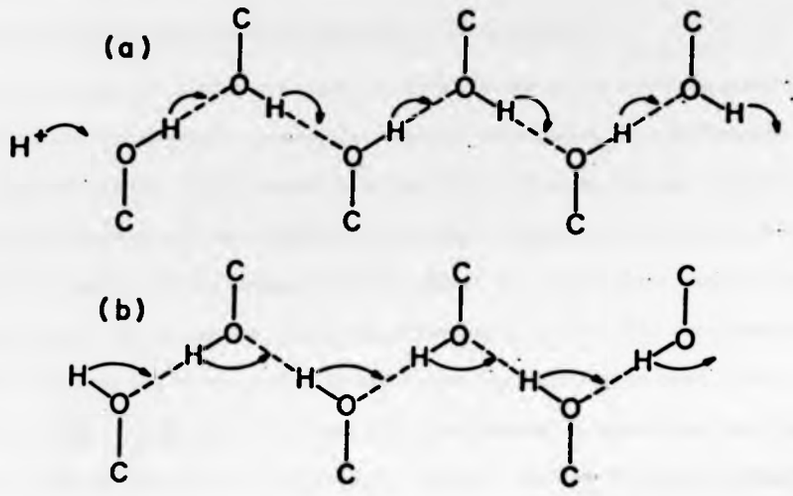


Figure 6.26 Mechanism of proton conduction in a proton wire.

(a) A proton enters from the left end to form an ionic defect that propagates to the right by sequential jumping of successive protons (shown by arrows) and ultimately reaches at the right end to give the state shown in (b).

(b) A negetive Bjerrum L fault forms on the right end by rotation around the CO axis and the defect propagates to the left via sequential rotation of successive hydroxyl groups to return to the ground state as shown in (a).

A cyclic conformational change in protein containing the amino acids which participate in making the hydrogen bonded chains may drive these reactions forward and backward and keep proton pump in active functional state.

6.26a & 6.26b). Excess protons which appear at the left end of the channel to form a positive - OH_2^+ ion moves to the right by a sequential hopping of the successive protons (shown by the arrows) from the left hand side of high potential to the right hand side of the site of action. The proton configuration in this stage is similar to Fig. 6.26a and clearly blocks the passage for the next proton by the same process. The next step suggested therefore, involves the rotation of OH dipolar groups, such as the one on the right hand end of the chain which is seen in ice where it is known as Bjerrum L (leer) fault. Such a fault then propagates by successive rotation of OH groups, shown by the arrows in Fig. 6.26b. and at the end of this process the proton configuration returns to its original state. The chain now becomes ready to transport another proton. Morowitz (1977) proposed that such hydrogen bonded chain "proton-wire" will have a low resistance which would allow a cell to perform "protochemistry" with "protodes" in a way comparable to that in which one performs electrochemistry with electrodes.

The second type of mechanism suggested is the formation of a voltage-gated ion channel like struture as has been proposed for antibiotic alamethicin (Fox & Richards, 1982). X-ray crystallography of this molecule has led the authors to propose a model (Fig. 6.27) where the oligomers of the amphiphilic molecules of alamethicin form solvent-filled channels. The interior of the channel has been shown to contain three bands of polar groups (Gln_7, Aib_{10}†, Gly_{22}) capable of hydrogen bonding to water. The occurance of a proline residue towards the centre of the channel causes the helix axis to bend away from the oligomer symmetry axis (Fig. 6.27) and gives the channel an appearance of a funnel. This proline induced bend results in two free carboxyls and one free amide group which provides a hydrophilic surface. Proton conductance studies using reconstituted subunit-9 in membrane vesicles (Schindler & Nelson, 1982) provide evidence in favour of a oligomeric voltage-gated ion channel like structure (see also Hoppe & Sebald, 1984). The occasional occurence of a charged residue within the hydrophobic segments, including those con-

† *Aib* (α- amino isobutyric acid)

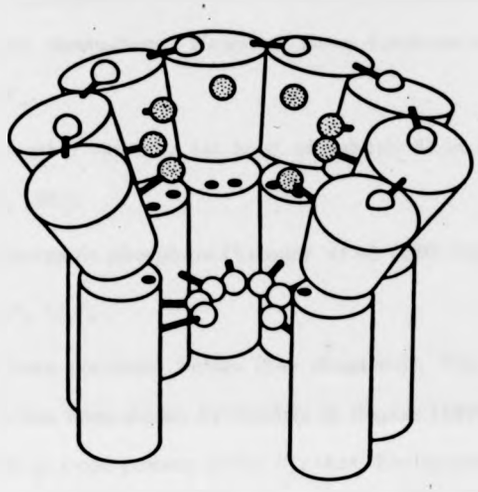


Figure 6.27 Schematic representation of the voltage-gated channel model for alamethicin (from Fox & Richards, 1982).

The alamethicin monomer has been shown as two cylindrical segments signifying the interruption of the α-helical hydrogen bonding introduced by the Pro_{14} residue. The stippled spheres at the mouth of the channel represents the Glu_{18} residue while the open spheres at the centre and top of the channel structure indicate the hydrogen bonded annulus of Gln_7 and Gln_{19} residues, respectively. The lower parallel cylinders represent the N-terminal domain, while upper cork shaped region represent the C-terminal domains and are tipped away from the channel n-fold axis. The black dots on the face of each cylinder represent the solvant accessible carboxyl groups of Aib_{10} and Gly_{11}.

served glutamic acids in subunit-6 and subunit-9 probably participate in the formation of a water cluster inside the ion channel and are directly involved in proton translocation.

The role of subunit-8 in the F_o

In the absence of the appropriate mutants we cannot demonstrate the direct participation of subunit-8 in proton conduction. However, three functons of this subunit could be demonstrated in the F_o.

(i) Assembly of other subunits (at least of subunit-6) in the F_o (Marazuki *et al*, 1983; Ray *et al*, 1984).

(ii) Binding of inorganic phosphate (Velours *et al*, 1980; Esparaza *et al*, 1981).

(iii) Binding of F_1 to F_o.

The first point has been discussed before (See chapter-5). The last two points deserve some explanation. It has been shown by Gueirin & Napias (1978), long before the acceptance of the subunit-8 as a component of the F_o, that this lipoprotein (due to its property of solubility in organic solvent) unlike other lipoprotein, subunit-9, can bind inorganic phosphate. But how this is connected to the energy conservaion system has not been elucidated. If it is proven that subunit-8, similar to the subunit-b of *E.coli* F_o sector, is involved in the binding of the F_1 sector by its C-terminal polar domain, the last two funcitons can be integrated into one on the following proposition: that during the catalysis activity of F_1, the basic polar domain of subunit-8 remains in immediate contact with the catalytic site and may provide bound inorganic phosphate to synthesise ATP from ADP. This later hypothetical function could be independent of this subunit's F_1 binding activity, but must be intimately related with the F_1 sector. In the future, by chemical cross-linking studies and using moloclonal antibodies, the hypothesis may be rationalised.

Recently it has been suggested that subunit-8 can conduct protons in reconstituted membrane vesicle in a voltage dependent manner (Dr. Velour, personal communication).

Probable involvement of other components in energy conservation of the F_o

Apart from the well defined subunits, discussed above, a few factors like "coupling factor-B" (Sanadi, 1982), F_6 (or factor-6) (Racker, 1979) have been shown to be associated with the O.S. ATPase complex. In fact, Sanadi *et al* (1984) have raised an antibody against coupling factor-B and have demonstrated its involvement in proton conduction of mitochondrial H^+ ATPase. But how exactly they are associated with the F_o and integrated to the function of energy conservation is not clear at present. In yeast, it has been discussed in the earlier chapter that a *mit* mutation (mit-175) downstream of subunit-6 structural gene, can stop growth of the organism on glycerol. So this locus, if it codes for any structural and/or catalytic factor, may also be associated with F_o and may play an important role in oxidative phosphorylation. With the lack of confirmation for these factors, and their stoichiometric ratio in the complex, and their topographic arrangement, it is very difficult to propose a total view of the F_o complex of the ATP synthase in mitochondria.

CHAPTER-7

A search for the candidate for non-mitochondrial cytoplasmic markers: Analysis of 3 μ DNA

INTRODUCTION

This chapter deviates from the mainstream of the experiments involving mit-DNA structure-function relationships. This is mainly due to one of our original aims, mentioned earlier, to establish a cytoplasmic candidate for some non-mitochondrial, non-nuclear inherited genetic markers involved in the energy transduction of mitochondria.

Genetics of cytoplasmically inherited characters

The biochemical genetic studies of oxidative phosphorylation from our laboratory have used different compounds that specifically inhibit mitochondrial respiration, electron transport, energy coupling and consequently block the growth of yeast cells on non-fermentative media (See Chapter-1). Some of the resistance alleles (e.g. VEN^R, TET^R, $Rh6G^R$) were demonstrated to be segregated from mitochondrial markers at about 45%-50% recombination rate (See Griffiths, 1976). It was shown (Griffiths *et al*, 1975), that the VEN^R and TET^R alleles were retained in a ρ^0 petite in which mt-DNA was absent. Similarly Rh6G resistant mutants were shown to possess resistivity against Rhodamine in the ρ° state (Carignani *et al*, 1977). All these alleles were shown not to be nuclear inherited and were assumed to be on a cytoplasmic genome other than mt-DNA. The genetic evidence for the existence of a cytoplasmic determinant other than mt-DNA comes from many other laboratories. For example, it has been shown that a factor, called [psi] which modulates the tRNA mediated ochre suppressor is inherited extra chromosomally but not located on mt-DNA (Cox, 1965, Liebman *et al*, 1975) of *Saccharomyces cerevisiae*. Inheritance of Janus green resistance in *S. cerevisiae* (Kruszewska & Szczesniak, 1978), Nalidixic acid resistance in *S. pombe* (Massardo & Del Giudice, 1982) also follow a extranuclear and extramitochondrial pattern.

Autonomously replicating entities in the cytoplasm of *S. cerevisiae*

Three classes of autonomously replicating bodies are generally found in the cytoplasm of *S. cerevisiae*: namely, 2 μ DNA (Clark-Walker, 1973, 1974), 3 μ DNA (Clark-Walker & Azad, 1980; Larinov *et al*, 1980) and ds RNAs (Buck *et al*, 1973; Herring & Bevan, 1974).

i) 2 μ plasmid DNA

2 μ DNA is a circular plasmid (originally named as omicron DNA or o-DNA) with a contour length of about 2 μ (about 6 kilobases), which is present in about 60 copies per cell of most yeast strains (Clark-Walker, 1974; Gubbins *et al*, 1977; Gerbaud & Guerineau, 1980). The most distinguishing feature of this species of plasmid is the presence of inverted repeat (IR) sequences of about 600bp separating a large (about 2.7 Kbp) and a small (about 2.3 Kbp) unique region (Cameron *et al*, 1977; Hollenberg *et al*, 1976). A reciprocal recombination event within the repeat produces a mixture of two sequence forms (A & B) of this plasmid, differing in the orientation of their unique regions (Broach, 1982). The observations, that this species of DNA could be isolated in a condensed nucleosome form, similar to the nuclear chromatin (Nelson & Fangman, 1979; Livingston & Hahne, 1979) and that it replicates only during the S-phase (Livingston & Kupfer, 1977) led to the conclusion that 2 μ DNA exists part of its life time in the nucleus and partly in the cytoplasm. Therefore it was suggested that 2 μ plasmid DNA could be a possible candidate for cytoplasmic genetic markers, not borne on mt-DNA (Guerineau *et al*, 1974; Griffiths *et al*, 1974).

However, the plasmid has been sequenced by Hartley & Donelson (1980) and has been shown to have reading frames which could code for three proteins of MW 33 000 (called Charlie), 43 000 (called Baker) and 48 000 (called Able). Evidence has been put forward that these gene products are expressed. They are involved in the reciprocal recombination within the inverted repeats and in the propagation of the plasmid itself (Broach, 1982). It is therefore, unlikely that this 2 μ DNA carries VEN^R, TET^R and $Rh6G^R$ markers. The cytoplasmic character [psi] has also been shown not to occur on 2 μ DNA (Tuite *et al*,

1982).

However, 2 μ DNA has been used extensively in designing yeast - *E.coli* shuttle vectors (Beggs, 1978) and for expression of foreign genes (Beggs *et al*, 1980; Hitzeman, 1981) in yeast. It has also been used in constructing integration vectors to aid in the location of genetic markers in the chromosomes of yeast (See Bostein & Davies, 1982).

ii) ds RNAs

ds RNAs or double stranded circular RNAs have been reported in the cytoplasm of *S. cerevisiae* (Buck *et al*, 1973; Herring & Bevan, 1974). Two types of ds RNAs are generally found in cytoplasm: (i) those involved in the "killer" phenomenon of yeast (Herring & Bevan, 1974) and (ii) those accumulated as a result of processing events of mitochondrial RNAs (Beilharz *et al*, 1982a).

ds RNAs associated with the killer phenomenon or rather killer-virus of yeast have been shown to be mainly of two types: L (mol. wt. 2.5×10^6 to 3.4×10^6 daltons) and M (mol. wt. 1.1×10^6 to 1.7×10^6 daltons). The details of these have been described in reviews by Wickner (1981) and Mitchell & Bevan (1983).

Tuite *et al* (1982) investigated killer-virus associated RNAs as a probable bearer of the cytoplasmic [psi] marker and have failed to show relationships among them. The other type of dsRNAs are not autonomously replicating entities, and occur transiently and therefore their candidature as bearers of the cytoplasmic genetic markers can be ruled out.

iii) 3μ DNA plasmid

3 μ DNA plasmids are another class of circular extrachromosomal DNA found in the cytoplasm of *S. cerevisiae* (Clark-Walker & Azad, 1980; Larinov *et al*, 1980) and they have been shown to contain sequences hybridizable to cytoplasmic rRNAs.

In the course of our investigation for a cytoplasmic determinant for the markers, VEN^R, TET^R, and $Rh6G^R$, we noticed that some of the laboratory yeast strains (derivative of parental D22 strain) contain a high copy number of 3 μ plasmid DNAs. Therefore, we

were interested in the determination of the structural organisation of this plasmid in respiratory competent (ρ^+) and respiratory deficient (ρ^- and ρ^0) strains as well as it's probable candidature for the above mentioned cytoplasmic genetic markers.

MATERIALS & METHODS

Yeast Strains

Most of the yeast strains have been described in chapter-2. Some of the strains which have not been mentioned earlier are given in Table 7.1.

Growth and culture of yeast strains

The growth media and culture condition of yeast strains have been described in Chapter-2.

Isolation of 3 μ DNA

Two types of method were generally employed to isolate the 3 μ plasmid.

The first method involves CsCl-ethidium bromide or CsCl-bisbenzimide density gradient centrifugation as described in Chapter 3. 3 μ DNAs appear as the bottom band (Fig. 7.4) which is collected either by puncturing the tube or by a narrow tipped pasteur pipette. The extraction of dye, dialysis and precipitation of DNA is essentially the same as described in Chapter 3.

The second method used involves the use of hydroxylapatite. The hydroxylapatite was made according to Miyazawa & Thomas (1965). The harvested cells were broken open by shaking with glass beads (0.25mm diameter) (Lang *et al.* 1977) in 0.25M Manitol, 10mMEDTA and 10mM Na-phosphate buffer (pH 7.5). After the removal of unbroken cells and glass beads by low centrifugation (4000 g for 5 mins) the supernatant was mixed with hydroxylapatite (about 0.5gm/ml) and kept for 5 minutes. Then, hydroxylapatite was removed by centrifugation. This step removes most free nuclear DNAs into buffer. The supernatant was then centrifuged at 16000 g for 30 minutes. The precipitated mem-

Table 7.1 Yeast Strains

Strain	Nuclear Genotype	Mitochondrial Genotype	Source
D22	a ade2	ρ	Dr. Wilkie
D22/72	a ade2	ρ *VEN*^{R} *TET*^{R} *Rh6G*^{R} *1799*^{R}	Carignani *et al* (1977)
D22/69	a ade2	ρ *VEN*^{R} *TET*^{R} *Rh6G*^{R} *1799*^{R}	Griffiths *et al* (1975)
D22/EC6	a ade2	*VEN*^{R} *TET*^{R} *Rh6G*^{R} *1799*^{R}	Griffiths *et al* (1975)
LP81	a leu kar-1	ρ *Oli2*^{R} *Oss1*^{R} pho9	Connerton *et al* (1984)

brane fraction was lysed in TE buffer with 1% SDS. The lysate was extracted with phenol:chloroform mix (1:1) and the DNAs were precipitated from the aqueous layer by adding two volumes of cold ethanol (- 20° C overnight). The DNA was redissolved in TE buffer (10mM Tris, 1mM EDTA). This crude preparation contains more than 95% rDNAs (open and closed circular forms of 3 μ plasmid) and mt-DNAs. After CsCl-bisbenzimide gradient centrifugation, the bottom fraction enriched with 3μ plasmids was isolated and extracted, as mentioned earlier. Use of hydroxylapatite enriches 3 μ DNA in the 16K (16000 g) fraction. This could be due to its association with membranes in the cytoplasm.

Restriction enzyme digest analysis and Southern hybridization

The restriction enzyme digestion and Southern hybridization methods have been described earlier (see Chapter 3 & Chapter 4).

Cloning of 3 μ plasmid in *E. coli*

3 μ plasmid was digested with *Hind*III and the resultant fragments were cloned in the *Hind*III site of pAT153. The experimental method has been described in Chapter 4. Briefly, the purified plasmid pAT153 was digested with *Hind*III and then treated with bacterial alkaline phosphatase (BAP) to prevent self ligation. Simultaneously purified 3μ plasmid from the yeast strain D22 was partially digested with *Hind*III. The reason for this will be described in the results section. Both vectors and target DNAs were ligated with T4 DNA ligase for 10-12 hrs at 15° C. The ligation mixture was used directly to transform the *E. coli* strain HB101. The positive clones were identified using a heterologous rDNA probe of *Xenopus laevis*.

Transformation of yeast cells

Transformation of yeast was done using the alkali cation induced method of Ito *et al* (1983).

Briefly, the yeast cells were grown aerobically in YPD. At the late log phase ($OD_{610} = 4$ to 8) the cells were harvested, washed once with TE Buffer and suspended in the same

buffer to a final concentration of 2 x 10^8 cells /ml. 0.5ml of this cell suspension was aliquoted into test tubes and mixed with an equal volume of 0.2M LiCl or lithium acetate. After 1 hour at 30^0 C with shaking, 100 µl of the cell suspension was incubated with 10-15 µl of a plasmid DNA solution (about 10 µg of DNA) in a 1.5ml microfuge tube and incubated at 30^0 C for 30 minutes in a standing position.

An equal volume of sterilized 70% polyethylene glycol (PEG-4000) was then added into the microfuge tube and mixed thoroughly on a vortex mixer. After standing for 1 hour at 30^0 C, the microfuge tube was immersed in water at 42° C and incubated for 5 minutes. The cells were cooled to room temperature, washed twice by centrifugation, with sterilised water at room temperature and plated directly onto test-plates.

Selection of yeast transformants

For the selection of the yeast transformants, the method of Chevallier & Aigle (1979) was employed. The method is based on the penicillinase producing properties of strains harbouring an Amp^R gene. In the procedure, test plates were made up of yeast nitrogen base (Difco) 0.65%, glucose 0.1%, soluble starch 0.2%, agar 2%, buffered with 0.02M phosphate at pH 6.8. After the cells had grown up on the test plate, a mixture of 4ml melted soft agar test medium (constitution is the same as the test plate but with 1% agar) plus 1.5ml reagent solution (3 mg/ml I_2, 15mg/ml KI, 0.02M phosphate buffer pH 7.0, 3 mg/ml ampicillin) was prepared, which gave a deep blue colour. The mixture was stirred and gently poured onto the test plates and the plates were left in the cold room (4^0 C) immediately for 15-60 minutes. Within this time strains carrying hybrid plasmid (i.e., recombinant of pAT153 and 3 µ plasmid) coding for ampicillin resistance show a well defined white halo which is easily distinguished from the rest. The halo is formed due to the reduction of the blue colour of starch - iodine complex by penicilloic acid which is produced by hydrolysis of penicillin (ampicillin) on test plates.

Chemicals and other materials

The chemicals and materials used have been described in earlier chapters. *X. laevis* clones of rDNAs were a gift from Dr. Alan Colman, Dept. of Biological Sciences, University of Warwick. pXl101 contains both 18S and 28S rDNA cistrons while pXl108 and pXl202 have 18S and 28S rDNA genes, respectively. pXl08 is a 5S rDNA gene specific clone. See Boseley (1979) and Moss (1982) for the detailed derivation of the clones.

RESULTS

1. Various species of cytoplasmic genomes in *S. cerevisiae*

When total cellular DNA preparation of *S. cerevisiae* (D22) was analysed by electrophoresis in a 1% agarose gel, 6 to 7 species of nucleic acids were observed (Fig. 7.1) The major band, is a mixture of nuclear DNA and mt-DNA which moves into a gel position corresponding to a size of about 24 Kbp-26 Kbp. DNase and RNase digestion demonstrates that two of them are double stranded (ds) RNAs whose molecular size corresponds to the viral M (1.9 Kbp) and L (3.4 Kbp) types. 2 μ DNA plasmid was analysed by its isolation from the gel and by restriction digests with *EcoR*1 and *Hind*III (data not shown). 3 μ plasmid DNA was identified initially by its molecular weight (about 9 Kbp on SmaI digestion) from the published results (Larinov *et al*, 1980).

Two more DNA bands of high molecular weight were visible above the nuclear and mitochondrial mixed DNA bands (Fig. 7.1). To detect whether these two high molecular weight bands are covalently closed circular (CCC) and open circular (OC) forms of the same DNA molecule, the gel was irradiated with UV for 5 minutes after the first electrophoresis and run in the second dimension. The principle behind this is that if they were the CCC and OC forms of the same molecule, after UV exposure both of the forms would be OC forms (due to nicks created in the CCC form) and consequently migrate the same distance in the second dimension. In fact, the observed result (Fig. 7.2) demonstrates that this is the case. The existence of such a high molecular weight plasmid has not been seen

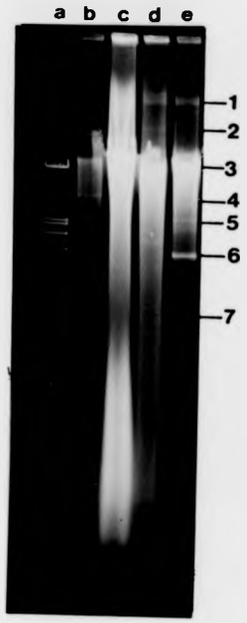


Figure 7.1 Various species of DNAs and RNAs observed in the whole cell DNA preparation of the strain D22 and it's derivatives.

The bands corresponding to the various numbers are: 1 & 2, a high molecular weight plasmid; 3, mixture of nuclear DNA and mt-DNA;4, 3 μ plasmid DNA (9 Kbp); 5, 2 μ plasmid DNA (6 Kbp);6, dsRNA (L, 3.4 Kbp); 7, dsRNA (M, 1.9 Kbp). Various bands shown were identified by the DNase, RNase and other restriction endonuclease enzyme digestion.

Lane a, λ (HindIII + EcoR1) digested marker DNA. Molecular size of the DNA fragments are (in Kbp): 21.7, 5.15, 5.00, 4.27, 3.48, 1.98, 1.90, 1.59, 1.37 0.94, 0.83, 0.56

Lane b, A plasmid preparation of strain D22 showing the 3 μ plasmid DNA.

Lane c, Sample lacking various plasmids and the double stranded RNAs (dsRNA)

Lane d, Sample lacking the dsRNAs

Lane e, Whole cell DNA from the parental strain D22.

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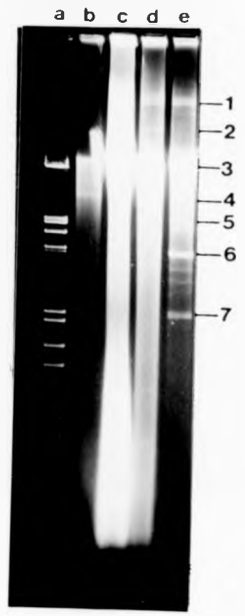

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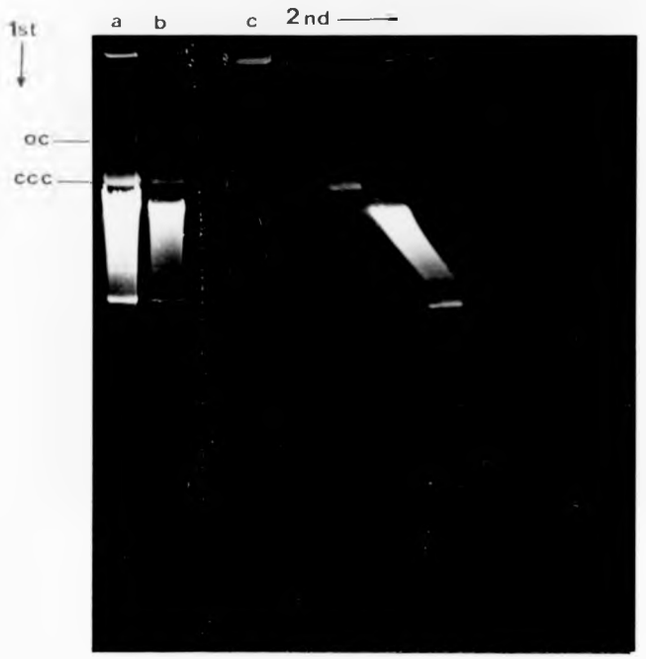
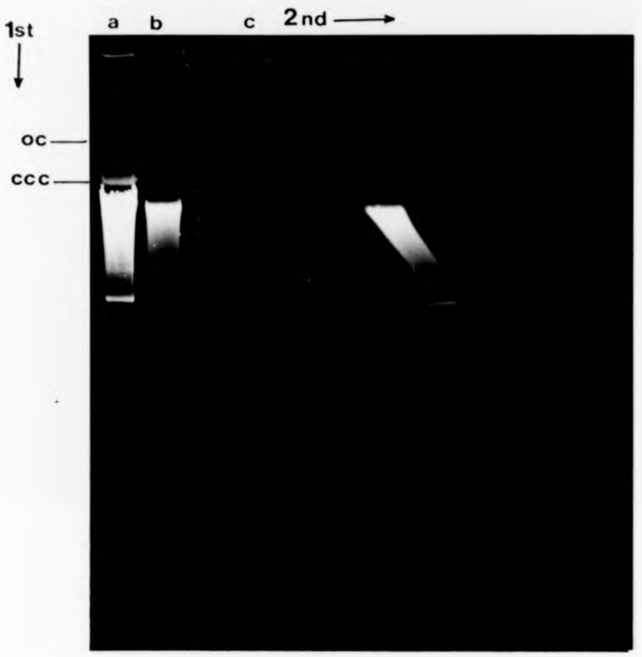


Figure 7.2 Two dimensional agarose gel electrophoresis for the identification of the high molecular weight plasmid from the strain D22

The total cellular DNAs isolated from the strain D22 were electrophoresed on a 0.8% agarose gel. The gel was stained with ethidium bromide and various species of DNAs were identified on a U V transilluminator and photographed. The gel was left on the U V illuminator for further 5 minutes and the samples of one lane was electrophoresed in the second direction (perpendicular to the first direction).

Bands marked as OC and CCC are the open circular and covalently closed circular molecules of the high molecular weight plasmid. The samples in the Lane a and b were run only in the 1st direction and the sample of the lane c was run in the 2nd direction. Note that in the 2nd dimension of the gel OC and CCC forms have moved to the same distance into the gel.



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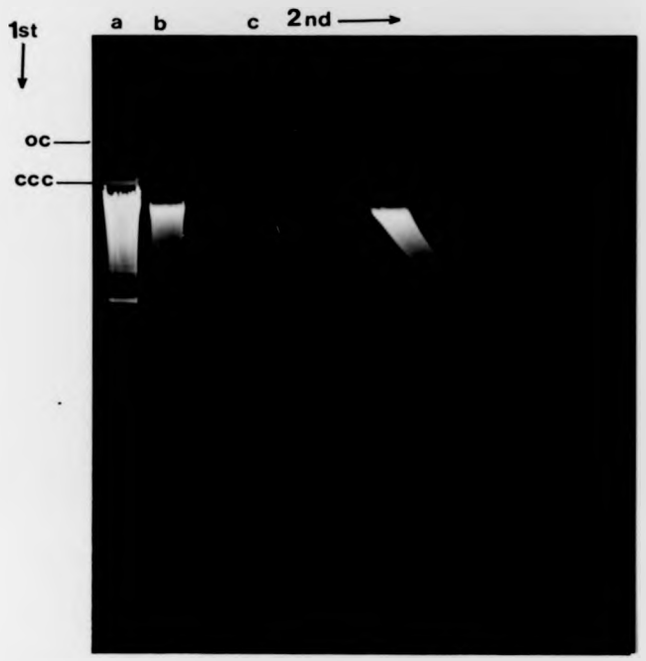

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in *S. cerevisiae* before. Southern hybridization study using labelled 2 μ DNA and 3 μ DNA as the probes, it was found out that this plasmid is not the oligomer of the 2 μ and 3 μ DNAs (data not shown). Neither did this band hybridize to mt-DNA. However, we were interested in studying 3 μ DNA in detail due to their high copy numbers in the parental strain, D22 (data not shown), of VEN^R, TET^R, and $Rh6G^R$ strains (See Table 7.1).

2. Study of 2μ DNA and dsRNAs from the yeast strains with VEN^R TET^R and $Rh6G^R$ genetic markers

Various strains possesing the above cytoplasmic markers (see Table 7.1) were analysed for their nucleic acid contents by agarose gel electrophoresis. All these strains are based on the parental grande D22 which contains hybridizable 2 μ and 3 μ DNA sequences and DNase and RNase resistant bands. When RNase digestion is performed in low salt conditions (10mM Tris-Cl, 1 mM EDTA, as opposed to 0.15-0.2M NaCl) the RNA band becomes sensitive, indicating the band is probably dsRNA. Strains D22/72, D22/69, and D22/EC6 are all cross resistant to the above genetic markers but possess different episomal characteristics. D22/72 is similar to wild type D22 in its nucleic acid content while D22/69 is devoid of dsRNAs and D22/EC6 is devoid of both dsRNAs and 2 μ sequences (Fig. 7.3). This evidence eliminates the latter genetic elements as candidates for the bearers of the resistant genotypes (VEN^R, TET^R and $Rh6G^R$) The remaining candidates are the high molecular weight plasmid (identified above) and the 3 μ DNAs.

3. Isolation and identification of 3 μ DNA

To characterise 3 μ DNA fully, it was necessary to purify the plasmid from other DNAs and RNAs. This was acheived by CsCl-bisbenzimide density gradient analysis (Fig. 7.4). Each band was identified by restriction digest analysis. On the basis of this, the 3 μ plasmid DNA was shown to form a band at 1.70 gms/cc region of the gradient. Identification of the 3 μ plasmid was achieved on the basis of electron microscopy and restriction digest analysis. EM study revealed that it had a contour length of about 3 μ

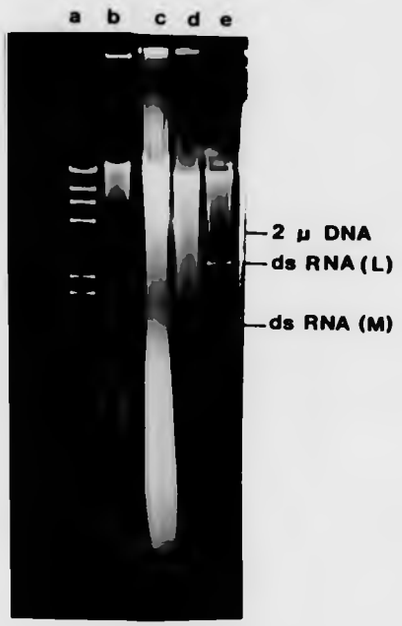
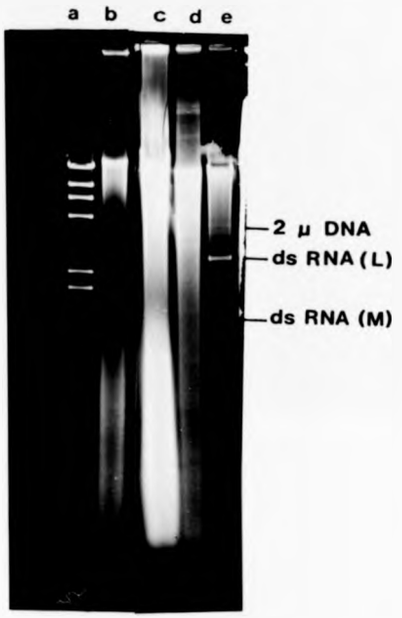


Figure 7.3 Evidence that the 2 μ plasmid DNA and ds RNAs do not carry the nonmitochondrial cytoplasmic markers.

Total DNAs were isolated from the 16K×g membrane fraction of the yeast cells under the present study and analysed on a 1% agarose gel.

Lane a, λ (HindIII digest) marker DNA; Lane b and c, D22/EC6 (VEN^R, TET^R, $R\lambda 6G^R$, 1799^R); Lane d, D22/69 (VEN^R, TET^R, $R\lambda 6G^R$, 1799^R); Lane e, parental strain D22.



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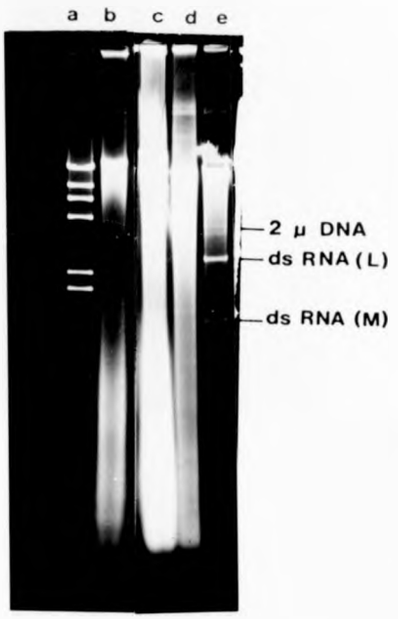

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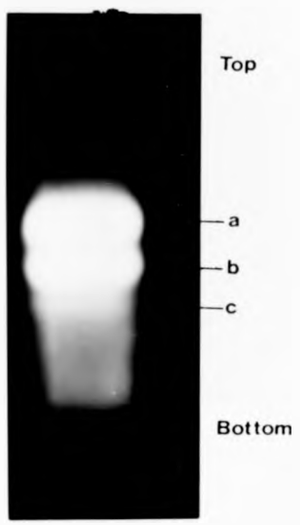
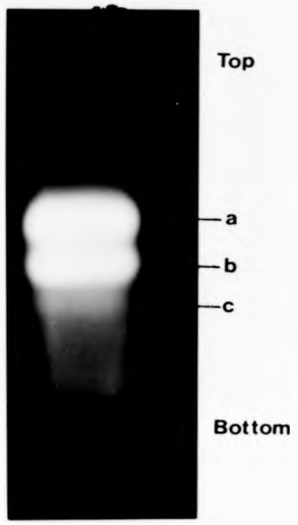


Figure 7.4. Caesium chloride-bisbenzimide density gradient centrifugation for the isolation of the 3 μ Plasmid DNA

The 16000 g (16 K) fraction of the membrane from the strain D22 was extracted with phenol/chloroform for the isolation of DNA which was then analysed on the CsCl:bisbenzimide gradient as described in the method.

band (a), mt-DNA; band (b), nuclear DNA; band (c) 3 μ plasmid DNA.



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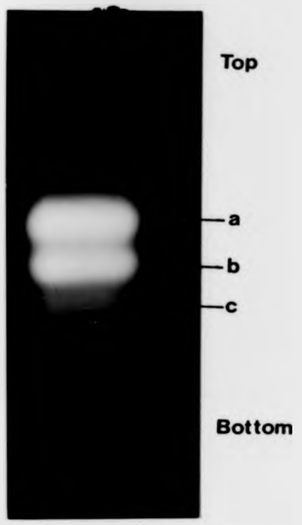

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(data not shown). When this species was digested with *EcoR*1 and *Hind*III restriction enzymes, it was observed that it has a similar restriction digest pattern to that described for genomic rDNA (Fig. 7.5). To confirm this, a heterologous rDNA probe from *Xenopus laevis* was used for Southern hybridization analysis. Both 18S rRNA and 28S rRNA gene containing probes (pXl 108 & pXl 212) can hybridize *EcoR*1 and *Hind*III generated fragments from *S. cerevisiae* (Fig. 7.6), but not the 5S rRNA gene containing probe (pXl 08). This demonstrates that the 3 μ DNA plasmid contains ribosomal genes for at least the 28S and 18S rRNA genes. Failure of the 5S rRNA gene of *Xenopus laevis* to show a positive hybridizable signal is due to divergent sequences of this gene from *S. cerevisiae* and *X. laevis*. A similar result was obtained by other workers (Petes *et al*, 1979; Bell *et al*, 1977; Gourse & Gerbi, 1980).

4. Cloning of 3 μ plasmid DNA

To understand the organisation of the 3 μ DNA plasmid, it was felt necessary to clone it in *E. coli* from where it could be isolated without contamination from nuclear rDNA genes. Restriction analyses showed that the 3 μ plasmid DNA has one unique restriction site for *Sma*I (*GGG*'CCC) and two for *Hind*III (*A*'AGCT). Because *Sma*I generates blunt ends and blunt end ligation is not as efficient as sticky end ligation, it was thought that a partial digestion of *Hind*III would be preferable to clone the whole 3 μ DNA in the *Hind*III site of pAT153. Using this strategy, a few rDNA positive clones were picked up (Fig. 7.7A). After screening the clones, two clones were chosen, one having the whole 3 μ DNA insert (named, pSC3H1) and the other having only a smaller *Hind*III fragment (named, pSC3H2). As expected, on *Hind*III digestion of the plasmids, pSC3H1 yielded the two expected *Hind*III fragments and pSC3H2 yielded one HindIII fragment of the 3 μ plasmid (Fig. 7.7B).

5. Restriction map of 3 μ plasmid based on pSC3H1

For the purpose of detailed characterisation of the 3 μ plasmid, it was desirable to generate a restriction map for it. The 3 μ plasmid isolated directly from yeast strains was

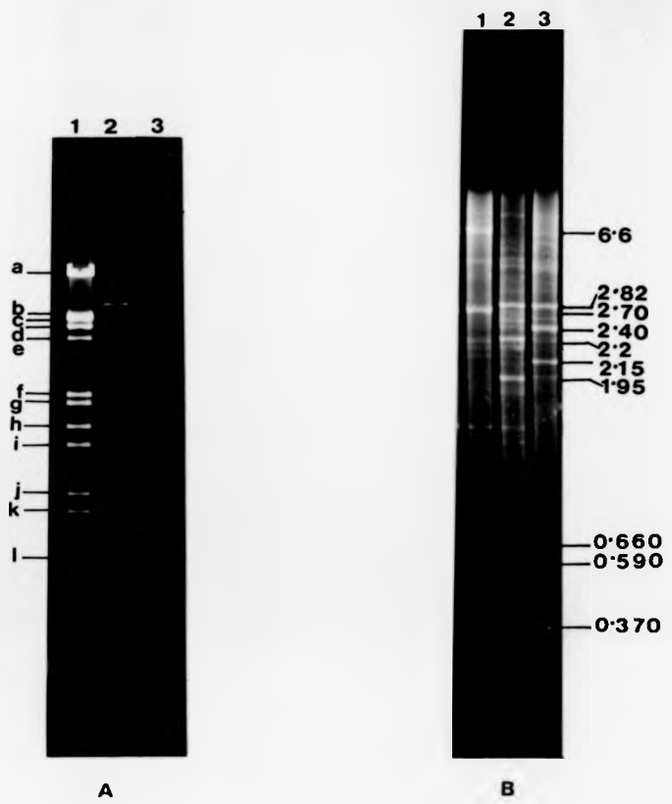


Figure 7.5 Gel electrophoretic pattern of the EcoR1 and HindIII digested 3 μ DNA and genomic DNA

The purified plasmid fraction and nuclear DNA was digested with the restriction endonucleases EcoR1 and HindIII and the samples were analysed on 0.8% agarose gel electrophoresis.

A) 3 μ plasmid DNA

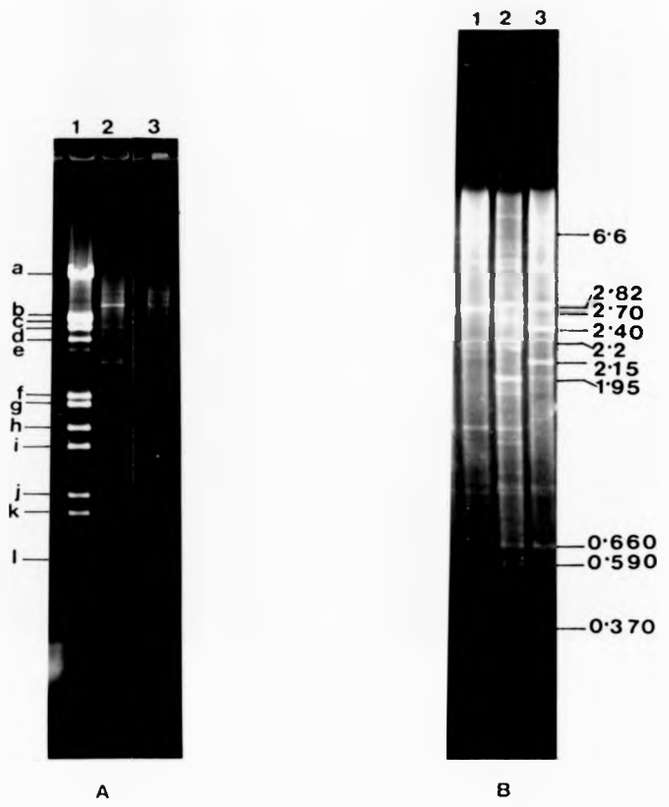
Lane 1, λ (HindIII + EcoR1) Digested marker DNA. The size of thefragments are: (a) 21.7, (b) 5.15, (c) 5.0 (d) 4.27, (e) 3.48, (f) 1.98, (g) 1.90, (h) 1.59, (i) 1.37, (j) 0.94, (k) 0.83, (l) 0.56

Lane 2, HindIII digested fragments of the 3 μ plasmid showing the 6.6 and 2.7 Kbp fragments.

Lane 3, EcoR1 digested fragments of the plasmid DNA (2.82, 2.4, 2.15, 660, 590 Kbp). The two smallest fragments (0.370 Kbp and 0.280 Kbp) have run out of the gel.

B) Nuclear DNA

Lane 1, HindIII digested fragments; Lane 2, HindIII + EcoR1 double digested fragments and Lane 3, EcoR1 digested fragments. The size of the EcoR1 and HindIII fragments are similar to those of the plasmid DNA. The sizesof the EcoR1 + HindIII double digest fragments are: 2.82, 2.22, 1.95, 660, 590 Kbp. The smallest fragments (0.370, 0.280, 0.200, 0.190 Kbp) have run out of the gel.



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Lane 2, HindIII digested fragments of the 3 μ plasmid showing the 6.6 and 2.7 Kbp fragments.

Lane 3, EcoR1 digested fragments of the plasmid DNA (2.82, 2.4, 2.15, 660, 590 Kbp). The two smallest fragments (0.370 Kbp and 0.280 Kbp) have run out of the gel.

B) Nuclear DNA

Lane 1, HindIII digested fragments; Lane 2, HindIII + EcoR1 double digested fragments and Lane 3, EcoR1 digested fragments. The size of the EcoR1 and HindIII fragments are similar to those of the plasmid DNA. The sizesof the EcoR1 + HindIII double digest fragments are: 2.82, 2.22, 1.95, 660, 590 Kbp. The smallest fragments (0.370, 0.280, 0.200, 0.190 Kbp) have run out of the gel.

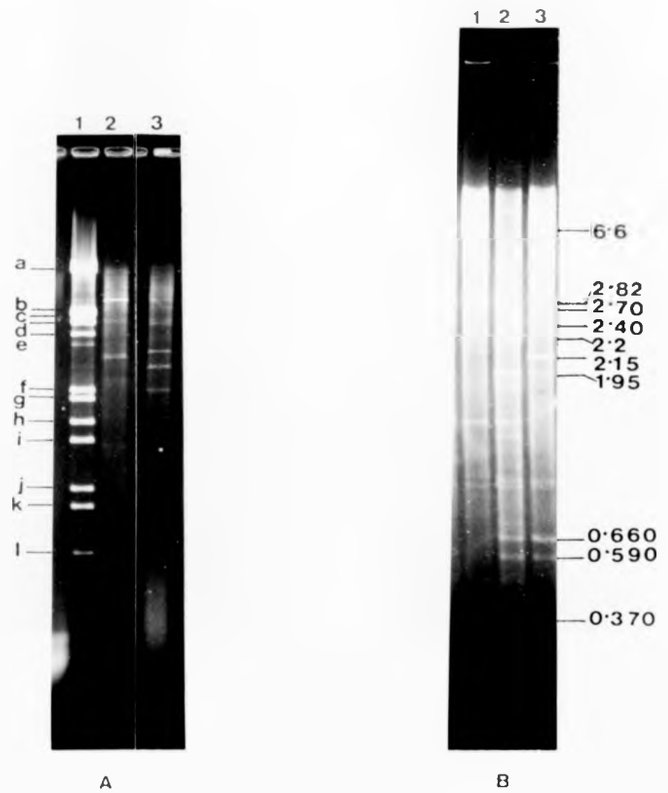

Figure 7.5 Gel electrophoretic pattern of the EcoR1 and HindIII digested 3 μ DNA and genomic DNA

The purified plasmid fraction and nuclear DNA was digested with the restriction endonucleases EcoR1 and HindIII and the samples were analysed on 0.8% agarose gel electrophoresis.

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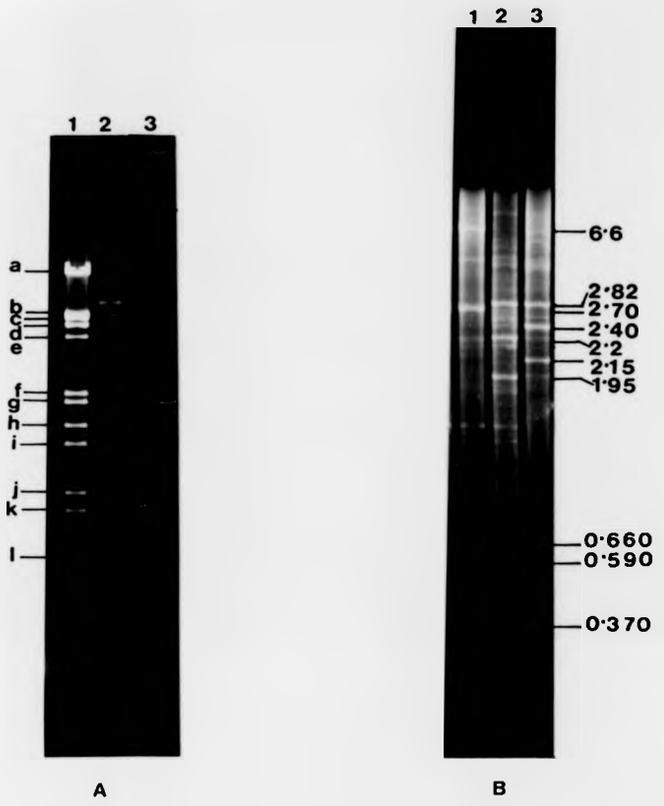

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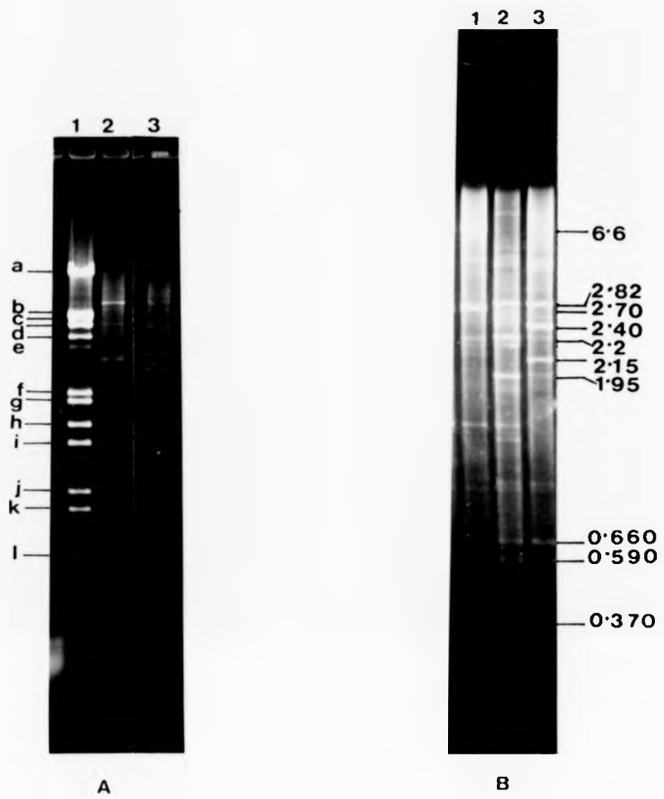

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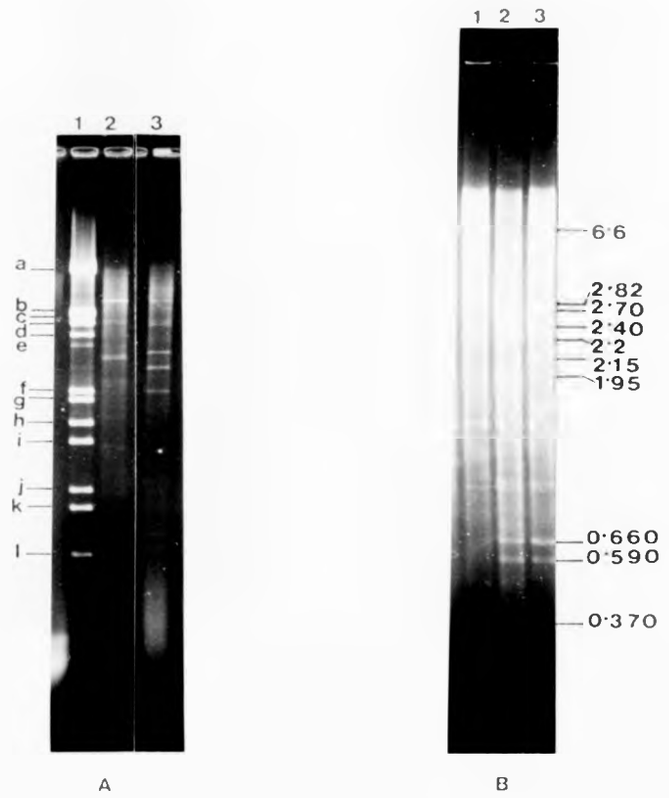

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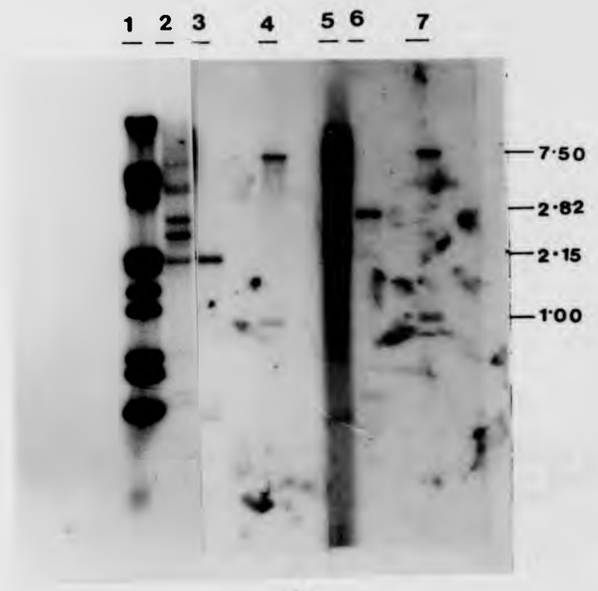
Lane 1. HindIII digested fragment. Lane 2. HindIII + EcoR1 double digested fragments and Lane 3. EcoR1 digested fragments. The size of the EcoR1 and HindIII fragments are similar to those of the plasmid DNA. The sizesof the EcoR1 + HindIII double digest fragments are: 2.82, 2.22, 1.95, 660, 590 Kbp. The smallest fragments (0.370, 0.280, 0.200, 0.190 Kbp) have run out of the gel.

Figure 7.6 Southern hybridization analysis of the 3 μ DNA

Purified 3 μ plasmid was digested with the restriction endonucleases and the samples were analysed on a 1% agarose gel. The DNA fragments were transferred onto a sheet of nitrocellulose filter and hybridized with the heterologous rDNA probes, 28S rRNA gene specific pXl212 (for lanes 6 & 7 of Fig. A and lanes 5 & 6 of Fig. B) and 18S rRNA gene specific pXl108 (for lanes 3 &4 of Fig. A and lanes 2 & 3 of Fig. B) from *Xenopus laevis*.

A) Lane 1, λ (HindIII + EcoR1) digested marker DNA; Lane 2, EcoR1 digested 3 μ DNA, probed with purified labelled 3 μ DNA; Lane 3 & 6, EcoR1 digested 3 μ DNA; Lane 4 & 7, Acc1 digested 3 μ DNA;Lane 5, λ HindIII digested marker DNA. The fragment sizes of the λ (HindIII + EcoR1) digested and HindIII digested fragments are given in the Appendix. Note that the largest EcoR1 fragment of the 3 μ DNA (2.82 Kbp) has 28S rRNA gene hybridizable sequences and the the fragment 1.95 Kbp has 18S rRNA gene hubridizable sequences.

B) Lane 1 & 4, λ HindIII digested marker DNA; Lane 2 & 4, HindIII digested 3 μ DNA; Lane 3 & 6, Sau3A digested 3 μ DNA. Note that the largest HindIII fragment (6.6 Kbp) has hybridizable sequences to both the 28S and 18S rRNA genes. Sau3A fragments shown here has not been analysed.



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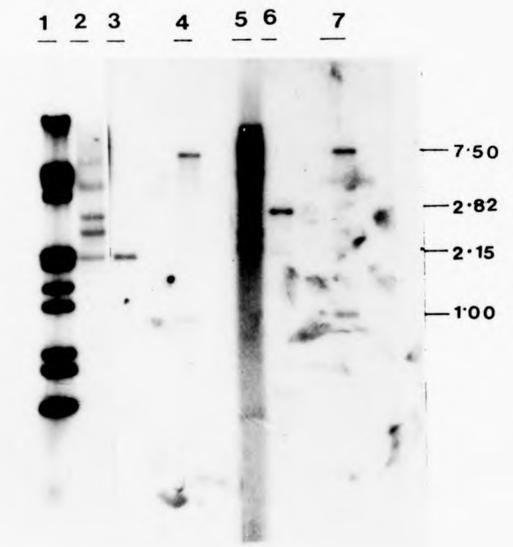
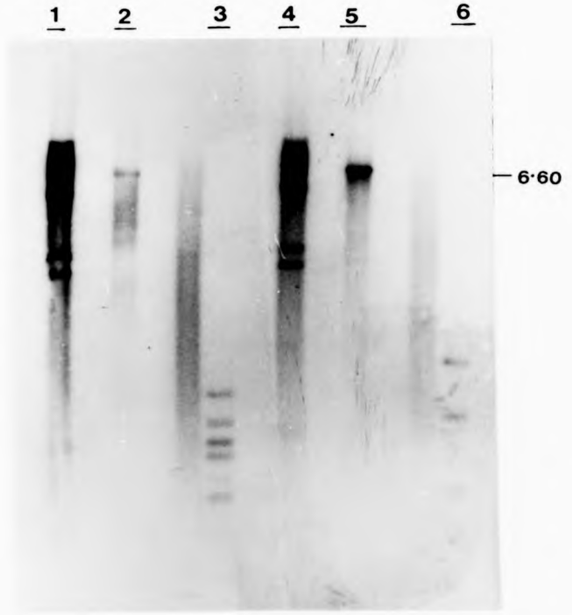
A) Lane 1, λ (HindIII + EcoR1) digested marker DNA; Lane 2, EcoR1 digested 3 μ DNA, probed with purified labelled 3 μ DNA; Lane 3 & 6, EcoR1 digested 3 μ DNA; Lane 4 & 7, Acc1 digested 3 μ DNA;Lane 5, λ HindIII digested marker DNA. The fragment sizes of the λ (HindIII + EcoR1) digested and HindIII digested fragments are given in the Appendix. Note that the largest EcoR1 fragment of the 3 μ DNA (2.82 Kbp) has 28S rRNA gene hybridizable sequences and the the fragment 1.95 Kbp has 18S rRNA gene hubridizable sequences.

B) Lane 1 & 4, λ HindIII digested marker DNA; Lane 2 & 4, HindIII digested 3 μ DNA; Lane 3 & 6, Sau3A digested 3 μ DNA. Note that the largest HindIII fragment (6.6 Kbp) has hybridizable sequences to both the 28S and 18S rRNA genes. Sau3A fragments shown here has not been analysed.



A



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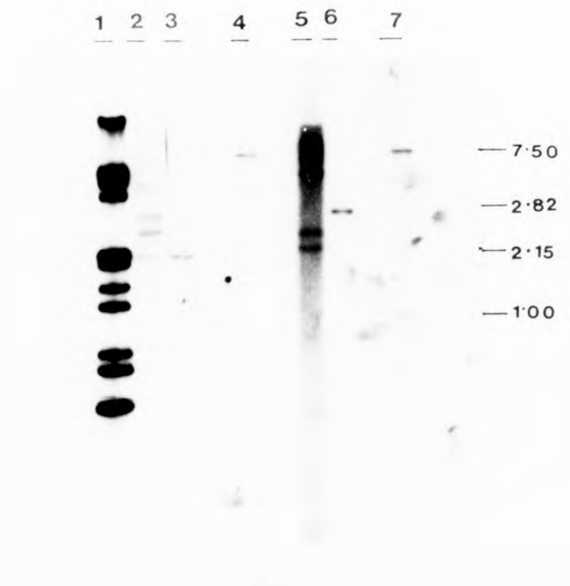


Figure 7.6 Southern hybridization analysis of the 3 μ DNA

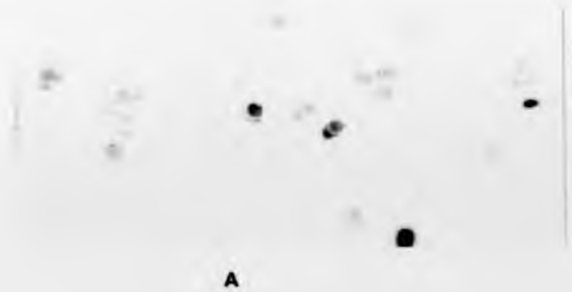
Purified 3 μ plasmid was digested with the restriction endonucleases and the samples were analysed on a 0.7 agarose gel. The DNA fragments were transferred onto a sheet of nitrocellulose filter and hybridized with the heterologous rDNA probes. 28S rRNA gene specific pXI212 (for lanes 6 & 7 of Fig. A and lanes 5 & 6 of Fig. B) and 18S rRNA gene specific pXI108 (for lanes 3 & 4 of Fig. A and lanes 2 & 3 of Fig. B) from *Xenopus laevis*.

A: Lane 1: λ (HindIII + EcoRI) digested marker DNA; Lane 2: EcoRI digested 3 μ DNA, purified with purified labelled 3 μ DNA; Lane 3 & 5: EcoRI digested 3 μ DNA; Lane 4 & 7: A+I digested 3 μ DNA; Lane 6: λ HindIII digested marker DNA. The fragment sizes of λ (HindIII + EcoRI) digested and HindIII digested markers are given in the Appendix. Note that the large EcoRI fragment of the 3 μ DNA (2.82 Kbp) has 28S rRNA gene hybridizable sequences and the fragment (1.95 Kbp) has 18S rRNA gene hybridizable sequences.

B: Lane 1 & 4: λ HindIII digested marker DNA; Lane 2 & 5: HindIII digested 3 μ DNA; Lane 3 & 6: Sau3A digested 3 μ DNA. Note the 6.6 Kbp HindIII fragment (6.6 Kbp) has both the hybridizable sequences (i.e. 28S rRNA and 18S rRNA gene). Sau3A fragments shown

Table 7.2 Fragment lengths of restriction enzyme digests of 3 μ DNA. Fragments were calibrated using (HindIII + EcoR1) digests of λ DNA and HaeIII digests of ϕX174RF (see Appendix).

Restriction enzyme	Fragment sizes (Kbp)
HindIII	6.750, 2.750
EcoR1	2.820, 2.400, 2.150, 0.660, 0.590, 0.370, 0.280
HindIII + EcoR1	2.820, 2.220, 1.950, 0.660, 0.590, 0.370, 0.280, 0.200, 0.170



A

B

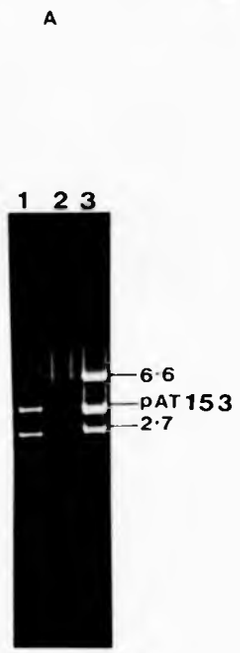
Figure 7.7 Cloning of the 3 μ DNA

The purified 3 μ plasmid was digested with HindIII and cloned in the HindIII site of the pAT 153.

A) Autoradiogram for the colony hybridisation filter for the 3 μ plasmid transformants. The heterologous probe (pXl212) was used to screen the positive transformants (dark spots).

B) HindIII digestion of the finally selected recombinant 3 μ plasmid clones (pSC3H1 and pSC3H2)

The purified plasmid from pSC3H1 (Lane 3) and pSC3H2 (Lane 1) were digested with HindIII and analysed on a 1% agarose gel, using HindIII digested 3 μ plasmid (Lane 2) as comparable marker. The HindIII fragments (6.6 & 2.7 Kbp) of the 3 μ DNA have been shown.



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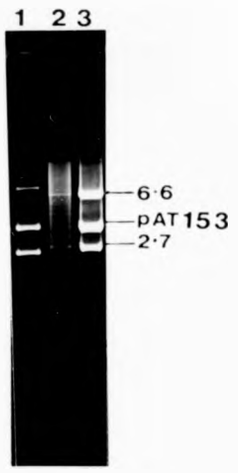

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used to generate initial *Hind*III and *EcoR*1 based maps. Table 7.2 shows the size of the restriction fragments, generated on *Hind*III and *EcoR*1 digestion of the plasmid (see also Fig. 7.5).

Based on the *Hind*III restriction sites on 3 μ DNA, pSC3H1 was first characterised for the orientation of the insert (Fig. 7.8). Then psSC3H1 was digested with *SstI, PstI, SalI, XbaI, Sma*I, *BamH*1, *EcoR*1 separately or with various combinations and with *Hind*III and the resultant fragments were analysed in 1% to 2% agarose gel elecrophoresis (Fig. 7.9). λ (*Hind*III + *EcoR*1) digests or ϕX174 RF (*Hae*III) markers were used to calibrate the molecular weight size of the fragments. Table 7.3 represents size distribution of the restriction fragments of pSC3H1 with various enzymes used. Based on this and other restriction data, obtained after secondary restriction digestion of isolated fragments from low melting point agarose gels, a restriction map was generated which is represented in Fig. 7.10. The most surprising feature of this map is the occurence of *Pst*1, *Sal*1 and *BamH*1 sites on this 3 μ plasmid. Previously it was reported that genomic rDNA does not contain any restriction site for *Pst*I, *Sal*I or *BamH*1 (Bell *et al*, 1977; Valenzuela, *et al*, 1977). Therefore it appears that a mutation and/or insertional phenomenon has occurred within the 3 μ DNA during its excision from chromosomal DNA. The insertion phenomenon looks more likely, for the reason that the genomic rDNA has been reported to have a repeat unit of about 9.060 Kbp (Fig. 7.12; See Warner, 1981) in contrast to the present analysis of 3 μ plasmid which has a 9.3 Kbp length. The insertion has most probably taken place within the non-transcribed spacer between the 5S rRNA and 18S rRNA genes (See next).

6. Organisation of rRNA genes on the 3 μ plasmid

The organisation of rRNA genes on the 3 μ plasmid was studied using a heterologous probe from *Xenopus laevis* (Fig.7.6). An 18S rRNA gene specific probe (clone pXl108) hybridized to the *EcoR*1 fragments 1.950 Kbp and 660 bp, while a 28S rRNA specific probe (pXl212) hybridized to the *EcoR*1 fragments, 2.820 Kbp, 0.590 Kbp 0.370 Kbp.

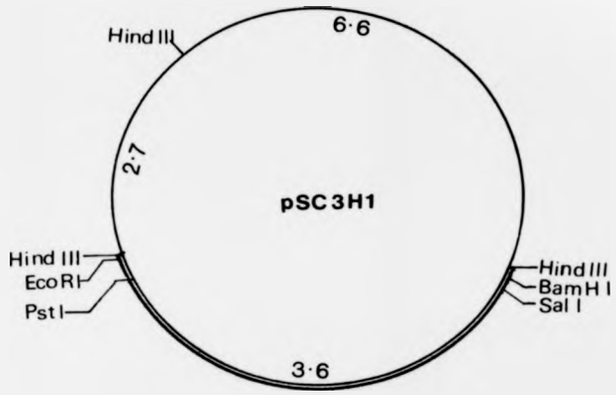

Figure 7.8 Orientation of the 3 μ plasmid DNA on pSC3H1

The circular map of pSC3H1 shows the orientation of the cloned 3 μ DNA on the recombinant plasmid pSC3H1. Double line region indicates the vector DNA (pAT153) and single line indicates the the cloned 3 μ DNA. The restriction sites (EcoR1, PstI, BamH1 and SalI) have been shown only for the vector DNA. No restriction sites except that for HindIII have been shown on the 3 μ DNA. The number indicates the fragment sizes (in Kbp) of the three HindIII fragments of pSC3H1. The map is not accurate to the scale.

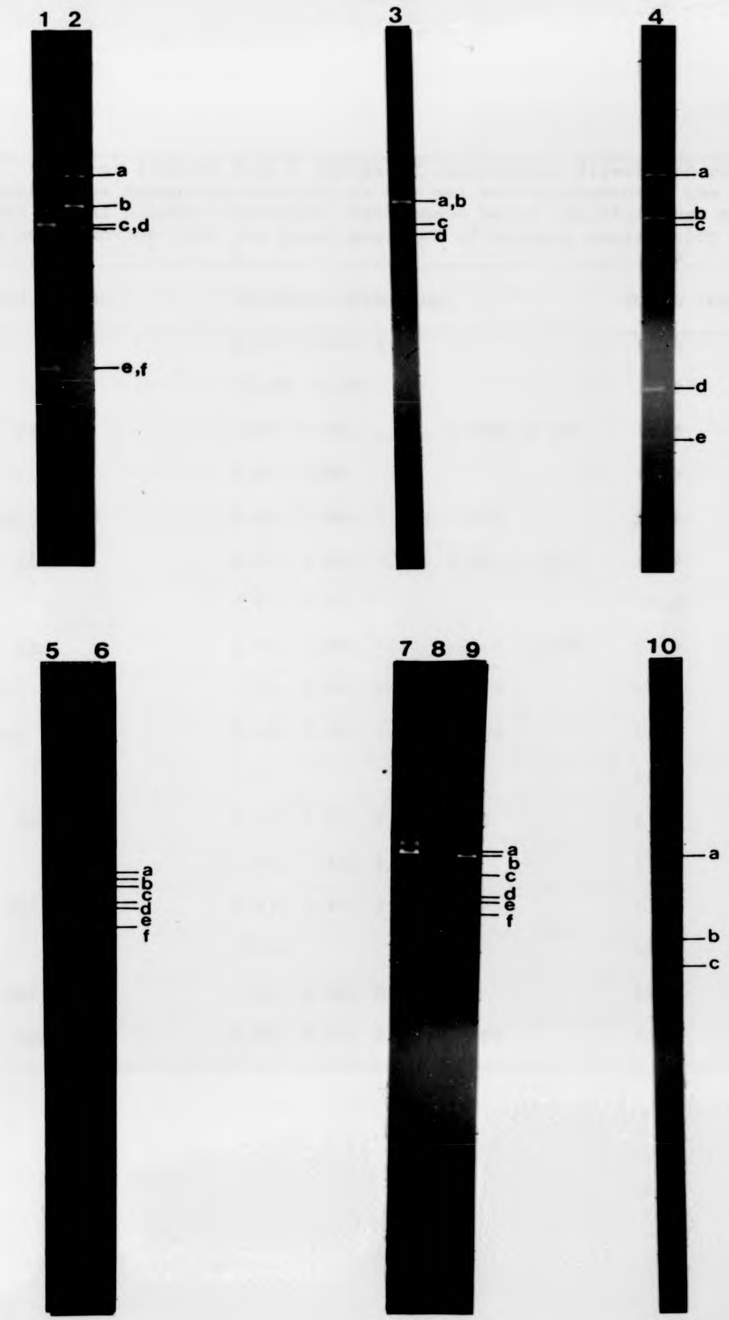

Figure 7.9 Gel electrophoretic pattern and molecular size of the restriction fragments of pSC3H1, produced by the restriction endonuclease HindIII (Lane 2), HindIII + PstI (Lane 1), HindIII + XbaI (Lane 3), HindIII + SalI (Lane 4), BamH1 + SstI (Lane 5), BamH1 (Lane 6), XbaI (Lane 7), XbaI + SalI (Lane 8), SalI (Lane 9) and PstI + SalI (Lane 10).

The purified plasmid from pSC3H1 was digested with various restriction endonucleases and analysed on 1% agarose slab gel. The molecular size of each fragment was determined using λ HindIII and (HindIII + EcoR1) digests as gel markers. Some bands showed anomalous mobility.

Mol. sizes of the fragments in Lane 1 & 2 are: (a) 6.6, (b) 3.6, (c) 2.82, (d) 2.70 (1.92), (e) and (f), each 0.780 Kbp

Lane 3: (a) & (b) 3.80 & 3.60, (c) 2.70, (d) 2.46 Kbp. The fragment of 0.340 Kbp is not visible in the gel.

Lane 4: (a) 6.60, (b) 2.98, (c) 2.20, (d) 0.620 and (e) 0.500 Kbp

Lane 5: (b) 5.53, (d) 3.40, (e) 2.40, (f) 1.0 Kbp

Lane 6: (a) 7.00, (c) 3.40, (d) 2.50 Kbp

Lane 7: (a) 9.10, (e) 3.80 Kbp

Lane 8: (c) 5.18, (e) 3.80, (d) 3.08 and (f) 0.840 Kbp

Lane 9: (b) 7.70, (d) 3.80 Kbp

Lane 10: (a) 7.72, (b) 2.70, (c) 2.20 Kbp. The smallest fragment 0.280 is not visible in the gel.

Note that pAT 153 itself has one restriction site for each of BamH1, HindIII, PstI and SalI. It has no restriction site for SstI.

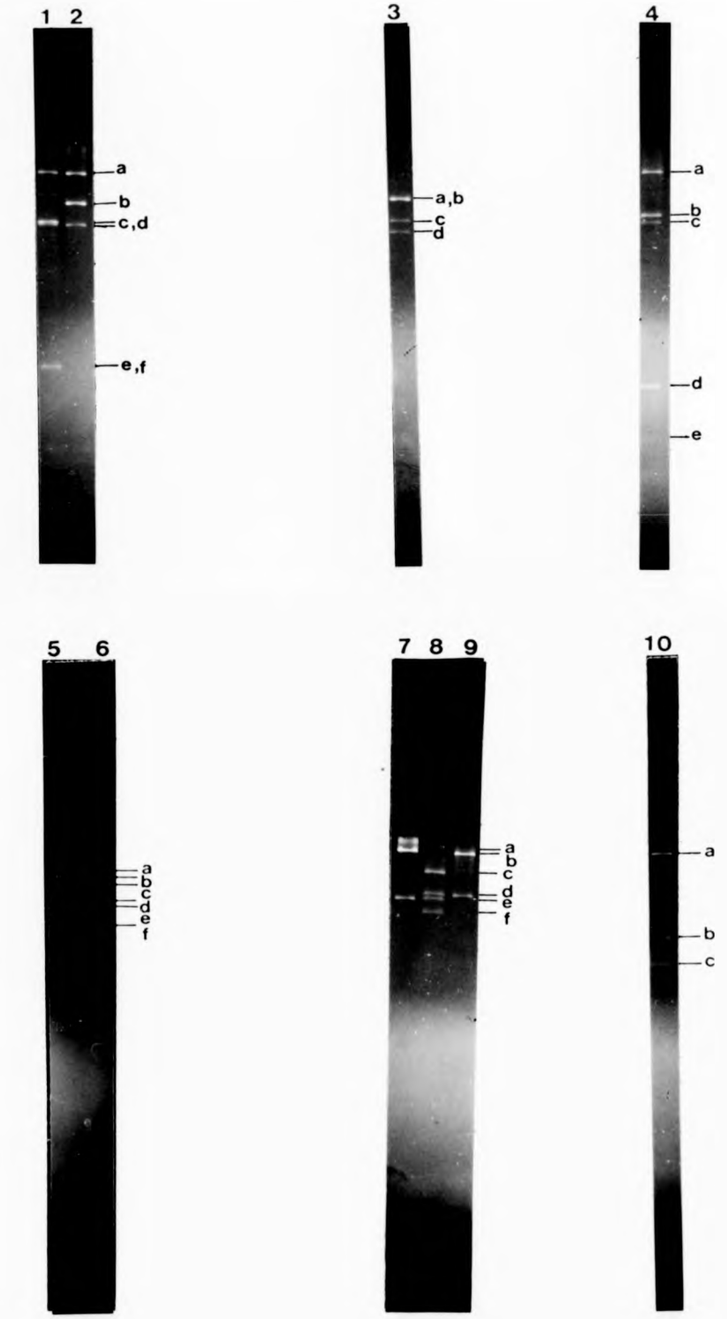

Figure 7.9 Gel electrophoretic pattern and molecular size of the restriction fragments of pSC3H1, produced by the restriction endonuclease HindIII (Lane 2), HindIII + PstI (Lane 1), HindIII + XbaI (Lane 3), HindIII + SalI (Lane 4), BamH1 + SstI (Lane 5), BamH1 (Lane 6), XbaI (Lane 7), XbaI + SalI (Lane 8), SalI (Lane 9) and PstI + SalI (Lane 10).

The purified plasmid from pSC3H1 was digested with various restriction endonucleases and analysed on $\frac{3}{4}$ agarose slab gel. The molecular size of each fragment was determined using λ HindIII and (HindIII + EcoR1) digests as gel markers. Some bands showed anomalous mobility.

Mol. sizes of the fragments in Lane 1 & 2 are: (a) 6.6, (b) 3.6, (c) 2.82, (d) 2.70 (1.92), (e) and (f), each 0.780 Kbp

Lane 3: (a) & (b) 3.80 & 3.60, (c) 2.70, (d) 2.16 Kbp. The fragment of 0.340 Kbp is not visible in the gel.

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Lane 5: (b) 5.53, (d) 3.40, (e) 2.40, (f) 1.0 Kbp

Lane 6: (a) 7.00, (c) 3.40, (d) 2.50 Kbp

Lane 7: (a) 9.10, (e) 3.80 Kbp

Lane 8: (c) 5.18, (e) 3.80, (d) 3.08 and (f) 0.840 Kbp

Lane 9: (b) 7.70, (d) 3.80 Kbp

Lane 10: (a) 7.72, (b) 2.70, (c) 2.20 Kbp. The smallest fragment 0.280 is not visible in the gel.

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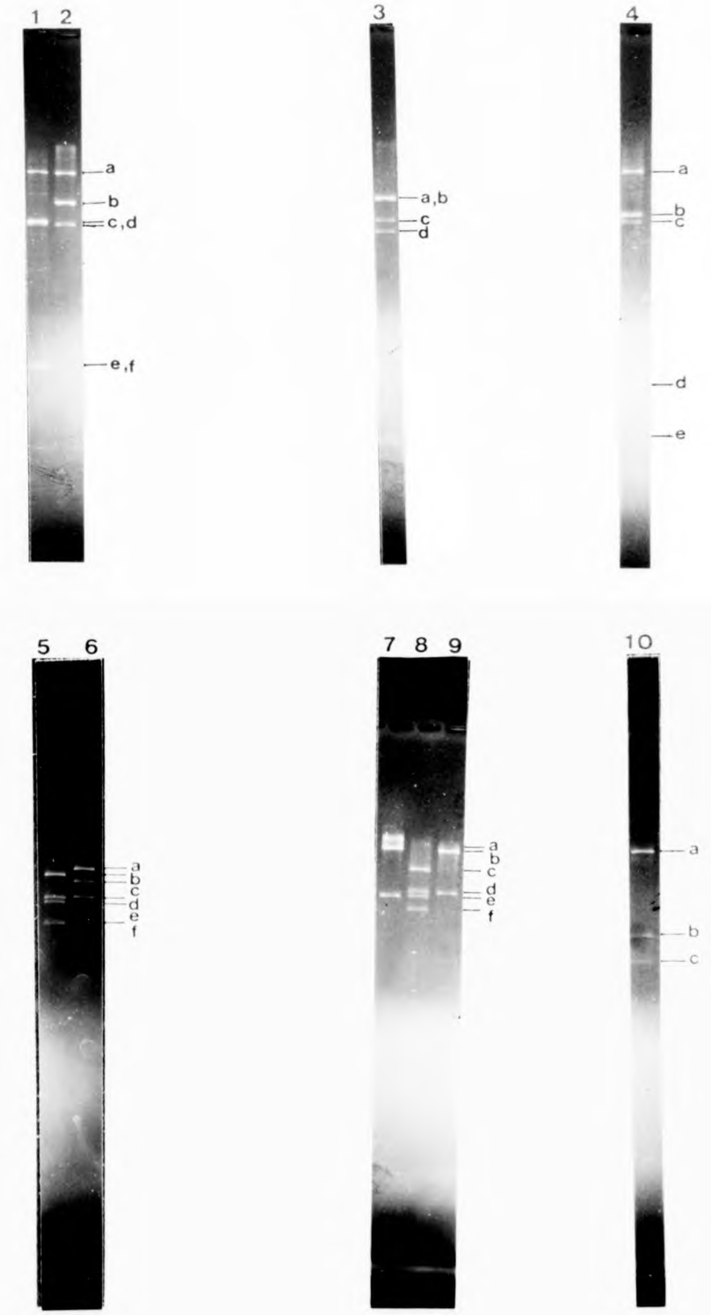

Figure 7.9 Gel electrophoretic pattern and molecular size of the restriction fragments of pSC3HH, produced by one restriction endonuclease: HindIII (Lane 2), HindIII + PstI (Lane 3), HindIII + XbaI (Lane 4), HindIII + SalI (Lane 5), BamHI + SstI (Lane 6), BamHI (Lane 7), XbaI (Lane 8), SalI (Lane 9), SalI + SalI (Lane 10), PstI + SalI (Lane 11).

The purified plasmid from pSC3HH was digested with various restriction endonucleases and analysed on $\frac{3}{4}$% agarose gel. The molecular size of each fragment was determined using λ-HindIII and HindIII + HaeIII digests as gel markers. Some bands showed co-migration mobility.

Molecular sizes of the fragments in Lane 1 (λ-2 are: a) 9.46, b) 3.68, c) 2.82, d) 2.70 (1.92), e) and f) 0.780 Kbp.

Lane 3: a) 9.46, b) 3.80, c) 3.50, d) 2.70, e) 1.95 Kbp. The fragment of 0.340 Kbp is not visible in the gel.

Lane 4: a) 9.60, b) 2.98, c) 2.20, d) 0.920 and e) 0.500 Kbp.

Lane 5: a) 9.60, b) 3.40, c) 2.80, d) 2.50 Kbp.

Lane 6: a) 7.00, b) 3.40, c) 2.50 Kbp.

Lane 7: a) 9.40, b) 3.80 Kbp.

Lane 8: a) 9.85, b) 3.98, c) 3.80, d) 1.98 and e) 0.840 Kbp.

Lane 9: a) 7.70, b) 3.80 Kbp.

Lane 10: a) 7.72, b) 2.70, c) 2.20 Kbp. The smallest fragment 0.280 is not visible in the gel.

Note that pVT 1.3 (cell line) has one restriction site for each of BamHI, HindIII, PstI and SalI. It has no restriction site for SstI.

Table 7.3 Fragment lengths from a series of restriction digests of pSC3H1. Some fragments show anomalous mobility in the gel and consequently the total size of the pSC3H1 plasmid increases. Data shown below therefore has been corrected by deduction from the known position of certain restriction sites.

RESTRICTION ENZYME	FRAGMENT SIZE (Kbp)	TOTAL (Kbp)
HindIII	6.60, 3.60, 2.70	12.9
PstI	10.20, 2.70	12.9
HindIII + PstI	6.60, 2.82, 1.92, 0.780 (x 2)	12.9
XbaI	9.10, 3.80	12.9
PstI + XbaI	5.28, 3.80, 3.08, 0.840	12.9
HindIII + XbaI	3.80, 3.60, 2.70, 2.46, 0.340	12.9
SalI	7.70, 5.20	12.9
HindIII + SalI	6.60, 2.98, 2.20, 0.620, 0.500	12.9
PstI + SalI	7.72, 2.70, 2.20, 0.280	12.9
XbaI + SalI	5.18, 3.80, 3.08, 0.840	12.9
SstI	12.9	12.9
HindIII + SstI	5.18, 3.60, 2.70, 1.42	12.9
BamHl	7.00, 3.40, 2.50	12.9
Bam Hl + SstI	5.53, 3.40, 2.50, 1.47	12.9
SmaI	12.90	12.9
BamHl + SmaI	7.00, 5.30, 0.600	12.9
HindIII + SmaI	6.60, 3.60, 2.04, 0.660	12.9

Because of highly diverged sequences, the 5S rRNA gene specific probe (pXl08) did not hybridize to 2.220 Kbp *EcoR*1 fragment. However, on the basis of the above results, and published results (Bell *et al*, 1977; Szostak & Wu, 1979), the organisation of rDNA genes on 3 μ plasmid is depicted in Fig. 7.11.

From Fig. 7.11 it is evident that the new restriction sites for *PstI, SalI*, and *BamH*1 are located in the region between 5S rRNA and 28S rRNA genes. Direct rRNA hybridization studies (Bell *et al*, 1977) with genomic rDNA revealed that this region is a nontrancribed spacer. Due to the similarity in the organisation of the rRNA genes on genomic rDNA (Fig. 7.12) and 3 μ DNA Fig. 7.11), it is likely that new restriction sites have been situated on the nontranscribed spacer (NTS) region. In the event of an insertion, the new piece of DNA is expected to carry these restriction sites.

7. Transformation of *S. cerevisiae* with pSC3H1 and pSC3H2

The occurrence of high copy numbers of 3 μ plasmid in the cytoplasm of *S. cerevisiae*, raised the possibility that the plasmid might have its own origin of replication or ARS element (Autonomous replicating sequences). To identify this ARS element, a laboratory yeast strain (D27) which has comparatively low copy numbers of 3 μ plasmid was transformed with pSC3H1 and pSC3H2. The transformation was selected on the basis of expression of β-lactamase from the ampicillin gene of pAT153 (Chevallier & Aigle, 1979). The frequency of transformation was seen to be moderate, about 300-500 per μg of DNA. Preliminary experiments suggested that the plasmid was stable and expressed its β-lactamase activity (data not shown). Both pSC3H1 (having the whole of the 3 μ plasmid) and pSC3H2 (having 2.7Kb small fragment of *Hind*III) can transform *S. cerevisiae* with the same efficiency. So it is assumed that there is at least one ARS like element on the 2.7 Kbp fragment of the 3 μ plasmid, which enables it to multiply in the cytoplasm.

8. 3 μ DNA plasmid in ρ cells:

To determine whether 3 μ plasmid undergoes any changes in respiratory deficient cells, a few ρ clones of *S. cerevisiae* were analysed for their 3 μ DNA. While ρ MR-53 did not

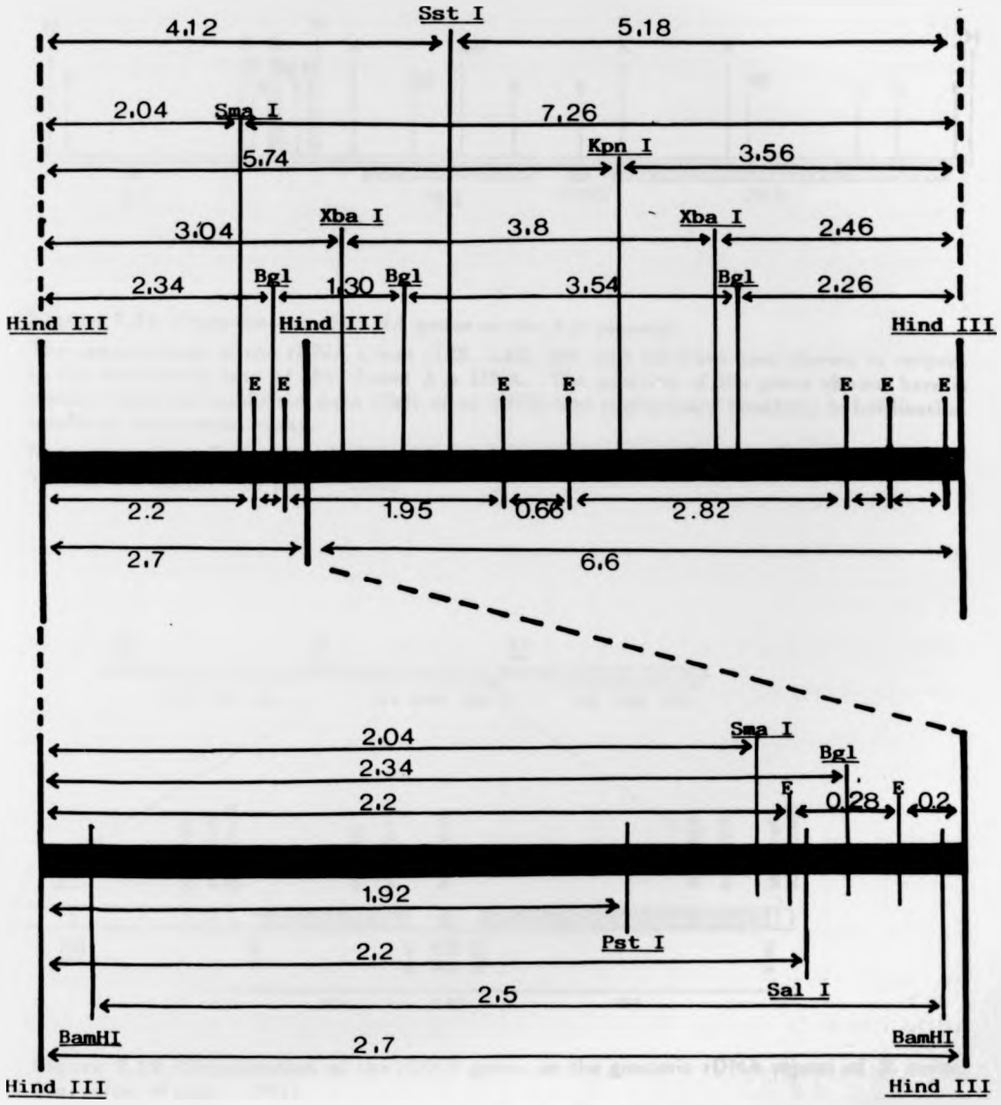

Figure 7.10 Restriction map of the cloned 3 μ DNA

The restriction map for the cloned 3 μ DNA has been drawn based on the restriction enzyme digests data of the whole recombinant plasmid pSC3H1 and published restriction sites (Bell *et al*, 1977). The known restriction sites have been shown above the 3 μ plasmid (solid bar), while at the bottom the small HindIII fragment (2.7 Kbp) has been enlarged to show the position of the new restriction sites (BamH1, PstI and SalI). The size of the fragments have been indicated in Kbp.

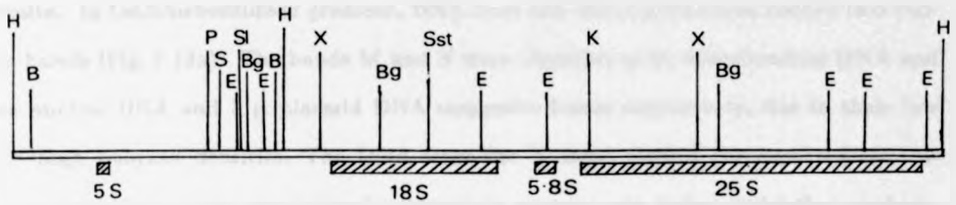

Figure 7.11 Organisation of rRNA genes on the 3 μ plasmid

The organisation of the rRNA genes (18S, 5.8S, 28S and 5S) have been shown in respect to the restriction map of the cloned 3 μ DNA. The position of the genes shown here is mainly based on published data (Bell *et al*, 1977) and preliminary Southern hybridisation results of the present study.

Restriction sites: E, EcoR1; H, HindIII; X, XbaI; S, SmaI; P, PstI; Sst, SstI; B, BamH1; Sl, SalI; K, KpnI; Bg, BglI + BglIII.

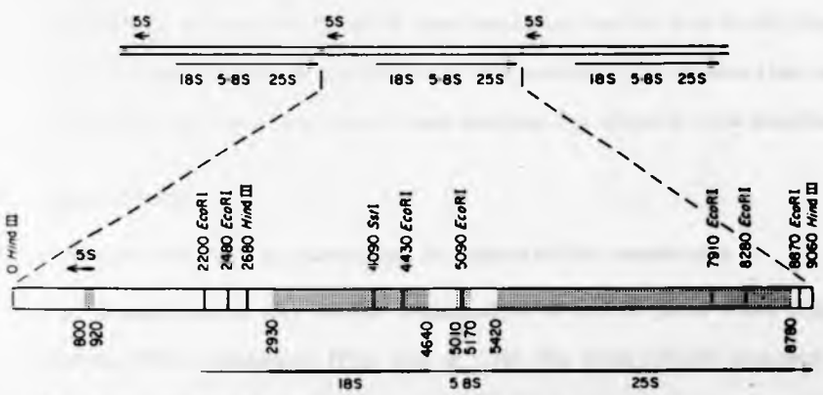

Figure 7.12 Organization of the rDNA genes on the genomic rDNA repeat of *S. cerevisiae* (after Warner, 1981).

The upper part illustrates the position of the 5S, 18S, 5.8S and 25S rRNA genes on the genome and the pattern of transcription in tandemly repeated rDNA units. The lower part represents one repeating unit with the linear co-ordinates (in nucleotide pairs) of the restriction sites and the coding region for the 4 rRNAs. The arrows indicate the direction of transcription.

show any structural alteration of the 3 μ DNA, LP-81 (ρ) produced some interesting results. In CsCl:bisbenzimide gradient, DNA from the 16K x g fractions yielded two visible bands (Fig. 7.13a). The bands M and N were expected to be mitochondrial DNA and the nuclear DNA and 3 μ plasmid DNA composite bands respectively, due to their low and high buoyant densities. The DNA from the N band yielded the expected electrophoretic pattern of the restriction fragments on agarose gels during restriction analysis. However, when the band-M DNA from LP-81 was digested with *EcoR*1, it was observed that the restriction pattern of the DNA on agarose gel electrophoresis is similar to the restriction fragments generated by the 3 μ plasmid DNA, except the second *EcoR*1 fragment (2.2 Kbp) which was missing (Fig. 7.13b). The buoyant density of the band-M DNA in a CsCl:bisbenzimide density gradient corresponds to the mt-DNA buoyant density. It has been mentioned in Chapter 3 that LP-81 has the kar-1 nuclear marker which interferes with replication and/or copy number of ρ mt-DNA molecules and in CsCl:bisbenzimide gradients we could not always detect mt-DNA. The occurrence of an aberrant or deleted 3 μ plasmid, without the 5S rRNA gene (assuming that the 2nd *EcoR*1 fragment contains 5S rRNA gene) in place of mt-DNA was very surprising. It appears that in this particular ρ strain, both normal 3 μ plasmid and aberrant 3 μ plasmid occur simultaneously.

DISCUSSION

1. Organisation of 3 μ plasmid and its nuclear rDNA counterpart

3 μ plasmid has a very similar organization of rDNA genes when compared with its nuclear rDNA counterpart (Fig. 7.11 & 7.12) The large *Hind*III fragment (6.6 Kbp) contains 18S, 28S and 5.8S rRNA genes while the Small *Hind*III fragment (2.7Kb) contains the gene for 5S rRNA. However, the occurrence of the *BamH*1, *Pst*I and *Sal*I restriction sites in the non-transcribed spacer between the 5S rRNA gene and 18S rRNA gene implies that there might be a small insertion of DNA within this region. This is also reflected in the total length of the plasmid, 9.30Kbp, in comparison to a genomic repeat length of 9.06 Kbp. An insertion of about 240 bp in this region is very interesting and may be a factor

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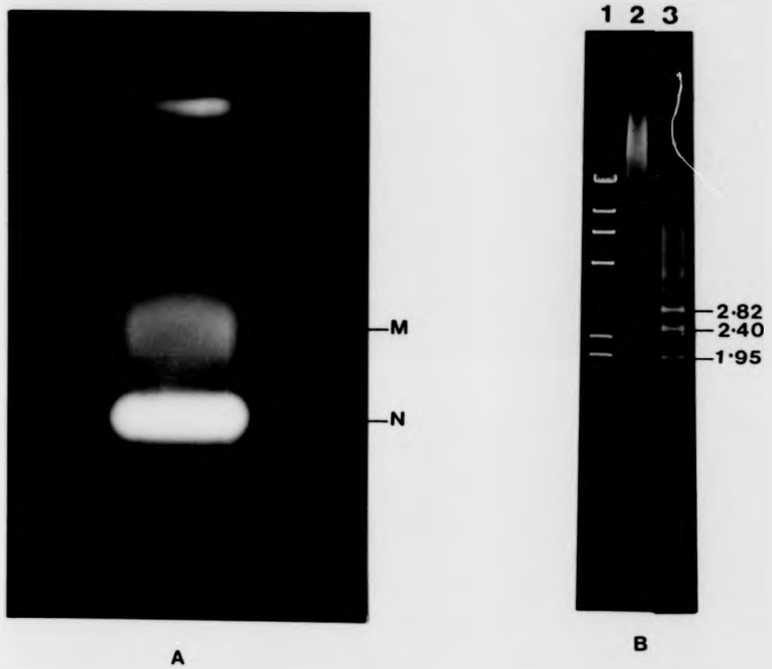
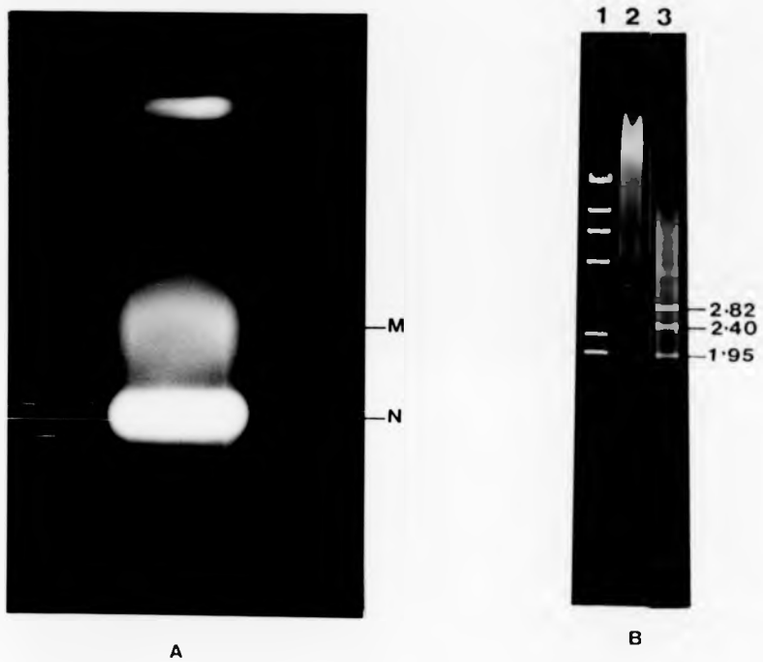


Figure 7.13 Deleted 3 μ plasmid from the ρ^- strain (LP81)

The DNA isolated from the 16 K membrane fraction of the ρ^- strain LP81 was analysed on a CsCl:bisbenzimide density gradient (**A**). The DNA band corresponding to the mitochondrial DNA (**M**) was purified and digested with the restriction enzyme EcoR1 and analysed on a 1% agarose gel (**B**)

A) CsCl:bisbenzimide gradient. M = mitochondrial fraction of DNA; N = Nuclear fraction of DNA.

B) Gel electrophoretic pattern of the EcoR1 digested DNA from the M and N fractions. Lane 1, λ HindIII digested marker DNA (see Appendix for the size of the marker fragments); Lane 2, EcoR1 digested M fraction; Lane 3, EcoR1 digested N fraction. Note that the EcoR1 digested M fraction shows only two major EcoR1 fragments (2.82 & 2.15 Kbp), while the N fraction shows all three fragments (2.82, 2.15 and 1.95 Kbp).



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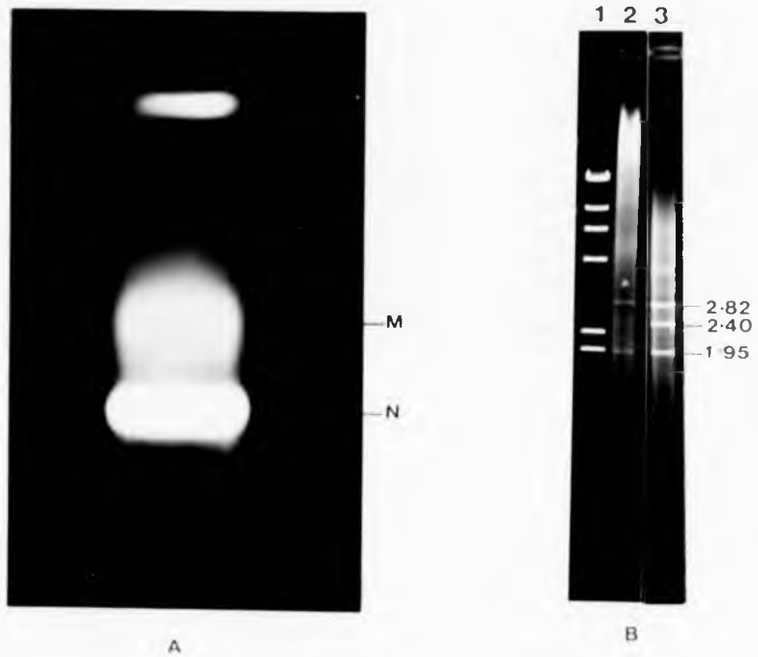

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in explaining why the rDNA plasmid in our laboratory strains of D22 origin have a very high copy number. As we have not mapped the position of rRNA transcripts in the 3 μ plasmid, it is very difficult to work out at present where exactly the insertion has taken place. DNA sequence analysis of this region may provide the nature and location of the insertion.

The length heterogeneity of rDNA gene spacers is common among vertebrates and arthropods (Long & Dawid, 1980), which is caused by their internally repetitous character. In *Xenopus laevis* and *Drosophila melanogaster* rDNA, it has been reported that a putative promotor region duplicates within the non-transcribed spacer (NTS) (Moss & Birnsteil, 1979; Simeone *et al*, 1982; Coen & Dover, 1982) and the transcription starts within the reiterated sequences. For these reasons the NTS of rDNA has been implicated in the regulation of transcription of the rDNA operon. Strain specific differences in rDNA has also been reported from *Schizophyllum commune*, where the length of the rDNA repeat unit increases from 9.20 Kbp to 9.60 Kbp due to insertion of 0.2 to 0.4 Kbp of DNA at a single site (Specht *et al*, 1984). The change observed within the spacer region in the cloned 3 μ plasmid (pSC3H1) may be a reflection of a new type of controlling element for the rDNA cistrons. Recently the NTS regions of yeast genomic rDNA have been sequenced (Skryabin *et al*, 1984) and have been shown to contain a variable region of about 250 bp, upstream of the RNA polymerase A binding site. The restriction map for the cloned 3 μ plasmid (Fig. 7.10), though preliminary, would be quite useful for future studies to investigate by appropriate genetic manipulation, what sort of control the NTS region exerts in replication and/or in transcription of rDNA, both *in vitro* and *in vivo*.

2. Autonomous replication of 3 μ DNA

Previously it has been reported that yeast genomic rDNA contains a sequence (ARS) capable of autonomous replication (Szostak & Wu, 1979). This implies that 3 μ DNA originates by the excision of a genomic rDNA repeat unit and after circularization, replicates autonomously in the cytoplasm by its inherent ARS activity. It is also possible that 3 μ

plasmid acquires some new element(s) during its formation which, besides providing an origin of replication, determines its own copy number and stability in the cytoplasm. Transformation experiments using pSC3H1 & pSC3H2 suggest that 3 μ DNA has its own origin of replication or ARS and at least one ARS element present within the smaller *Hin-dIII* fragment (2.7Kbp). Larinov and Shubochkina (1982) have demonsrated that ARS activity is present within the *EcoR*1 B fragment (2.22 Kbp) of genomic rDNA. By analysing three *Sau*3A subfragments of this *EcoR*1 B fragment, Skryabin *et al* (1984) narrowed down the ARS activities to a small region of about 570bp of NTS. Unfortunately, this ARS appeared to be comparatively weaker. The question therefore remains unresolved, whether the same ARS is involved in the propagation of 3μ plasmid or some new insertional event occurs during the generation of 3 μ plasmid in the cytoplasm.

3. Deleted 3 μ DNA of ρ cell

Observation of a deleted 3 μ DNA in a ρ (LP-81) strain is very interesting. It occurs along with the normal 3 μ plasmids. Therefore its occurrence reflects either an aberrant excision from the nucleus or incidental deletion during ethidium bromide treatment of the yeast cells. In the absence of any further data, it is very difficult to speculate whether it has any significance in the physiology of ρ cells. Sedimentation of the deleted 3 μ plasmid in the mitochondrial fraction of the CsCl:bisbenzimide density gradient was unexpected. Bisbenzimide is an (A+T) specific dye and hence, it should bind (G+C) rich rDNAs comparatively less and the 3 μ plasmid should not move to a buoyant density of the gradient similar to mt-DNA. One report from *Schizosaccharomyces pombe*, however, suggests (Fournier *et al*, 1982) that rDNA plasmid may band with mt-DNA in the CsCl:bisbenzimide density gradient. The reason for this unusual buoyant density is far from clear.

4. The possibility of any cytoplasmic genetic markers on the 3 μ DNA

Study of the organisation of 3 μ plasmid DNA shows that it contains the repeat unit of rDNA seen in chromosomal rDNA clusters. The only exception is a small insertion of

about 250 bp in the NTS region between the 18S and 5S rRNA genes. Therefore it seems unlikely that 3 μ DNA would contain any cytoplasmic genetic markers. However, it has been reported recently by Dai *et al* (1984) that a [psi] strain can be transformed into [psi]$^+$ strain with 3 μ plasmid. This interesting report does not explain in depth how 3 μ plasmid complements the function of [psi] . However, it would be interesting to find out what function of the 3 μ DNA can modulate the suppression dependent translation in [psi]$^+$ strains and whether any other functions of the 3 μ plasmid are involved in modulating the genetic characters of yeast strains.

5. Evidence for a new cytoplasmic element

Experimental results provided earlier indicate that neither 2 μ plasmid, nor dsRNAs are involved in the expression of non-mitochondrial cytoplasmic genetic characters, VEN^R, TET^R and $Rh6G^R$. A strain can show rhodamine resistivity when its 2 μ DNA and dsRNAs are missing (Fig. 7.3). A study of the organisation of the 3 μ plasmid also does not provide any strong evidence that it carries the VEN^R, TET^R or $Rh6G^R$ alleles. Therefore, the search for a physical entity for these characters still remains open. Evidence for a high molecular weight plasmid (Fig. 7.2) in the cytoplasm of yeast raises the possibility that this could be a probable candidate. However, the inconsistency of its occurrence in the cytoplasm makes it difficult for systematic searching. Reports on the existence of extra chromosomal circular copies of Ty 1 elements (Ballario *et al*, 1983) also culminates with the confusing identification of certain DNA species. Isolation and identification of mitochondrial plasmids from *Neurospora* (Collins *et al*, 1980; Natvig *et al*, 1984), maize (Weissinger *et al*, 1982), *Brassica* (Palmer *et al*, 1983) still maintains optimism for identification of a similar plasmid in *S. cerevisiae* where they might be related with the mitochondrial functions.

CHAPTER-8

GENERAL DISCUSSION

1. Nuclear control of petite mitochondrial DNA in Yeast

The nuclear genome undoubtedly makes a major contribution towards the normal functioning of mitochondria. Therefore it is essential to isolate mitochondrial mutants in an isonuclear background for their comparative biochemical studies. For this reason the nuclear fusion mutation *kar-1* (Conde & Fink, 1976) had been used in this laboratory for cytoduction of various mitochondrial mutations into a common nuclear background. As a result of this most of the yeast strains used in the present study had *kar-1* mutation on the nuclear genomes. The results obtained in Chapter-3 show that the mutation *kar-1* has an effect on the transmission and copy number of petite (ρ^-) mitochondrial genomes. This unexpected finding was a major problem for molecular characterisation at the DNA level, of various petites generated from the grande strain CD40 by petite deletion mapping (Chapter-2). Though this has been bypassed by cloning of the relevent portion of the mitochondrial genome into the plasmid vector pAT153 of *E. coli* (Chapter-4), the problem as such remains unsolved regarding the mechanism of action of *kar-1*.

The poor yields of mt-DNA from the petites with the *kar-1* mutation point to the fact that the problem is associated with the copy number or replication of ρ^- mt-DNAs. However, the observation that some of the cells of these petites (e.g. MR53, MR23, MR50, MR60) had ρ^0 appearance (as evidenced by the lack of chondriolytes in DAPI stained cells) suggests that the problem may also be associated with the transmission of the ρ^- genomes. Probably during the transmission of mt-genome, a few copies of mt-DNA are disproportionately distributed among the daughter cells and a result of this a certain fraction of cells always become ρ^0. Therefore the quantity of mt-DNA isolated from a petite with *kar-1* represents the DNA of a fraction of the cell population.

A report by Delgado & Conde (1984) shows that Benomyl, a commercial fungicide and

known inhibitor of fungal microtubule polymerization (Quinlan *et al*, 1980), can prevent nuclear fusion and can be used in the production of cytoductants in yeast. The effect of *kar-1* could be analogous. Both of them somehow disrupt the microtubule associated cytoskeletal network and the spindle polar body (SPB) (Quinlan *et al*, 1980) and affect not only the fusion of nuclear membrane, but also the transmission of mt-genome by altering the organisation of the mitochondria in the cells. This explanation finds support from the DNA sequence analysis of a cloned kar-1 gene from Dr. Fink's laboratory (Rose & Fink, 1984). The polypeptide deduced from the DNA sequence shows the charecteristics of the microtubule associated proteins (MAP) (M. Rose. personal communication). But why this effect is limited to the petite (ρ) mt-genome and, what is the function of normal allele of *kar-1* in the cell can not be explained. Obviously more detailed study would be needed to understand the underlying mechanism.

2. Organisation of mutations of oxidative phosphorylation on the mitochondrial genome of *Saccharomyces cerevisiae*

Mutations of the ATPase complex involved in oxidative phosphorylation have been demonstrated to cluster around two distinct regions: Oli-1 and Oli-2, separated from each other by a complex locus, Cob-box, on the mitochondrial genome of *Saccharomyces cerevisiae*. Combined genetic, biochemical and molecular analyses of the Oli-1 region (which includes the genetic loci Oli1, Oli3, VEN^{R}_{81}, and pho2) established that this region codes for subunit-9 or the DCCD binding protein of the O.S. ATPase (Sebald *et al*, 1979; Macino & Tzagoloff, 1979; Hensgens *et al*, 1979). It was of interest therefore, to study the Oli-2 region (in which many genetic loci have been mapped) to find out whether it codes for one or more proteins. Two types of mutations have been employed: antibiotic resistant ($Oli2^{R}$, $Oss1^{R}$) and *mit* (pho8, pho9, mit-175) mutations. While both antibiotic resistant mutations have been mapped in the structural gene of subunit-6 (or the Oli-2 gene), two *mit* mutations (pho8 and and pho9) have been mapped upstream and one mit-mutation (mit-175) has been mapped downstream of the Oli-2 gene. These three genetic

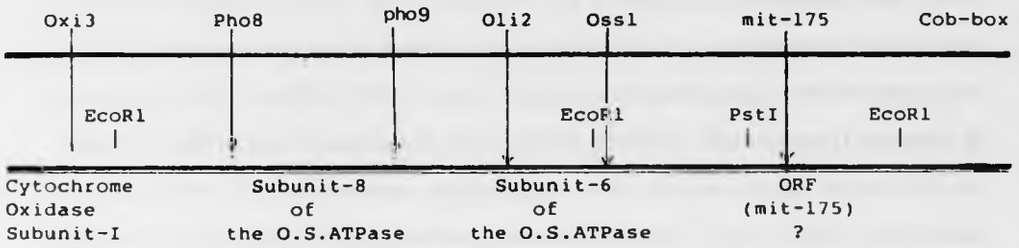
loci, distinct from each other in petites, were assumed to be on separate structural genes either coding for subunits of the O.S. ATPase or a regulatory gene(s) controlling the expression of the subunit-6 gene (see Chapter-2, Connerton *et al*, 1984). From the present study it is apparent that the Oli-2 region containing these three discriminative genetic loci encode two and probably three gene products related to oxidative phosphorylation.

The known organisation of genes for oxidative phosphorylation on the mitochondrial DNA indicates that they are not contained in an operon (i.e. all functionally related genes would be clustered into a single region and controlled by a single regulatory gene,or operator) as would be expected for their origin from archaebacteria following endosymbiosis (Margulis, 1970; See also Woese, 1981; Gray, 1982; Gray & Doolittle, 1982; Wallace, 1982). In *E.coli*, the *atp* or *unc* operon consists of all the genes for the subunits of the ATP synthase (homologous to the O.S. ATPase of mitochondria) (see Walker *et al*, 1984). The distinctive difference in the organisation of the two systems establishes that while mitochondria might have their origin in endosymbiotic prokaryotes, they undoubtedly have undergone their own evolution in a quite distinct way from their ancestors, at least at the level of genomic organisation. The complexity of the cytoplasmic environment of eukaryotes, which has evolved gradually, would have demanded change in organisation of the harbouring mt-genome. This implies that, the structure and number of components constituting any functionally equivalent system should also change. The oligomycin insensitivity of prokaryotic ATP synthase partly supports this presumption. The fine structure genetic map established for the Oli-2 region of *Saccharomyces cerevisiae* (Fig. 2.2, Chapter-2) provides the opportunity to substantiate this presumption as well as to obtain information on the role of different components of O.S.ATP synthase of mitochondria in oxidative phosphorylation.

3. Physical and genetic map correlations with various mutations in the Oli-2 region.

DNA sequence analysis of the Oli-2 region (3000 bp downstream of Oxi-3) demonstrates

Figure 8.1 Genetic and Physical map Correlation of Various markers in the Oli-2 region.



that this region codes for two subunits, subunit-6 and subunit-8, and possibly one more downstream of the subunit-6 gene. Antibiotic markers (*Oli*2^R, *Oss*1^R), as expected, have been shown to be located within the reading frame of subunit-6. The pho9 mutation, however, has been shown to lie neither within the subunit-6 gene nor within the subunit-8 gene and possibly, therefore lies in the intergenic regulatory region. The mutation mit-175 has been located downstream of the Oli2 based on partial DNA sequence from this strain. This demonstrates agreement between genetic data and their physical location on mt-DNA (Fig. 8.1). One particular locus however is at variance, that of the *Oss*1^R genetic marker. Earlier studies by Lancashire and Mattoon (1979b) demonstrated that the relative position of *Oli*2^R, *Oli*4^R and *Oss*1^R on mitochondrial DNA were Oli2 _ Oss1 _ Oli4. DNA sequence analysis however demonstrates that the orientation of these markers on the mt-DNA is Oli2 _ Oli_4 _Oss1. This observed discrepancy could be due to the variable recombination frequencies in this region of mt-DNA. The authors (Lancashire & Mattoon, 1979b) took the average recombination values obtained in this region to interpret their genetic data and to position them on the map. The recombination values within the mitochondrial genome cannot always be accepted as a proper estimation of physical distance between two loci within the same structural gene. For example, in the subunit-9 gene, two distinctive genetic loci Oli1 and Oli3 have been shown to lie apart by a recombination value of 1.5% (Lancashire *et al*, 1975; Trembath *et al*, 1976). But when subunit-9 from two Oli1 resistant mutants (D273-10B/A21 & D273-10B/A31) and one Oli3 resistant mutant (D273-10B/A68) were sequenced, it was shown that while A21 and A31 Oli1 resistivity were due to a change in the 53rd aminoacid and 65th aminoacid respectively, the Oli3 (A68) resistivity was due to change in the 57th aminoacid of subunit-9 (Sebald *et al*, 1979). Therefore, the order of the loci in terms of physical location, Oli1 (A21) - Oli3 (A68) - Oli-1 (A31), was quite unexpected in view of recombination analysis by Lancashire *et al* (1975) and Trembath *et al* (1976). However, in the present study overall mapping data for the location of pho8, pho9, Oli-2, Oss-1 and mit-175 was in agreement with the DNA sequence data.

4. Changes in the subunit-6 gene associated with drug (Oligomycin & Ossamycin) resistance.

The DNA sequence analysis of mt-DNA from oligomycin and ossamycin resistant mutants, genetically linked in the Oli-2 region, establishes that a single point mutation in an otherwise conserved region of the subunit-6 gene can offer resistance to a particular drug (Fig. 5.19, Chapter-5). Oligomycin resistance in $Oli2^R$-76 is due to a change in the 171st aminoacid, (isoleucine → phenyl alanine). Ossamycin resistance in $Oss1^R$-92 is due to a change in the 254th aminoacid residue (aspartic acid → asparagine) (See Table 8.1). The location of the $Oli2^R$-76 resistance allele in the 171st aminoacid is of interest as Macino & Tzagoloff (1980) located the $Oli2^R$-118 allele in the same aminoacid residue, but the change was isoleucine → methionine. These two alleles however, recombine with each other with a value of 0.01% to 0.1% (Connerton *et al*, 1984b). This extremely high rate of recombination is difficult to explain, but points out the fact that recombination values should not be used as the sole criterion of the physical location of any allele on the mt-genome.

Recently, Novitski *et al* (1984) have sequenced another Oli2 mutation ($Oli2^R$-23 allele) and found an aminoacid substitution in the 175th aminoacid residue, serine → threonine. All these results suggest that this particular region of subunit-6 may play an important role in the functional properties of the F_o complex of mitochondria, as it is known that F_o is the site of action of oligomycin. Oligomycin probably does not bind solely to these residues (171st & 175th aminoacid residues), but also binds to another region of subunit-6, a genetically distinct site called Oli-4. Macino & Tzagoloff (1980) have demonstrated a change at the 232nd position (isoleucine → phenylalanine) in a $Oli4^R$ mutation. Slott *et al* (1983) also demonstrated, from DNA sequence analysis of an oligomycin resistant mouse cell line, a change in the 243rd residue (valine → glutamic acid) of the corresponding subunit-6 gene. This position appears to be a highly conserved valine residue, when comparisons are made with other organisms (See Fig. 5.19 & 5.21). This particular region of subunit-6 (Oli-4 Site) together with the Oli-2 Site (this thesis; Macino & Tzagoloff, 1980;

Table 8.1 Changes associated with the Oligomycin and Ossamycin resistance

Mutation	Affected gene	Observed Changes			
		Nucleotide	Codon	Amino acid	Location
$Oli2^R$-76	Subunit-6	A → T	ATT→TTT	Isoleucine → Phenyl alanine	171st amino acid residue
$Oss1^R$-92	Subunit-6	G → A	GAT→AAT	Aspartic acid → Asparagine	254th amino acid residue

Novitski *et al*, 1984) are probably involved in the binding of oligomycin and leading to inhibition of proton translocation across the membrane sector or F_O of the O.S. ATPase. The oligomycin binding site however, appears to overlap with the ossamycin binding site(s). Most of the oligomycin resistant mutants of yeast are cross resistant to ossamycin both *in vitro* and *in vivo* (Griffiths & Houghton, 1974). *Oli*2^R-118 strain (Macino & Tzagoloff, 1980) also has cross resistance to ossamycin. Therefore the location of ossamycin resistivity in the present study at the 254th aminoacid residue (asparagine in place of aspartic acid) of the subunit-6 gene points out that ossamycin interacts both in the oligomycin reacting site (as evidenced by their cross reactivity) as well as in other sites, away from this site. Ossamycin possibly interacts inside the membrane with the hydrophobic groups of the oligomycin binding site and outside the membrane with a charged residue, aspartic acid which is presumed to lie outside the membrane (see Fig. 6.11, Chapter-6).

5. Identification problems of subunit-6 gene

Concerted genetic and molecular analysis prove beyond doubt that the Oli-2 locus of mt-DNA is the structural gene for subunit-6 (see Roberts *et al*, 1979). However the reading frame identified in the Oli-2 locus has a coding capability of a much larger protein (28.2 kilodalton) than the protein identified as subunit-6 (20.5 kilodalton) in SDS gels (Tzagoloff & Meagher, 1971; Roberts *et al*, 1979). To rationalise this discrepancy two arguments have been put forward: the protein undergoes post translational modificaion by selective degradation of a larger precursor molecule, and/or the hydrophobic protein makes an unusual complex with SDS whose mobility becomes faster than usual. A third possibility has been raised in this thesis (Chapter 5), i.e., that the 2nd ATG codon of the 777 nucleotide long reading frame of the Oli-2 locus might be the initiation site for translation of the protein. In this case the product would be about 178 aminoacid residues long and would give a molecular weight of about 19 kilodaltons. The molar percentages of the aminoacid sequence of the 2nd reading frame is similar to the values which have been experimentally

obtained for subunit-6 from *Neurospora* by protein analysis or DNA sequence analysis (Table 5.4, chapter-5) However, in a recent meeting on Yeast Genetics and Molecular Biology of Yeast (1984), Lukins *et al* (1984) reported a *mit* mutation in the Oli-2 region which is located within the first 36 to 55 aminoacid residues. First 36 aminoacids, as deduced from the DNA sequence of the subunit-6 gene in this *mit* mutant, are similar to those of the wild type but the 37th to 55th aminoacid residues are different from the wild type. The reading frame then terminates by a nonsense codon and therefore, does not express subunit-6. The location of the *mit* mutation in this region, calls into question the possibility of the 2nd ATG codon being the translation initiation site. However, the analysis of subunit-6 by cyanogen bromide fragmentation and antibodies raised against the NH_2 terminal aminoacids of subunit-6 should give a definitive answer to this problem.

The existence of two forms of subunit-6 was demonstrated in one dimensional gels by Todd & Douglas (1983) and in two dimensional gels by Stephenson *et al* (1981). Papain-generated peptides of subunit-6 revealed that the two forms, 6a & 6b were identical except for the mobility of a single fragment (Todd & Douglas, 1983). Hence the possibility that both the 1st ATG and 2nd ATG might operate as translational initiation sites remains open. The occasional location of an oligomycin resistant mutation in the nucleus (Rank and Bech-Hansen, 1973; Rank *etal*, 1975; Mitchell *et al*, 1973; Cohen & Eaton, 1979) might provide another explanation for the observed variants of subunit-6, in which case a nuclear counterpart of subunit-6 is synthesised in the cytoplasm and then transported to the mitochondrion. However, all these explanations remain unproven.

5. Location of the pho9 mutation

The evidence from the DNA sequence analysis that the mutation pho9 does not lie within the genes for the subunit-6 and subunit-8 is very interesting. Genetic evidence suggested (Chapter-2) that the mutation was located upstream of the Oli2 and could be discriminated in petites from the Oli2 locus, and therefore could be on a separate gene, regulatory or structural (see Connerton *et al*, 1984). During this work DNA sequence analysis

by Macreadie *et al* (1983) and protein sequence analysis by Velour *et al* (1984) have shown that in fact, there is a gene between the Oxi-3 and Oli2, which codes for the subunit-8 of the O.S. ATPase. DNA sequence analysis of this region in the present study also indicates the existence of the same gene which is in perfect homology with the DNA sequence data of Macreadie *et al* (1983) and protein sequence data of Velour *et al* (1984). However, one of the earlier presumptions that the pho9 mutation may be located on the subunit-8 gene (Chapter-2) does not appear to be true. However, the possibility that the mutation is a regulatory one and controls the expression of the subunit-6 and subunit-8 (as evidenced by the absence of these subunits in the O.S.ATPase of the mutant strain) raises the hope for a system to study the regulatory sequence on mitochondrial genome of yeast which is little understood so far.

7. Location of the mit-175 mutation

Genetic evidence in Chapter-2 shows that mit-175 is located downstream of the Oli-2. Mit-175 has typical *mit* characteristics, with normal cytochrome spectra and ATPase activity (I.F. Connerton, unpublished result), but is unable to grow on nonfermentative media. It has a normal subunit-6, subunit-8 and subunit-9 in the O.S. ATPase (Ray *et al*, 1984). The sequence analysis of the relevant portion of mt-DNA from the wild type has revealed several reading frames, which poses a problem in the identification of the gene. One probable reading frame has been analysed (See Fig.5.17, Chapter-5) which can give rise to a protein product of 16.2 kilodalton. This reading frame uses ATA as an initiator codon which is why the gene could not be confirmed. The relevent portion of the mt-DNA from the mutant strain (mit-175) has now been cloned and future DNA sequence analysis of this region would help to confirm the reading frame.

If the reading frame codes for a structural protein of the O.S. ATPase, why it is not detected in the gel electrophoretic analysis is not known. The possibility that the gene codes for a regulatory protein which is active in trace amounts and remains undetectable at the level of sensitivity of the method, or that it remains masked by some other mito-

chondrially synthesised protein remains to be established. The transcription of a RNA from this region of mt-DNA indicates that this gene is expressed at least at the transcriptional level (see Chapter 5). Therefore, another possiblity is that the RNA plays some role in the catalytic activity of the O.S.ATPase. Evidence for the role of RNA as catalyst is now accumulating in the literature (Guerrier-Takada *et al*, 1983; Huang *et al*, 1984). Also recent evidence has indicated that RNA is required for the import of precursor proteins into mitochondria (Firgaira *et al*, 1984). This observation could be of significance in respect to the mit-175 locus.

8. The relative organisation of the genes for subunit-8, subunit-6 and mit-175 in the mt-DNA

The relative spatial organisation of the subunit-8 gene, subunit-6 gene and mit-175 in *Saccharomyces cerevisiae* is represented in Fig. 8.2. The figure demonstrates that the organisation of the genes in this segment of the mt-genome follows the same general pattern observed elsewhere in the mt-genome of *S. cerevisiae*: the structural genes are separated by long (A+T) rich spacers with clustered (G+C) sequences within them (Prunell and Bernardi, 1977a; 1977b). When comparing the organisation of this segment of the genome with those from other organisms (Fig. 8.3), it is found that the relative distance between the subunit-8 and subunit-6 genes has gradually shortened during the course of evolution. The implication of this is not understood at present. Cellular economy has no doubt, compelled them to dispense with the luxury of spacers between these two genes which are otherwise involved in a similar function in F_o, but overlapping of the genes in mammals might have something to do with the control and expression of two essential components of the O.S.ATPase. However, the occurance of a 42 bp intron at the beginning of the subunit-8 gene in *Neurospora* (in fact 14 aminoacids are in frame with the first ATG coding f-met) is interesting (Morelli & Macino, 1984). It is not yet established whether this first 42 nucleotide inframe-sequence is an intron or, just an extended N-terminal end of the same protein.

Figure 8.2 Organisation of DNA at the Oli-2 region of mt-DNA. The data are based on the strain CD40. The data beyond the last EcoR1 site has been taken from Macino & Tzagoloff (1980). The positions of the genes and spacers are not according to the scale.

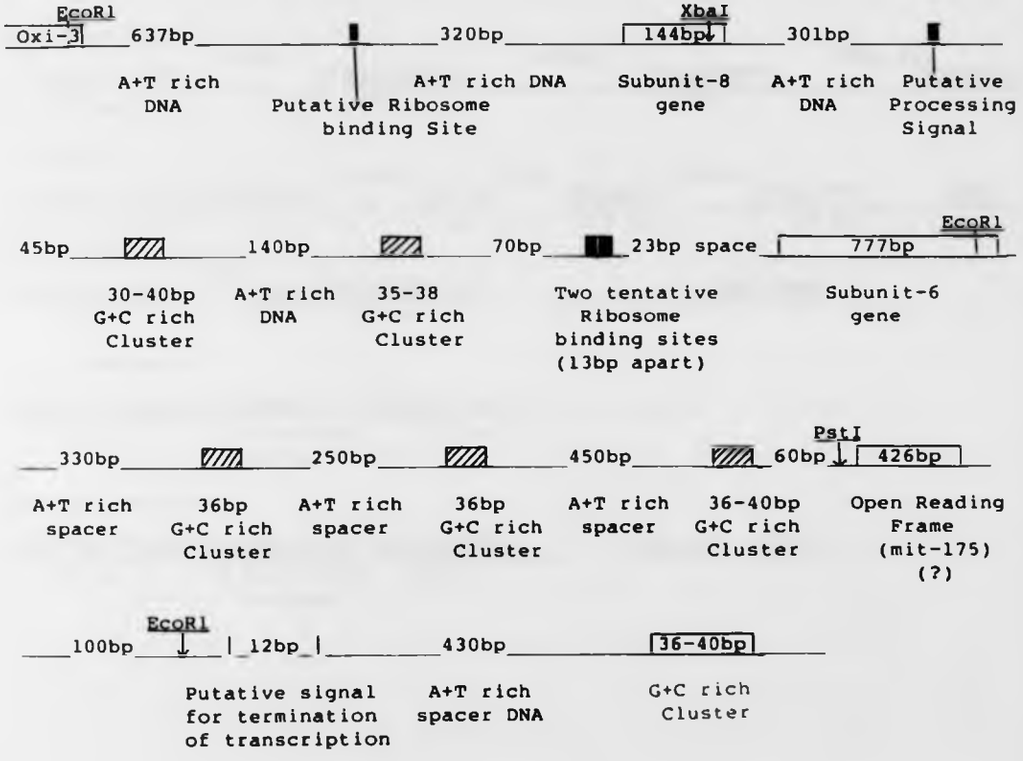
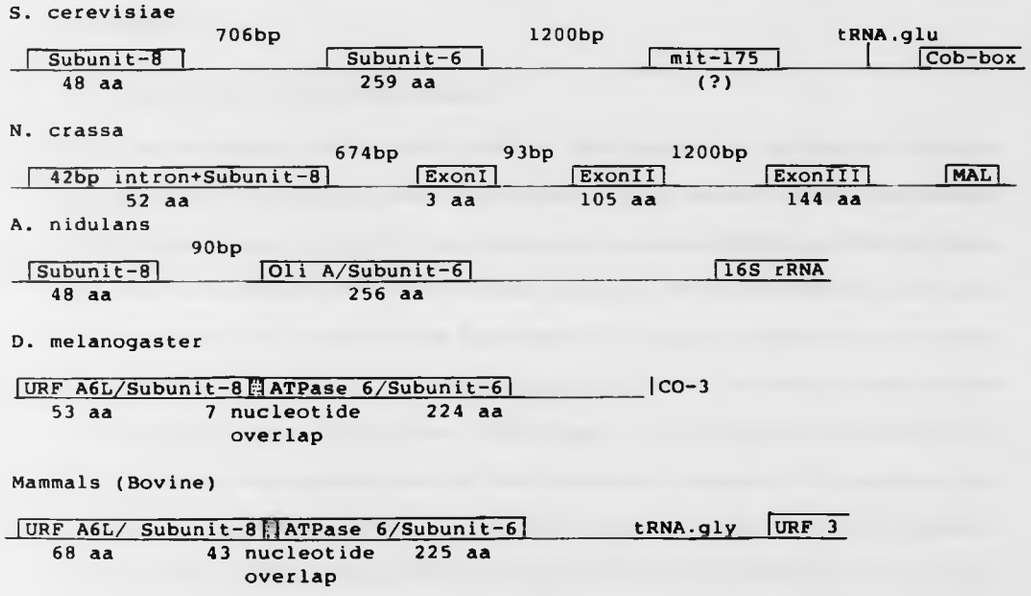


Figure 8.3 Relative position of the genes for subunit-6 and subunit-8 in various organisms.



The comparative organisation of the Oli-2 region from various organisms, however, does not reveal any URF, downstream of the gene for subunit-6 which could be similar to the tentative reading frame suggested for mit-175 locus found in *Saccharomyces cerevisiae*. Other URFs found in the same region of *S. cerevisiae* also do not have any substantial homology with the URFs found in mammals (bovine, mouse, rat and human), Insects (*D. melanogaster, D.yakuba*, or other fungi (*A. nidulans, N. crassa*). With the lack of total sequencing data from mit-175, it would be very difficult to propose any particular URF as the real gene.

9. Transcription of the Oli-2 region

Three major species of RNAs (5100, 4800 and 3000 nucleotides) are found to hybridize with the Oli-2 region specific probe (See chapter-5), which probably contains the messege for the subunit-6 and subunit-8. Two other smaller transcripts (800 bp and 600 bp) which hybridize to downstream of the Oli2 locus are also found, which are relatively weak in our gels (Fig. 5.18). The occurence of the higher molecular weight species of RNAs in the gels are probably the precursors of mRNAs for this region. In fact, the transcriptional studies *in vitro* (Christianson & Rabinowitz, 1983; Osinga *et al*, 1984) have indicated that the Oli-2 region is co-transcribed with the Oxi-3 (the gene for subunit 1 of cytochrome oxidase) locus. The resultant RNA is then probably processed to give rise to three abundant species of RNA (5100, 4800 and 3000 nucleotides) for the Oli-2 region which contain the information for both subunit-8 and subunit-6. Of these three species 3000 nucleotides species is probably the matured mRNA (which is very strong positive band in Fig.5.18, Chapter-5). It is not known whether this mRNA undergoes further processing to produce separate transcripts for subunit-6 and subunit-8.

Using a cloned mt-DNA fragment on a bacterial plasmid as a substrate for transcription with purified mitochondrial RNA polymerase, it has been suggested that there is no putative promotor between the Oxi-3 and subunit-8 genes (Edwards *et al*, 1983). When the DNA sequences between these two regions were searched for the occurence of putative

mitochondial promotor sequences (ATATAAGTA) (Tabak *et al*, 1983), at 198bp upstream of the subunit-8 gene a slightly different sequence (ATAAATGTA) was found. Only two differences are found in the 4th and 6th places of the putative nonanucleotide promotor. Whether this sequence can act as a promotor for the Oli-2 region is yet to be established. The abundance of 5100 nucleotide and 4800 nucleotide mRNA species for subunit-6 and subunit-8 could be due to a new initiation of the transcript from this promotor. Some petites which lack the so called promoter have been shown to transcribe their genome (Cobon *et al*, 1982).

The occurrence of 800 nucleotide and 600 nucleotide RNA transcripts which hybridize to 585 bp and 450 bp *HpaII* fragments of Eco-7 (see Chapter-5 and Cobon *et al*, 1982) indicate that this region is expressed at the transcriptional level. The possibility that the small transcripts for this region are the secondary products of normal processing from larger transcripts of the Oli-2 region without any functional significance can be counter-argued for two reasons: (i) genetic mapping of the locus mit-175 in this region, and (ii) the discrete size of the transcripts as opposed to random products. However to find out the proper significance of the transcripts, studies of the translation of this RNA *in vitro* and sequencing of the mit-175 mt-DNA from this region are necessary.

Osinga *et al* (1984) have shown that a dodecamer sequence (5'-AATAATATTCTT-3') which is present downstream of nearly all protein coding genes on yeast mt-DNA, occurs 19 bp downstream of the *EcoR*1 site of 1.7Kb *EcoR*1 fragment. The motif is situated 100 bp downstream of the proposed functional URF of this region. This dodecamer motif may act as a processing point in the generation of 3'-ends of the mature mRNAs or act as a signal for the termination of transcription.

Processing of transcripts from the Oli-2 region is probably signalled by the secondary structures of (G+C) rich clusters observed in this region. No other mechanism has been proposed so far. However, the hepta-nucleotide sequence (ATTCTTA) which has been demonstrated by Thalenfeld *et al* (1983) to be a processing signal between the Oli1 (subunit-9) gene and $tRNA^{arg}$ gene, can be found between the genes for subunit-8 and

subunit-6 (See Fig. 5.8, Chapter-5), which might also be significant in the processing step.

10. Ribosome binding sites

No Shine & Dalgarno (SD) sequence has been demonstrated to occur in the 3' end of the 15S rRNA of yeast mitochondria. However, a 10 nucleotide sequence at the 3' end of the 15S rRNA of yeast mitochondria has been shown to occur upstream of mRNAs of various mitochondrial genes and on the basis of this, 5' - AAATTCTATA - 3' has been proposed to be analogous to SD sequence, which can potentially bind mRNA to the ribosome (Li *et al*, 1982, Tzagoloff, 1982). Li *et al* (1982) pointed out that an 8 bp sequence (TAAGATAT) which occurs 50 bp upstream of the ATG codon of the subunit-6 gene and is homologous to the 3' end of 16S rRNA (see Fig. 5.15, Chapter-5) is the putative ribosome binding site and may subserve the function of an SD sequences. In addition Novitski *et al* (1984) have claimed that an 11bp sequence (TATTTATAAGT) which occurs 26bp upstream of the 1st ATG codon of subunit-6 gene might also be a potential ribosome binding site with 25% greater estimated hydrogen bond stability (ΔG= -16kcal/mole).

Novitski *et al* also pointed out another 9 bp potential ribosome binding site, 309bp upstream of subunit-8 gene, which we also find in our sequence data (Fig. 5.15). This seems to be an unlikely candidate due to its location, far away from the initiating ATG codon of the gene for subunit-8.

However, we find another 11 bp sequence, 11bp upstream of the 2nd ATG codon of subunit-6 gene, which is complementary to the 3' end of mitochondrial 15S rRNA (Fig. 5.15d, Chapter-5). Within this 11 bp sequence 10 nucleotides are complementary to the 15S rRNA. Whether this has a role in initiation of translation from this ATG codon is not clear, as has been argued in Chapter-5 and section-5 of the present chapter. In any event, occurrence of these sequences complementary to the 3' end of the 15S rRNA could be fortuitous due to the (A+T) richness of mitochondrial DNA. Hence, we shall have to wait for direct experimental evidence for their functional significance, if any, in the initiation of translation.

11. Secondary and tertiary structure analyses of various subunits of the F_O

Secondary structure analysis based on hydrophobicity plots (Kyte & Doolittle, 1982) and a modified Chou & Fasman's prediction method (Chapter-6) have been used to propose tentative models for subunit-6, subunit-8 and subunit-9 of yeast mitochondrial ATP synthase (Fig. 6.11, 6.14, 6.20, Chapter-6). In subunit-6 seven hydrophobic segments span the lipid bilayer, a general feature similar to that found in bacteriorhodopsin (Fig. 6.6). All these hydrophobic segments have a potential α-helix structure which would be energetically favourable in the lipid bilayer. In contrast, subunit-9 has 2 hydrophobic intramembrane segments giving it a hair-pin like structure in the membrane (Fig. 6.20) and subunit-8 has only one membrane spanning segment (Fig. 6.14). How these subunits, interact with each other and assemble to form the F_o "proton channel" is not known. Subunit-6 with its seven membrane spanning segments, may potentially form a proton channel like bacteriorhodopsin, but no experiment has been done so far to demonstrate this, using isolated subunit-6 reconstituted into lipid vesicles. However, reconstitution experiments in membrane vesicles using subunit-9 from yeast (Schindler & Nelson, 1982) has demonstrated that this subunit alone in oligomeric form can make a selective proton channel, similar to the homologous subunit from chloroplasts (Nelson *et al*, 1977). The authors suggested that a dimer of subunit-9 can form a single channel and that hexameric subunit-9 observed in preparations of the O.S. ATPase, probably forms three proton channels in the membrane (See Chapter-6). This suggests a stoichiometric relationship with the 3α and 3β subunits (catalytic site of ATP synthase) found to occur in F_1 (Todd *et al*, 1979) and may be relevent to the 3 H^+/ATP ratio observed in oxidative phpsphorylation (McCarty, 1978). However, this presumption does not explain how they are related to the membrane spanning segments of subunit-6 and whether or not subunit-6 contributes to the formation of a channel *in vivo* and/or whether subunit-6 makes a separate channel in association with the channels formed by the subunit-9. Secondary structure analyses suggest that it may form a separate channel, the structure of which would be similar to that of bacteriorhodopsin. Biochemical data from this laboratory suggest that

there might be two channels: one driving protons from outside of the mitochondria to the inside during ATP synthesis and the other from outside to inside during ATP hydrolysis. The latter reaction can be selectively inhibited while the former reaction continues in the presence of the organotin compound Ve2383 (Emmanuel, 1981; Emmanuel *et al*, 1984).

The candidature of the subunit-8 in the formation of the proton channel has not been addressed as yet due to its recent inclusion in the membership book of the F_o sector. Preliminary experiments by Dr. Velour (personal communication) however, suggest that subunit-8 can also conduct protons across the membrane in a voltage dependent manner. Therefore, the tertiary and quaternary structure of the F_o components and their topography in the mitochondrial membrane is very difficult to envisage. In the present thesis (Chapter 6) however, it has been argued that the amino terminal end of subunit-6 (similar to subunit-a of *E.coli*; see Sebald et al, 1984), the carboxyl terminal end of subunit-8 (similar to subunit-b of *E.coli*), and the interhydrophobic segment of subunit-9, would protrude into the mitochondrial matrix and interact with the F_1 sector of the O.S. ATPase (Fig. 8.4). In this picture, both subunit-6 and subunit-9 contribute to the formation of the proton channel while subunit-8 helps stabilise them into the membrane after initiating their assembly process. The carboxyl end of the subunit-8 might interact with F_1 components, analogous to the subunit-b of *E.coli*. Whether the *in vitro* phosphate binding activity of the subunit-8 is of any relevence (Velour *et al*, 1980; Esparza *et al*, 1981) to the catalytic site of the F_1 complex for the sythesis of ATP molecules remains to be established. The participation of other known factors (coupling factor-B, F_8) and unknown factors (e.g. from the mit-175 genetic locus) in this model can be envisage as a loose and/or temporary participation in the $F_o.F_1$ complex. However, more physical, chemical and biochemical studies would be needed to establish a definitive model for the O.S.ATPase complex and it is pointless to base speculations on proton conducting pathways on *in vitro* experiments with single polypeptide components of F_o.

12. Oligomycin and ossamycin inhibition of oxidative phosphorylation

Analyses of DNA sequences from oligomycin and ossamycin resistant strains have identified the probable aminoacids involved in the binding of these drugs (this thesis; Macino & Tzagoloff, 1980; Sebald *et al*, 1979). Based on the model proposed in this thesis (Fig. 6.24, Chapter-6) it is found that nearly all oligomycin binding sites would lie within the membrane. It has also been proposed that the binding sites of the subunits involved would be located in the lipid facing surface of the membrane spanning α-helices (Figs. 6.22, 6.23, 6.24, Chapter-6). This suggests that oligomycin binds to the F_o within the membrane and probably blocks the proton conduction through it by steric modification of the proton conducting channel. The conserved charged residues (glutamic acid) observed near oligomycin binding sites face towards the proton channel and are probably directly involved in the proton conduction. Chemical modificaton of charged residues and mutational studies of subunit-c from *E.coli* support this view (Hoppe & Sebald, 1984). This also explains why oligomycin resistant mutants do not show altered oxidative phosphorylation both *in vitro* and *in vivo* (Griffiths & Houghton 1974).

In one reported case of oligomycin resistance (Sebald *et al*, 1979) the altered residue of subunit-9 lies outside the membrane, a situation similar to that suspected for the ossamycin binding site of subunit-6. This suggests that oligomycin and ossamycin probably interact mainly within the membrane but some of their chemical groups have possible surface interaction also. Alteration in these residues, therefore, prevents binding of these drugs to the membrane without affecting oxidative phosphorylation. Ossamycin however, seems to interact with a membrane exposed charged residue, as modification of aspartic acid residue (D_{254}) subunit-6 leads to ossamycin resistance. There is no evidence for a similar interaction with oligomycin. DCCD however, directly reacts with the membrane embedded glutamic acid of subunit-9, which has been shown in the model (Fig. 6.24) to face the hydrophilic proton channel.

A few DCCD resistant mutations have been recorded in *E.coli*, where they cluster around the charged residue, aspartic acid similar to oligomycin resistant mutations. It was

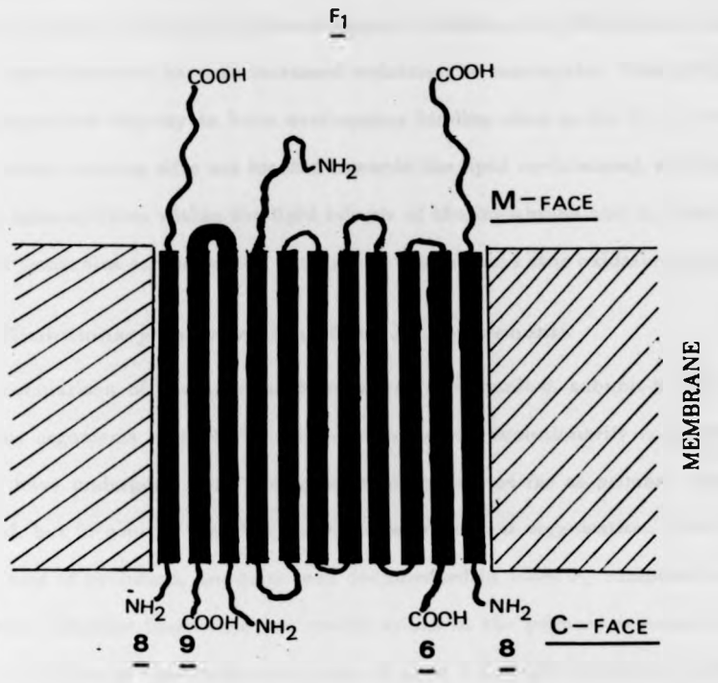


Figure 8.4 Predicted polarity of the three subunits (subunit-6, subunit-8 and subunit-9) of the O.S.ATPase complex in mitochondrial membrane. M-face is the matrix side and C-face is the periplasmic or cytoplasmic side of mitochondria. 6, 8 and 9 indicates respective subunits. Two subunit-8 have been shown in the diagram to illustrate the fact that, a dimer of subunit-8 probably works as a single functional component in the F_o

observed that oligomycin resistant mutants of Oli-1 locus show an increased resistance to DCCD (Avner & Griffiths, 1970). These evidences suggest that the DCCD binding site in the F_o probably overlaps with the oligomycin binding site. Oligomycin resistant mutants of *S. cerevisiae* also have an increased resistance to ossamycin. This also points out that ossamycin and oligomycin have overlapping binding sites in the F_o. In most cases however, these reacting sites are located towards the lipid environment which means all these drugs interact from within the lipid bilayer of the membrane and in doing so they modify or put constraint on the proton conducting channel and stop oxidative phosphorylation.

13. Evolutionary conservation of the F_O components

The comparison of the aminoacid sequences of subunit-6, subunit-8 and subunit-9 from various organisms suggest that these proteins are evolutionarily conserved (Chapter 5). They have undergone some divergent modification as far as primary sequences are concerned, but in general they have a common structural organisation. Unity and diversity, two faces of evolution, are quite well documented in these F_o components. The question, however, whether these observed modifications in the primary sequences of the subunits are a reflection of their continued adaptation for more efficient energy production remains to be solved. Two types of mechanism have been proposed to operate on functionally equivalent proteins during the course of evolution: selective neutral mutations and adaptive mutations. An assessment of the relative importance of one upon the other needs careful study of the physiological demands of the species and their success to fulfil that demand. Two main arguments are generally provided to support neutral mutations. Kimura (1968) suggested that the substitutional load due to the high rate of evolution, would have been intolerable unless most of the mutations had been neutral in natural selection. King & Jukes (1969) argued that the rates of evolution of individual proteins are uniform and most aminoacid differences between species are functionally insignificant. This uniform rate and the apparent nonsignificant random genetic change at the molecular level has been demonstrated to hold for cytochrome c, haemoglobin, histone and insu-

lins (Markussen, 1974; King & Jukes, 1969). However Blundell and Wood (1978) have argued for the evolution of insulin in terms of "adaptive process". They have critically analysed the relationship of sequence changes of the insulin molecule to its three dimensional structure and the role of various parts of this structure in the conversion of the proinsulin molecule to the active form, the storage of insulin, its transport to the site of action and its interaction with receptors. The authors did not exclude the posibility of some neutral changes along with the adaptive modification of the insulin molecule according to the physiological demand of the species.

In analysing the sequence changes observed in subunit-6 (subunit-a of *E. coli*), subunit-8 (analogous to subunit-b of *E. coli*), subunit-9 (subunit-c of *E. coli*), it has become apparent that "neutral changes" cannot account for all the observed changes in these subunits from various species.

Analysis of subunit-6, for instance, reveals that the aminoacid sequences in the last three hydrophobic segments V, VI, and VII (see Fig. 6.12, Chapter-6) are relatively more conserved than in the first four hydrophobic segments. In *E. coli*, the first four segments carry more charged aminoacid residues than its eukaryotic counterparts. Studying the mutational sites in yeast subunit-6, it appears that the last three hydrophobic segments (segments V, VI and VII) are functionally important and probably related to proton conduction. Therefore, the altered aminoacid sequences observed in these segments could be due to selectively neutral mutation. But why have the first four segments undergone so much change? It is possible that these segments do not play a major role in oxidative phosphorylation and provide only the basis for initial assembly of the subunit into the membrane or they may have some special adaptive features, still unknown. Indirect evidence for the first proposal comes from the study of a *mit* mutation (mb-12) from Dr. Lukins's laboratory (Dr. H.B. Lukins, personal communication) where the carboxyl terminal 14 aminoacids from subunit-6 is missing. Subunit-6 from this mutant does not, however, fail to assemble into the membrane, suggesting that the NH_2 terminal end has the potentiality to assemble into the membrane. N-terminal segments might also have a role

in providing the basis for interaction with temporary components, if any, of the $F_o.F_1$ complex. *E. coli* being prokaryotic in nature, might do well with three primitive, but basic subunits of F_o (subunit-a, subunit-b, subunit-c). Eukaryotic ATP synthetase complex appears to have a more complicated structure by the presence of the ATPase inhibitor protein (Pullman & Monroy, 1963), coupling factor -B (Sanadi, 1982) and Factor-6 or F_6 (Racker, 1979). These associated factors may provide a better regulatory mechanism for oxidative phosphorylation, but probably create a more complex mechanism of ATP synthesis. In concert with the evolving complex mechanism, primary components of the F_o will have to adapt for interaction with newly evolved components by sequence changes within structural, catalytically dispensable parts. This supports the second view that the observed changes in the primary structure of the protein may have adaptive value.

Various Subunit-8 species also show divergence in primary sequence such that this subunit probably subserves certain functions, besides its unknown primary function(s) which are species specific, or rather phylogenetic group specific. This is evident by the fact that primary sequences of this subunit within a particular phylogenetic group (e.g. mammals, Insects, Fungi) are very similar (see Fig. 5.22, Chapter-5).

Subunit-9, however, shows a more conserved structure and sequence (Fig. 6.21, Chapter-6) which is probably due to their essential primary function related to proton conduction. It is probably true that during the evolution of a complex protein those parts or subunits which remain conserved are essential for the primary function of the complex and therefore selectively neutral mutations are the cause of primary sequence changes. On the contrary, the other parts or other subunits which are not direct participants in the primary function of the protein or protein complex, undergo adaptive modifications according to the necessity of the organisms. And those parts of proteins would show diverged aminoacids in their primary sequences. However, to substantiate these ideas we would have to analyse the role (s) of different parts of each subunit by functonal dissection (e.g. by selective degradation or modifications, site directed mutagenesis). In this respect, the O.S. ATPase would be a good candidate to measure the rate of evolution (in terms of adaptive

modifications) in a complex enzyme system which is found in all aerobic organisms.

14. Non-nuclear and non-mitochondrial components in the control of mitochondrial functons

Studies reported in Chapter 7 of this thesis reveal that neither 2 μ DNA nor dsRNAs found in the cytoplasm of *Saccharomyces cerevisiae* carry non-nuclear, non-mitochondrial genetic markers, such as $Rh6G^R$, VEN^R, TET^R. The candidature of 3 μ DNA is also not clear. The 3 μ DNA plasmid which has been studied here, has an apparent insertion in the NTS (non transcribing spacer) sequences. The possibility that this insertion carries the above mentioned markers should be proven by analysing 3 μ DNA from a ρ^o VEN^R, TET^R, or ρ^o $Rh6G^R$ strains. Recent findings by Dai *et al* (1984) provides evidence that the 3 μ DNA plasmid bears [psi] factor, a similar non-mitochondral cytoplasmic genetic marker. The location of this marker on the 3 μ plasmid is not known, nor how it modulates translation of protein.

Evidence for the existence of a high molecular weight plasmid in the cytoplasm of some strains of *Saccharomyces cerevisiae*, also deserves attention. Future molecular analyses of this plasmid would be interesting. Discovery of mitochondrial plasmids from various species of *Neurospora* (Collins *et al*, 1981; Natvig *et al*,1984), maize (Weissinger *et al*, 1982), *Brassica* (Palmer *et al*, 1983) and the promiscuity of DNA (Ellis, 1982) within the cellular organelles has evoked a new and interesting aspect of the control of mitochondrial functions by several mechanisms. The multiple control of mitochondrial functions along with the mitochondrion's own role in regulating the others, might throw some light on complex phenomena, viz., cell surface changes associated with the ρ state of yeast, different survival rates of ρ and ρ^o cells (Wilkie, 1983), senescence associated changes of mt-DNA in *Podospora* (Esser *et al*, 1983), maternal inheritance of cell surface antigens (Lindahl, 1983).

The study of 3 μ DNA may also be of biotechnological interest as this high copy number plasmid (probably due to an insertional event in the NTS sequences between the 5S gene

and the 18S & 28S rRNA genes) may be useful in industrial applications for the expression of foreign genes in *Saccharomyces cerevisiae*.

APPENDIX

I. Growth media for yeast

A. Liquid

1. NO:
1% (W/V) yeast extract (Difco)
1% (W/V) Bactopeptone
2% Glucose
50mM Na/K - Phosphate buffer, pH 6.25
(10mM Na_2HPO_4
40mM KH_2PO_4)

N.B.: i) Glucose is autoclaved separately in a concentrated form (20%-25%) and added to the sterilised media to make a final concentration of 2%. ii) 0.01% adenine sulphate was added, when required.

2. N3: 1% (W/V) yeast extract (Difco)
1% (W/V) Bactopeptone
3% (W/V) glycerol
50mM Na/K Phosphate buffer, pH 6.25

3. YPD: Same as NO but without 50mM Na/K-Phosphate buffer.

4. YPD: Same as N3 but without 50mM Na/K Phosphate buffer.

5. N3OI: Same as N3 plus oligomycin (1.0 μg/ml)

6. N3OS: Same as N3 plus ossamycin (2.0 μg/ml

7. YP5 and YP10: same as YPD but with 5% and 10% glucose respectively

8. WO: 0.67% (W/V) yeast nitrogen base (Difco), 2% glucose.

B. Solid

For plates and slants of solid media, 2.3% agar (Difco) was added to the liquid media mentioned above.

II. Growth media for *E. coli*

A. Liquid

1. LB (Luria-Bertani) Medium:

Bactotryptone (W/V) 1%
Bacto-yeast extract (W/V) 0.5%
NaCl 1%
pH was adjusted to 7.5 with NaOH

2. 2XTY

Bactotryptone 1.6%
yeast extract 1.0%
NaCl 0.5%

B. Solid:

1. LB Plates: same as LB, but with 1.5% agar (Difco)
2. 2XTY Plates: same as 2XTY, but with 1.5% agar.
3. H-Top agar:

Bactotryptone 1.0%
Difco agar 0.8%
NaCl 0.8%

4. Minimal plates (+ glucose):

i) Prepare sterile 5X minimal "A" salts:

K_2HPO_4 5.25gm/100ml
KH_2PO_4 2.25gm/100ml
$(NH_4)_2SO_4$ 0.50gm/100ml
Na-citrate, $2H_2O$ 0.25gm/100ml

ii) Autoclave 390ml H_2O with 7.5 gm agar. Then, when cool add:

100ml 5X"A" salts
0.5ml 1M Mg SO (sterile)
0.25ml 1% Thiamine hydrochloride (Vit B_1)
100ml sterile 20% glucose (autoclaved separately)

iii) Mix and pour onto plates

Note: all media were autoclaved at 120° C and at 15 psi unless otherwise mentioned.

III. ANTIBIOTICS

1. Ampicillin

Stock solutions: 25mg/ml of the sodium salt of ampicillin was made in water. Sterilisation by filtration and stored in aliquots at - 20° C.

Working solutions: Final concentration in the liquid media or in solid plates is 35-50 μg/ml

2. Chloramphenicol

Stock solutions: 3.4mg/ml in 100% ethanol. Stored at - 20° C

Working solutions: For amplificaton of plasmids, 170 μg/ml for selection of resistant bacteria 30 μg/ml

3. Tetracycline

Stock solutions: 12.5mg/ml (hydrochloride form) in ethanol/water (50% V/V) Store at - 20° C

Note: because tetracycline is light sensitive, solutions and plates containing the antibiotics were stored in the dark and handled with wrapped aluminium foil.

Working solutions: 12.5 μg/ml. Because magnesium ions are antagonists of tetracycline, media used was always without magnesium salts for selection of bacteria resistant to tetracycline.

Some important stock solutions used in this study:

1. Denhardt's solution (100X)

Ficoll 20gms
Polyvinylpyrolidone 20gms
Bovine serum albumin (BSA) 20gms
H_2O to 100ml

2. 20XSSC:

3M NaCl
0.3M Na-citrate
pH adjusted to 7.0 or 7.5 using NaOH

3. 20XSSPE:

3.6M NaCl
200mM NaH_2PO_4 (pH7.4)
200mM EDTA (pH 7.4)

4. TE (pH 7.5):

10mM Tris-Cl (pH 7.5)
1mM EDTA (pH 7.5)

5. TSE (pH 7.50

10mM Tris-Cl (pH 7.5)
10mM NaCl
0.1mM EDTA (pH 8.0)

6. 10X ligase buffer:

700mM Tris-Cl, pH 7.5
70mM $MgCl_2$
150mM DTT
10mM ATP

7. 10X Nick-translation buffer

0.5mM Tris-Cl, pH 7.2
0.1M $MgSO_4$
1mM DTT
500mM BSA

8. 10XT4 DNA polymerase buffer

0.33M Tris-acetate, pH 7.9
0.66M Potassium acetate
0.10M Magnesium acetate
5mM DTT
1mg/ml BSA

9. 10 X Polynucleotide kinase (PNK) buffer

0.5M Tris-Cl, pH 7.5
0.1M $MgCl_2$
0.1M DTT

10. 10 X Bal-31 buffer

0.2M Tris-Cl (pH 8.0)
0.2M NaCl
0.12M $MgCl_2$
0.12M $CaCl_2$
10mM EDTA

11. 10 X BAP buffer

0.5M Tris-Cl pH 8.0
10mM $MgCl_2$

12. 10 X CIP buffer

0.5M Tris-Cl pH 9.0
10mM $MgCl_2$
1mM $ZnCl_2$
10mM Spermidine

13. CIP inactivating buffer (TNE buffer)

100mM Tris-Cl, pH 8.0
1M NaCl
10mM EGTA

V. ELECROPHORETIC BUFFERS

1. Tris-borate (TBE) buffer, pH 8.3

Stock solutions: (10 X)
1M Tris
1M Boric Acid
20mM EDTA

Working solutions:

For restriction analysis of DNA fragments:

20 times diluted solution (50mM Tris-borate, 1mM EDTA),

and for sequencing analysis:

10 times diluted solution (100mM Tris-borate, 2mM EDTA), were used.

2. Tris-acetate (TAE)

Working solutions

0.04 M Tris-acetate, 0.002mM EDTA

Concentrated stock solutions (Per litre) (10 X):

Tris base 242 gm
glacial acetic acid 57.1 ml
0.25M EDTA (pH 8.0) 200 ml

VI Gel Loading Buffer

1. For restriction analysis

10 X buffer

50% glycerol
25mM EDTA
0.25% bromophenol blue

2. For sequencing analysis
Formamide-dye mix

0.1% (W/V) Xylenecyannol FF
0.1% (W/V) Bromophenol blue
10mM EDTA
95% (V/V) deionized formamide
store at 4° C.

3. Tris-glycine buffer (2 X)

Tris base 3 gm
glycine 14 gm
Water to make 500 ml
(pH 8.2 - 8.4)

VII. Some useful chemical solutions for M-13 cloning & sequencing.

A. Acrylamide stock solutions

1. For DNA sequencing analysis (40%)

Acrylamide - 38 gm
Bis-acrylamide 0.825 gm
Water to make 100 ml
Store in dark bottle at 4°

2. For other purposes (30%)

Acrylamide 30 gm
bis-acrylamide 0.825 gm
water to make 100 ml

B. Other solutions

1. IPTG (isopropyl -β-D-thiogalactopyranoside) 25 mg/ml in water, store at - 20° C.

2. BCIG (5-bromo-4chloro-3 indolyl-β-galactoside, 20 mg/ml in dimethyl formamide

3. dNTP solutions

Stock solution:

10mM at 10mM Tris-Cl (p.H. 7.5)
0.1mM EDTA

Stored at - 20°C.

working solutions (0.5mM):

Diluted 20 times with distilled water
stored at - 20° C.

4. dideoxy (ddNTPs) solutions:

Stock solutions: 10mM at 5mM Tris-Cl (pH 7.0)

working solutions

i) ddA (1.0mM): diluted 1:10 in distilled water. Stored at - 20° C.
ii) ddG (0.7mM): diluted 1:14.5 in distilled water. Stored at -20° C.
iii) ddT (2mM): diluted 1:5 in distilled water. Stored at - 20° C.
iv) ddC (0.35mM): diluted 1:28.6 in distilled water. Stored at - 20° C.

5. N^0 mix for the sequencing reaction (for using $\alpha-^{32}$PdGTP only)

A^0 mix:

20 µl 0.5 mM dTTP
20 µl 0.5 mM dCTP
1 µl 0.5 mM dATP
1 µl 0.05mM dGTP
20 µl polymerase reaction buffer (10 X)

G^0 mix:

20 µl O.5 mM dATP
20 µl 0.5 mM dTTP
20 µl 0.5 mM dCTP
1 µl 0.05mM dGTP
20 µl polymerase reaction buffer concentrated (10 X)

T^0 mix:

20 µl 0.5 mM dATP
20 µl 0.5 mM dTTP
1 µl 0.5 mM dTTP
1 µl 0.05mM dGTP
20 µl polymerase reaction buffer concentrated (10 X)

C^0 mix:

20 µl 0.5 mM dATP
20 µl 0.5 mM dTTP
1 µl 0.5 mM dCTP
1 µl 0.05mM dGTP
20 µl polymerase reaction buffer concentrated (10 X)

6. 10 X concentrated polymerase reaction buffer

70 mM Tris-HCl, pH 7.5
70 mM $MgCl_2$
500 mM NaCl

VIII. Restriction enzymes used in this study with their recognition sequences and assay buffer:

Aha III

Storage buffer

20 mM Tris.Cl, pH 7.2
7 mM β-mercaptoethanol
50% glycerol (V/V)
Incubaation at 37° C.

Assay buffer

10 mM Tris.Cl, pH 7.2
80 mM NaCl
10 mM $MgCl_2$
100 µg/ml BSA
Incubation at 37° C.

Sal I

5'-G$^{\downarrow}$TCGA C-3'
3'-C AGCT$_{\uparrow}$G-5'

Storage Conditions:
20 mM KPO_4 (pH 7.0)
50 mM NaCl
1 mM Na_2EDTA
1 mM NaN_3
1 mM Dithiothreitol
500 µg/ml BSA
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
8 mM Tris·HCl (pH 7.6)
6 mM $MgCl_2$
150 mM NaCl
0.2 mM Na_2EDTA
Assay at 37°C

Comments:
Glycerol concentrations greater than 5% may alter recognition sequence specificity

Does *not* cleave DNA when the deoxyadenosine or internal deoxycytosine nucleotide is methylated

Sma I

5'-CCC$^{\downarrow}$GGG-3'
3'-GGG$_{\uparrow}$CCC-5'

Storage Conditions:
20 mM Tris·HCl (pH 7.5)
1 mM Na_2EDTA
7 mM 2-mercaptoethanol
500 µg/ml BSA
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
15 mM Tris·HCl (pH 8.0)
6 mM $MgCl_2$
15 mM KCl
Assay at 30°C

Comments:
KCl is absolutely required for enzyme activity. NaCl *cannot* be substituted

BRL recommends assaying *Sma* I at 30°C. At 37°C, *Sma* I activity is reduced approximately 50%

Does *not* cleave DNA when only the internal (CCCGGG) deoxycytosine nucleotide is methylated

*Sau*3A I

5'-$^{\downarrow}$GATC-3'
3'-CTAG$_{\uparrow}$-5'

Storage Conditions:
10 mM Tris·HCl (pH 7.4)
50 mM KCl
0.1 mM Na_2EDTA
1 mM Dithiothreitol
500 µg/ml BSA
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
6 mM Tris·HCl (pH 7.5)
6 mM $MgCl_2$
50 mM NaCl
100 µg/ml BSA
Assay at 37°C

Comments:
Glycerol content greater than 7.5% may reduce the specificity of the enzyme

Does cleave DNA when the deoxyadenosine nucleotide is methylated

Sst I

5'-G AGCT$^{\downarrow}$C-3'
3'-C$_{\uparrow}$TCGA G-5'

Storage Conditions:
20 mM Tris·HCl (pH 8.0)
150 mM KCl
500 µg/ml BSA
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
CORE BUFFER
Assay at 37°C

Comments:
Glycerol concentrations greater than 5% may alter the recognition sequence specificity

Taq I

5'-T$^{\downarrow}$CG A-3'
3'-A GC$_{\uparrow}$T-5'

Storage Conditions:
50 mM Tris·HCl (pH 7.5)
0.1 mM Na_2EDTA
10 mM 2-mercaptoethanol
500 µg/ml BSA
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
CORE BUFFER
Assay at 65°C

Comments:
Does cleave DNA when the deoxycytosine nucleotide is methylated, but does *not* cleave DNA when the deoxyadenosine nucleotide is methylated. This occurs in the site forming the boundary of the C and F fragments of pBR322

Mbo II (Mapping Grade)

5'-GAAGA(N)$_8$↓-3'
3'-CTTCT(N)$_7$↑-5'

Storage Conditions:
10 mM KPO_4 (pH 7.4)
0.1 mM Na_2EDTA
7 mM 2-mercaptoethanol
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
10 mM Tris·HCl (pH 7.9)
10 mM $MgCl_2$
6 mM KCl
1 mM Dithiothreitol
100 µg/ml BSA
Assay at 37°C

Comments:
Does *not* cleave DNA when the deoxycytosine nucleotides are methylated or when the 3' deoxyadenosine nucleotide (GAAG *A*) is methylated

Mbo I

5'-↓GATC-3'
3'-CTAG↑-5'

Storage Conditions:
10 mM Tris·HCl (pH 7.4)
50 mM KCl
0.1 mM Na_2EDTA
1 mM Dithiothreitol
500 µg/ml BSA
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
CORE BUFFER
Assay at 37°C

Pst I

5'-C TGCA↓G-3'
3'-G↑ACGT C-5'

Storage Conditions:
10 mM Tris·HCl (pH 7.4)
50 mM NaCl
0.1 mM Na_2EDTA
1 mM Dithiothreitol
0.15% (v/v) Triton x-100
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
CORE BUFFER
Assay at 37°C

Comments:
Glycerol concentrations greater than 5% may alter the recognition sequence specificity

Xba I

5'-T↓CTAG A-3'
3'-A GATC↑T-5'

Storage Conditions:
50 mM Tris·HCl (pH 7.4)
0.1 mM Na_2EDTA
1 mM Dithiothreitol
500 µg/ml BSA
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
6 mM Tris·HCl (pH 7.4)
6 mM $MgCl_2$
100 mM NaCl
Assay at 37°C

Comments:
Glycerol concentrations greater than 5% may alter recognition sequence specificity

Does not cleave DNA when the deoxycytosine nucleotide is methylated

Xho I

5'-C↓TCGA G-3'
3'-G AGCT↑C-5'

Storage Conditions:
50 mM Tris·HCl (pH 7.4)
200 mM KCl
0.1 mM Na_2EDTA
5 mM 2-mercaptoethanol
500 µg/ml BSA
50% (v/v) glycerol
Store at −20°C

Assay Conditions:
CORE BUFFER
Assay at 37°C

Comments:
Does *not* cleave DNA when the internal deoxycytosine or the deoxyadenosine nucleotide is methylated

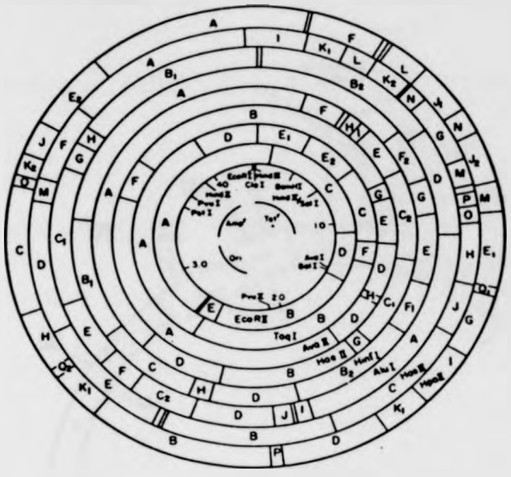
IX. Molecular size of various marker DNAs used in the study.

λ HindIII digests (in Kbp)	λ EcoR1 digests (in Kbp)	λ HindIII & EcoR1 (in Kbp)	ϕx174RF HaeIII (in bp)
23.130	21.70	21.226	1353
9.416	7.52	5.15	1078
6.682	5.83	5.10	872
4.361	5.64	4.27	603
2.322	4.85	3.48	310
2.027	3.48	1.98	*271
0.56		1.90	*281
		1.59	234
		1.37	194
		0.94	118
		0.83	72
		0.56	

* Anomalous mobility of these two fragments has been confirmed by physical mapping and complete sequence analysis of this duplex DNA of 5380 bp (Sanger *et al*, 1977).

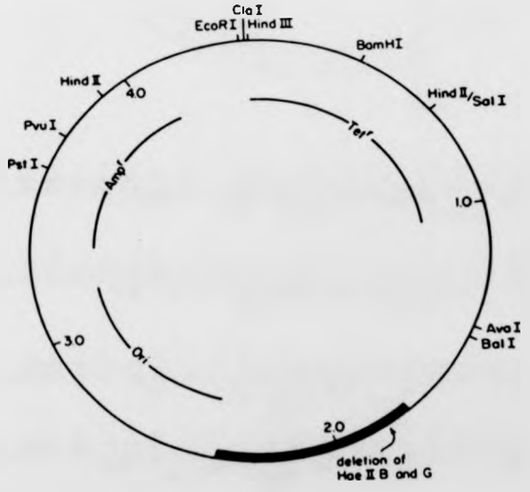
Sanger, F., Barrel, B.G., Air, G.N., Brown, N.L., Coulson, A.R., Fiddes, J.C., Hutchinson, A, III., Slocombe, P.M., and Smith, M., (1977) in Nature, 265, pp 687

X. Genetic and physical map for various vector DNA used in the study.



pBR322

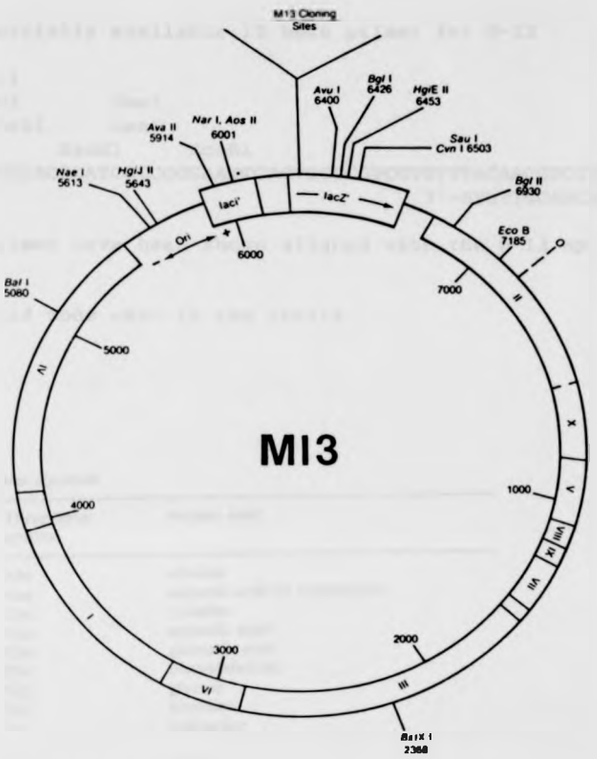
Size: 4.3 kb
Replicon: ColE1, relaxed
Selective markers: Amp^r, Tet^r
Single sites: Ava I, Pst I, BamHI, Pvu II, Cla I, Sal I, EcoRI, Hind III
Insertional inactivation: Amp^r - Pst I
Tet^r - BamHI, Hind III (variable), Sal I
References: Bolivar et al (1977); Sutcliffe (1978, 1979).
Comments: pBR322 is the most versatile of the plasmid cloning vectors. Its complete nucleotide sequence is known (Sutcliffe 1979).



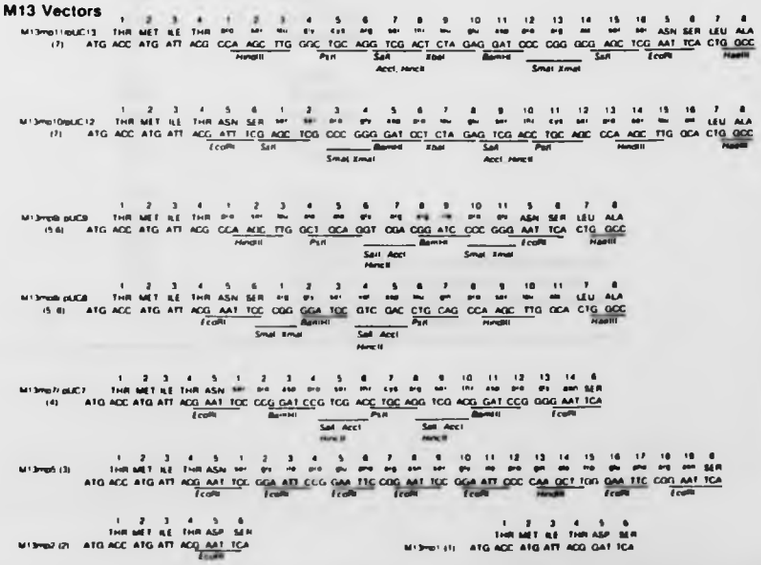
pAT153

Size: 3.6 kb
Replicon: ColE1, relaxed
Selective markers: Amp^r, Tet^r
Single sites: Ava I, Pst I, BamHI, Cla I, Sal I, EcoRI, Hind III
Insertional inactivation: Amp^r - Pst I
Tet^r - BamHI, Hind III (variable), Sal I
Reference: Twigg and Sherratt (1980)
Comment: A high-copy variant of pBR322

Restriction Map of M13 RF DNA



M13 Vectors



XI. DNA sequenc of commercially available 15 base primer for M-13 sequencing

```
                    SalI
                    AccI        SmaI
                    HincII      XmaI
6211     HindIII PstI        BamHl      EcoRl
 ATTACGCCAAGCTTGGCTGCAGGTCGACGGATCCCCGGGAATTCACTGGCCGTCGTTTTTACAACGTCGTGACT-3'
                                                                3'-ATGTTGCAGCACTGA-5'
                                                                              6298
```

The nucleotides of the primer have been shown aligned with the M-13 mp9 vector DNA.

XII. One letter amino acid code used in the thesis

The One-Letter Symbols

One-letter symbol	Three-letter symbol	Amino acid
A	Ala	alanine
B	Asx	aspartic acid or asparagine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
X	Xaa	unknown or 'other' amino acid
Y	Tyr	tyrosine
Z	Glx	glutamic acid or glutamine (or substances such as 4-carboxyglutamic acid and 5-oxoproline that yield glutamic acid on acid hydrolysis of peptides)

XIII. Alogarithm for α-helix and β-sheet plot of protein

XIII. Alogarithm for α-helix and β-sheet plot of protein

```
      $RESET NOBINDINFO                                                                 00000010
      $RESET FREE                                                                       00000020
      FILE  60(KIND=REMOTE)                                                             00000030
      FILE  10(KIND=DISK,TITLE='ATPSU/PROTEIN',MYUSE=IN)                                00000040
      FILE  9(KIND=DISK,TITLE='ATPSU/PROTEIN',MYUSE=IN)                                 00000050
      FILE  7(KIND=DISK,TITLE='AVG/PROTEIN', MAXRECSIZE=14,                             00000060
     +      BLOCKSIZE=420,AREASIZE=120, MYUSE=OUT)                                     00000070
      FILE  8(KIND=DISK,TITLE='AVG/PROTEIN', MAXRECSIZE=14,                             00000080
     +      BLOCKSIZE=420,AREASIZE=120, MYUSE=OUT)                                     00000090
      FILE  3(KIND=DISK,TITLE='TABLE/1',MYUSE=IN)                                       00000100
      FILE  4(KIND=DISK,TITLE='TABLE/1',MYUSE=IN)                                       00000110
      $INCLUDE'*WARWICK/GHOST80F'                                                       00000120
            DIMENSION AMINO(500),A(3),FILNAM(5),TBL(3),NAM(10),PIC(10)                  00000130
            DIMENSION ORG(3),PROT(3)                                                    00000140
            REAL AVA(500), AVB(500),ALPHA(20),BETA(20),AVPT1(500),AVPT2(500)            00000150
            INTEGER BS(500),SUB                                                         00000160
            NG=0                                                                        00000170
            CALL PAPER(1)                                                               00000180
            WRITE(60,7)                                                                 00000190
      7     FORMAT(1X,'Enter the number of graphs to be plotted',/                      00000200
     *           ' on a paper(maximum 2):')                                             00000210
            READ(5,/)IT                                                                 00000220
            DO 8 IDSET=1,IT                                                             00000230
            WRITE(60,477) IDSET                                                         00000240
       477  FORMAT(1X,'GRAPH  NO.  : ',I1)                                             00000250
            IF(IDSET.EQ.1) GO TO 478                                                    00000260
            WRITE(60,479)                                                               00000270
       479  FORMAT(1X,'Do you want to plot this graph ?',/                             00000280
     *       ' Enter 1 if you want to plot, otherwise',/                                00000290
     *      ' enter 2',/)                                                               00000300
            READ(5,/)NG                                                                 00000310
            IF(NG.EQ.2) GO TO 501                                                       00000320
      478   WRITE(60,40)                                                              00000330
      40    FORMAT(1X,' What input sequence do you want to process',/                   00000340
     *           '  in exactly six characters, e.g. YEAST6:'/)                         00000350
            READ(5,41) A(1)                                                             00000360
      41    FORMAT(A6)                                                                00000370
            WRITE(FILNAM,42) A(1)                                                       00000380
      42    FORMAT('ATPSU/',A6,'.')                                                   00000390
            ID=IDSET+8                                                                  00000400
            CHANGE (ID,TITLE=FILNAM)                                                    00000410
            READ(ID,/) N                                                                00000420
            READ(ID,30) (AMINO(I), I=1,N)                                               00000430
      30    FORMAT(1X,A1)                                                             00000440
            WRITE(60,77)                                                                00000450
      77    FORMAT(7X,'TABLE  1',7X,'TABLE 2',7X,'TABLE 3',//                         00000460
     *       6X,'ALPHA  BETA    ALPHA  BETA    ALPHA  BETA',/                         00000470
     *1X,'E',4X,' 1.53  0.26    1.51  0.37    1.60  0.50',/                           00000480
     *1X,'M',4X,' 1.20  1.67    1.45  1.05    1.60  1.20',/                           00000490
     *1X,'A',4X,' 1.45  0.97    1.42  0.83    1.60  0.80',/                           00000500
     *1X,'L',4X,' 1.34  1.22    1.21  1.30    1.60  1.20',/                           00000510
     *1X,'K',4X,' 1.07  0.74    1.16  0.74    1.20  0.70',/                           00000520
     *1X,'F',4X,' 1.12  1.28    1.13  1.38    1.20  1.20',/                           00000530
     *1X,'Q',4X,' 1.17  1.23    1.11  1.10    1.20  1.20',/                           00000540
     *1X,'W',4X,' 1.14  1.19    1.08  1.37    1.20  1.20',/                           00000550
     *1X,'I',4X,' 1.00  1.60    1.08  1.60    1.20  1.60',/                           00000560
     *1X,'V',4X,' 1.14  1.65    1.06  1.70    1.20  1.60',/                           00000570
     *1X,'D',4X,' 0.98  0.80    1.01  0.54    1.00  0.50',/                           00000580
     *1X,'H',4X,' 1.24  0.71    1.00  0.87    1.00  0.50',/                           00000590
     *1X,'R',4X,' 0.79  0.90    0.98  0.93    1.00  0.80',/                           00000600
```

```
      *1X,'T',4X,' 0.82  1.20      0.83  1.19      0.80  1.20',/                00000610
      *1X,'S',4X,' 0.79  0.72      0.77  0.75      0.80  0.70',/                00000620
      *1X,'C',4X,' 0.77  1.30      0.70  1.19      0.80  1.20',/                00000630
      *1X,'Y',4X,' 0.61  1.29      0.69  1.47      0.70  1.60',/                00000640
      *1X,'N',4X,' 0.73  0.65      0.67  0.89      0.70  0.80',/                00000650
      *1X,'P',4X,' 0.59  0.62      0.57  0.55      0.50  0.50',/                00000660
      *1X,'G',4X,' 0.53  0.81      0.57  0.75      0.50  0.70',/                00000670
      * ' Which of the above tables would you like to use(1,2,3):')             00000680
       READ(5,48) A(2)                                                         00000690
48     FORMAT(A1)                                                              00000700
       IB=IDSET+2                                                              00000710
       WRITE(TBL,49) A(2)                                                      00000720
49     FORMAT('TABLE/',A1,'.')                                                 00000730
       CHANGE(IB,TITLE=TBL)                                                    00000740
       DO 71 K=1,20                                                            00000750
       READ(IB,/) ALPHA(K)                                                     00000760
       READ(IB,/) BETA(K)                                                      00000770
71     CONTINUE                                                                00000780
       WRITE(60,61)                                                            00000790
61     FORMAT(1X,' How many amino acids to be taken for average',/             00000800
     *          ' (4,5 or 6) :'/)                                             00000810
       READ(5,/) NC                                                            00000820
       IF (NC.EQ.3) A(3)='3'                                                   00000830
       IF (NC.EQ.4) A(3)='4'                                                   00000840
       IF (NC.EQ.5) A(3)='5'                                                   00000850
       IF (NC.EQ.6) A(3)='6'                                                   00000860
       IF (NC.EQ.7) A(3)='7'                                                   00000870
       IF (NC.EQ.8) A(3)='8'                                                   00000880
       IF (NC.EQ.9) A(3)='9'                                                   00000890
       WRITE(NAM,62) A                                                         00000900
62     FORMAT('AVG/',A6,2A1,'.')                                               00000910
       IO=IDSET+6                                                              00000920
       CHANGE(IO, TITLE=NAM)                                                   00000930
       J=1                                                                     00000940
300    SUMA=0.0                                                                00000950
       SUMB=0.0                                                                00000960
       SUMPT1=0.0                                                              00000970
       SUMPT2=0.0                                                              00000980
       DO 200 I=J,J+NC-1                                                       00000990
       SEQ=AMINO(I)                                                            00001000
       IF (SEQ.EQ.'E') GO TO 101                                               00001010
       IF (SEQ.EQ.'M') GO TO 102                                               00001020
       IF (SEQ.EQ.'A') GO TO 103                                               00001030
       IF (SEQ.EQ.'L') GO TO 104                                               00001040
       IF (SEQ.EQ.'K') GO TO 105                                               00001050
       IF (SEQ.EQ.'F') GO TO 106                                               00001060
       IF (SEQ.EQ.'Q') GO TO 107                                               00001070
       IF (SEQ.EQ.'W') GO TO 108                                               00001080
       IF (SEQ.EQ.'I') GO TO 109                                               00001090
       IF (SEQ.EQ.'V') GO TO 110                                               00001100
       IF (SEQ.EQ.'D') GO TO 111                                               00001110
       IF (SEQ.EQ.'H') GO TO 112                                               00001120
       IF (SEQ.EQ.'R') GO TO 113                                               00001130
       IF (SEQ.EQ.'T') GO TO 114                                               00001140
       IF (SEQ.EQ.'S') GO TO 115                                               00001150
       IF (SEQ.EQ.'C') GO TO 116                                               00001160
       IF (SEQ.EQ.'Y') GO TO 117                                               00001170
       IF (SEQ.EQ.'N') GO TO 118                                               00001180
       IF (SEQ.EQ.'P') GO TO 119                                               00001190
       IF (SEQ.EQ.'G') GO TO 120                                               00001200
101    PL=ALPHA(1)                                                             00001210
       PB=BETA(1)                                                              00001220
       PT1=0.95                                                                00001230
```

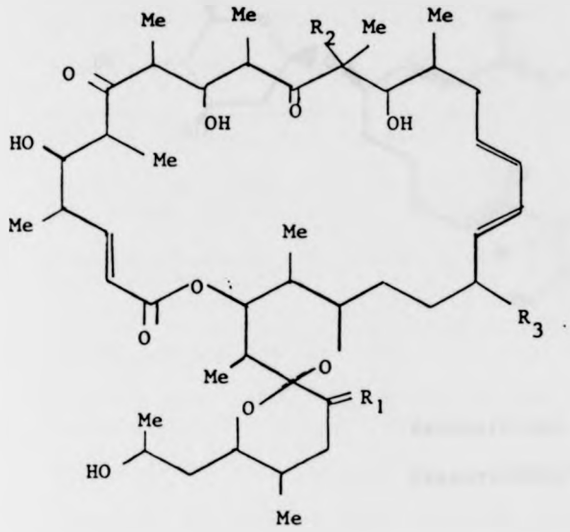
```
1240          PT2=0.80                                                  00001240
1250          GO TO 150                                                 00001250
1260     102  PL=ALPHA(2)                                               00001260
1270          PB=BETA(2)                                                00001270
1280          PT1=0.60                                                  00001280
1290          PT2=0.51                                                  00001290
1300          GO TO 150                                                 00001300
1310     103  PL=ALPHA(3)                                               00001310
1320          PB=BETA(3)                                                00001320
1330          PT1=0.74                                                  00001330
1340          PT2=0.64                                                  00001340
1350          GO TO 150                                                 00001350
1360     104  PL=ALPHA(4)                                               00001360
1370          PB=BETA(4)                                                00001370
1380          PT1=0.50                                                  00001380
1390          PT2=0.36                                                  00001390
1400          GO TO 150                                                 00001400
1410     105  PL=ALPHA(5)                                               00001410
1420          PB=BETA(5)                                                00001420
1430          PT1=1.19                                                  00001430
1440          PT2=1.13                                                  00001440
1450          GO TO 150                                                 00001450
1460     106  PL=ALPHA(6)                                               00001460
1470          PB=BETA(6)                                                00001470
1480          PT1=0.66                                                  00001480
1490          PT2=0.62                                                  00001490
1500          GO TO 150                                                 00001500
1510     107  PL=ALPHA(7)                                               00001510
1520          PB=BETA(7)                                                00001520
1530          PT1=0.96                                                  00001530
1540          PT2=0.84                                                  00001540
1550          GO TO 150                                                 00001550
1560     108  PL=ALPHA(8)                                               00001560
1570          PB=BETA(8)                                                00001570
1580          PT1=0.60                                                  00001580
1590          PT2=0.48                                                  00001590
1600          GO TO 150                                                 00001600
1610     109  PL=ALPHA(9)                                               00001610
1620          PB=BETA(9)                                                00001620
1630          PT1=0.47                                                  00001630
1640          PT2=0.29                                                  00001640
1650          GO TO 150                                                 00001650
1652     110  PL=ALPHA(10)                                              00001652
1654          PB=BETA(10)                                               00001654
1656          PT1=0.59                                                  00001656
1658          PT2=0.43                                                  00001658
1660          GO TO 150                                                 00001660
1662     111  PL=ALPHA(11)                                              00001662
1664          PB=BETA(11)                                               00001664
1666          PT1=1.52                                                  00001666
1668          PT2=1.61                                                  00001668
1670          GO TO 150                                                 00001670
1672     112  PL=ALPHA(12)                                              00001672
1674          PB=BETA(12)                                               00001674
1676          PT1=0.95                                                  00001676
1678          PT2=0.77                                                  00001678
1680          GO TO 150                                                 00001680
1682     113  PL=ALPHA(13)                                              00001682
1684          PB=BETA(13)                                               00001684
1686          PT1=1.01                                                  00001686
1688          PT2=1.05                                                  00001688
1690          GO TO 150                                                 00001690
1860     114   PL=ALPHA(14)                                             00001860
```

```
1870          PB=BETA(14)                                                    00001870
1880          PT1=0.98                                                       00001880
1890          PT2=0.98                                                       00001890
1900          GO TO 150                                                      00001900
1910     115  PL=ALPHA(15)                                                   00001910
1920          PB=BETA(15)                                                    00001920
1930          PT1=1.43                                                       00001930
1940          PT2=1.52                                                       00001940
1950          GO TO 150                                                      00001950
1960     116  PL=ALPHA(16)                                                   00001960
1970          PB=BETA(16)                                                    00001970
1980          PT1=0.96                                                       00001980
1990          PT2=0.92                                                       00001990
2000          GO TO 150                                                      00002000
2010     117  PL=ALPHA(17)                                                   00002010
2020          PB=BETA(17)                                                    00002020
2030          PT1=1.14                                                       00002030
2040          PT2=1.08                                                       00002040
2050          GO TO 150                                                      00002050
2060     118  PL=ALPHA(18)                                                   00002060
2070          PB=BETA(18)                                                    00002070
2080          PT1=1.46                                                       00002080
2090          PT2=1.56                                                       00002090
2100          GO TO 150                                                      00002100
2110     119  PL=ALPHA(19)                                                   00002110
2120          PB=BETA(19)                                                    00002120
2130          PT1=1.56                                                       00002130
2140          PT2=2.04                                                       00002140
2150          GO TO 150                                                      00002150
2160     120  PL=ALPHA(20)                                                   00002160
2170          PB=BETA(20)                                                    00002170
2180          PT1=1.56                                                       00002180
2190          PT2=1.63                                                       00002190
2200     150  SUMA=SUMA+PL                                                   00002200
2210          SUMB=SUMB+PB                                                   00002210
2220          SUMPT1=SUMPT1+PT1                                              00002220
2230          SUMPT2=SUMPT2+PT2                                              00002230
2240     200  CONTINUE                                                       00002240
2250          AVA(J)=SUMA/NC                                                 00002250
2260          AVB(J)=SUMB/NC                                                 00002260
2270          AVPT1(J)=SUMPT1/NC                                             00002270
2280          AVPT2(J)=SUMPT2/NC                                             00002280
2290          J=J+1                                                          00002290
2300          IF ((N-J).EQ.(NC-2)) GO TO 400                                 00002300
2310          GO TO 300                                                      00002310
2320     400  WRITE(60,401)                                                  00002320
2330     401  FORMAT(1X,'Write the name of the organism this',/              00002330
2340         *         ' graph is to represent, e.g., YEAST: ')              00002340
2350          READ(5,402) ORG                                                00002350
2360     402  FORMAT(3A6)                                                    00002360
2370          WRITE(60,403)                                                  00002370
2380     403  FORMAT(1X,'Write the name of the protein, e.g., ATPase:')      00002380
2390          READ(5,404) PROT                                               00002390
2400     404  FORMAT(3A6)                                                    00002400
2410          WRITE(60,405)                                                  00002410
2420     405  FORMAT(1X,'Enter subunit, if any (Enter 0 if none) : ')        00002420
2430          READ(5,/) SUB                                                  00002430
2440          CALL RADIAN                                                    00002440
2450          CALL BLKPEN                                                    00002450
2460          IF(IDSET.EQ.1) YR=0.63                                         00002460
2462          IF(IDSET.EQ.2) YR=0.15                                         00002462
2464          IF(IT.EQ.1) YR=0.45                                            00002464
2466          CALL PSPACE(0.05,0.565,YR,(YR+0.37))                           00002466
```

```
2468          CALL MAP(0., 1., 0., 1.)                                          00002468
2470          CALL BORDER                                                       00002470
2472          CALL CSPACE(0.05,0.565,YR,(YR+0.37))                              00002472
2474          CALL CTRMAG(9)                                                    00002474
2476     C* WRITE TITLE AND SPECIFY AXES                                        00002476
2478          CALL PLOTCS(0.30,0.90,'ALPHA HELIX AND BETA SHEET PLOT',31)       00002478
2480          CALL CTRMAG(6)                                                    00002480
2482          CALL PLOTCS(0.55, 0.025, 'SEQUENCE POSITION', 17)                 00002482
2484          CALL POSITN(0.025,0.28)                                           00002484
2486          CALL CTRORI(1.0)                                                  00002486
2488          CALL TYPECS('RELATIVE PROBABILITY',20)                            00002488
2490          CALL CTRORI(0.0)                                                  00002490
2492          CALL CTRMAG(5)                                                    00002492
2494          CALL PLACE(0, 0)                                                  00002494
2495          CALL CRLNFD                                                       00002495
2496          CALL CRLNFD                                                       00002496
2498          CALL CRLNFD                                                       00002498
2499          CALL CRLNFD                                                       00002499
2500          CALL SPACE(1)                                                     00002500
2502          CALL TYPECS('ORGANISM : ',11)                                     00002502
2504          CALL TYPECS(ORG,18)                                               00002504
2506          CALL CRLNFD                                                       00002506
2508          CALL CRLNFD                                                       00002508
2510          CALL SPACE(1)                                                     00002510
2512          CALL TYPECS('PROTEIN : ',10)                                      00002512
2514          CALL TYPECS(PROT,18)                                              00002514
2516          CALL CRLNFD                                                       00002516
2518          CALL CRLNFD                                                       00002518
2520          IF(SUB.EQ.0) GO TO 591                                            00002520
2522          CALL SPACE(1)                                                     00002522
2524          CALL TYPECS('SUBUNIT : ',10)                                      00002524
2526          CALL TYPENI(SUB)                                                  00002526
2528          CALL CRLNFD                                                       00002528
2530          CALL REDPEN                                                       00002530
2532          CALL POSITN(0.83,.94)                                             00002532
2534          CALL LINE(0.05,0.0)                                               00002534
2536          CALL CTRMAG(5)                                                    00002536
2538          CALL BLKPEN                                                       00002538
2540          CALL TYPECS(' ALPHA HELIX',12)                                    00002540
2542          CALL BROKEN(3,3,3,3)                                              00002542
2544          CALL BLUPEN                                                       00002544
2546          CALL POSITN(0.83,0.91)                                            00002546
2548          CALL LINE(0.05,0.0)                                               00002548
2550          CALL BLKPEN                                                       00002550
2552          CALL TYPECS(' BETA SHEET',11)                                     00002552
2554          CALL FULL                                                         00002554
2740     591  RMAX=0.                                                           00002740
2750          RMIN=0.3                                                          00002750
2760          DO 600 I=1,N-NC+2                                                 00002760
2770          BS(I)=I-1+INT((NC+1)/2)                                           00002770
2780          IF (AVA(I).GT.RMAX) RMAX=AVA(I)                                   00002780
2805          IF (AVB(I).GT.RMAX) RMAX=AVB(I)                                   00002805
2820     600  CONTINUE                                                          00002820
2840      499 FORMAT(2X,'SEQ. NUMBER',5X,'ALPHA AVERAGE',4X,                    00002840
2850         *         'BETA AVERAGE',4X,'B.TURN1 ',4X,'B.TURN2 ')              00002850
2890          WRITE(IO,499)                                                     00002890
2900          DO 552 I=1,N-NC+1                                                 00002900
2910          WRITE(IO,500) BS(I), AVA(I),AVB(I),AVPT1(I),AVPT2(I)              00002910
2920      552 CONTINUE                                                          00002920
2930      500 FORMAT(6X,I3,10X,F6.3,10X,F6.3,10X,F6.3,10X,F6.3)                 00002930
2940          LOCK IO                                                           00002940
2950          MAX=N-NC+2                                                        00002950
2960          MIN=0                                                             00002960
```

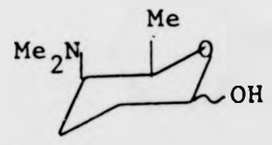
```
2970    C* DEFINE NEW PLOTTER SPACE AND MATH SPACE                              00002970
2971          IF(IDSET.EQ.1) YR1=0.5                                            00002971
2972          IF(IDSET.EQ.2) YR1=0.0                                            00002972
2973          CALL PSPACE(0.09,0.505,(YR+0.04),(YR+0.30))                       00002973
2974          CALL CSPACE(0.09,0.505,(YR+0.04),(YR+0.30))                       00002974
2975          CALL MAP(MIN, MAX, RMIN, RMAX)                                    00002975
2976          CALL CTRMAG(5)                                                    00002976
2977    C* DRAW AND ANNOTATE AXES                                               00002977
3010          CALL MARK(MAX-MIN,SX)                                             00003010
3020          CALL MARK(RMAX-RMIN,SY)                                           00003020
3030          CALL AXESSI(SX,SY)                                                00003030
3040          CALL REDPEN                                                       00003040
3050          CALL POSITN(MIN,RMIN)                                             00003050
3060          DO 700 I=1,N-NC+2                                                 00003060
3070          CALL JOIN(BS(I),AVA(I))                                           00003070
3080    700   CONTINUE                                                          00003080
3090          CALL BLUPEN                                                       00003090
3100          CALL BROKEN(3,3,3,3)                                              00003100
3110          CALL POSITN(MIN,RMIN)                                             00003110
3120          DO 750 I=1,N-NC+2                                                 00003120
3130          CALL JOIN(BS(I),AVB(I))                                           00003130
3140    750   CONTINUE                                                          00003140
3150          CALL BLKPEN                                                       00003150
3160          CALL POSITN(0.0,1.0)                                              00003160
3170          CALL LINE(MAX,0.0)                                                00003170
3175          CALL FULL                                                         00003175
3180     8    CONTINUE                                                          00003180
3190    501   WRITE(PIC,751) A                                                  00003190
3200     751  FORMAT('PIC1/',A6,2A1,'.')                                        00003200
3210          CHANGE(83, TITLE=PIC)                                             00003210
3220          CALL GREND                                                        00003220
3230          STOP                                                              00003230
3240          END                                                               00003240
3250          SUBROUTINE MARK(RANGE,STEP)                                       00003250
3260          IB=IFIX(ALOG10(RANGE))                                            00003260
3270          A=RANGE/10**IB                                                    00003270
3280          IF ((A.GE.1.).AND.(A.LT.3.)) STEP=2.*10.**(IB-1)                  00003280
3290          IF ((A.GE.3.).AND.(A.LT.7.)) STEP=5.*10.**(IB-1)                  00003290
3300          IF ((A.GE.7.).AND.(A.LT.10.)) STEP=1.*10.**IB                     00003300
3310          RETURN                                                            00003310
3320          END                                                               00003320
```

XIV. Structure of some of the mitochondrial ATPase inhibitors



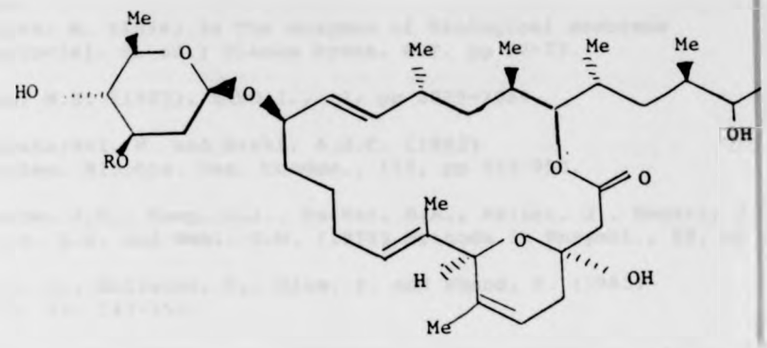
	R_1	R_2	R_3
Oligomycin A:	H_2	OH	Et
B:	O	OH	Et
C:	H_2	H	Et
D:	O	OH	Me

STRUCTURE OF OLIGOMYCIN (after Von Glehn *et al* (1972)



STRUCTURE OF OSSAMYCIN

N.B. The structure of ossamycin is not known. The structure of ossamine, which is an acid hydrolysis product of ossamycin, is shown above. This is sufficient to differentiate ossamycin from the oligomycin family.



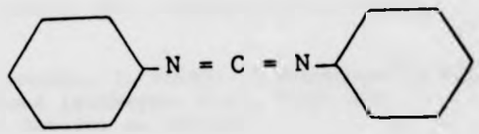
Venturicidin A (R = NH_2CO)

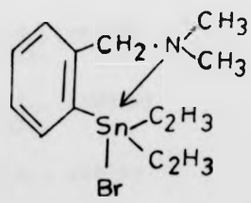
Venturicidin B (R = H)

STRUCTURE OF VENTURICIDIN

N,N'-Dicyclohexylcarbodiimide (DCCD)

Structure





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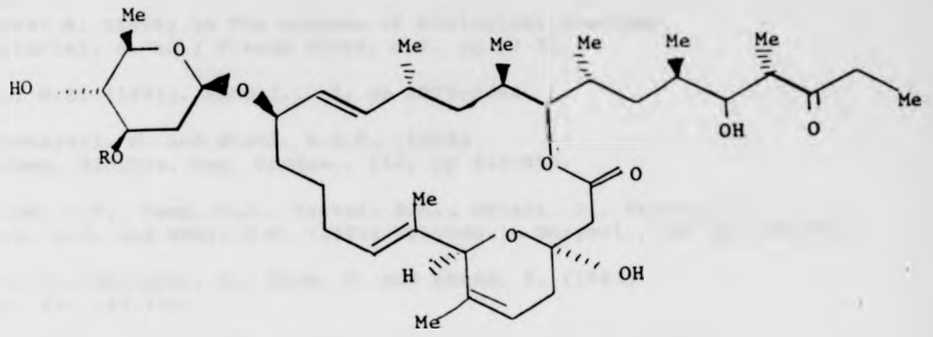


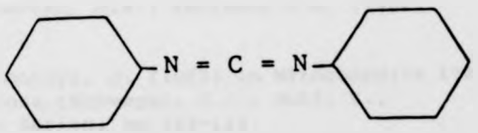
Venturicidin A (R = NH_2CO)

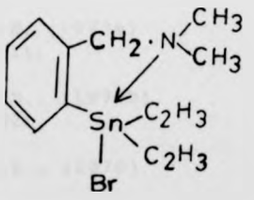
Venturicidin B (R = H)

STRUCTURE OF VENTURICIDIN

N,N'-Dicyclohexylcarbodiimide (DCCD)

Structure





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