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MITOCHONDRIAL GENES AND THEIR
EXPRESSION IN SACCHAROMYCES CEREVISIAE

*A Thesis submitted in fulfilment of the
requirements for the degree of Doctor of
Philosophy of the University of Warwick*

by

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September 1976

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Mr. Jenkins shook his head. "I thought he was making things up with his big words. I thought he was trying to show off. I didn't know there really were mitochondria."

A Wind in the Door,

Madeleine L'Engle.

ACKNOWLEDGMENTS

I wish to thank Dr. D. E. Griffiths for his supervision and advice during the course of this work; also my friends and colleagues in this department.

I am also extremely grateful to Professor R. A. Butow and his colleagues for their kind hospitality and use of facilities in South Western Medical School.

I would also like to thank Drs. D. H. Williamson, A. H. Scragg and D. H. Thomas for advice and help with nucleic acid techniques.

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ABBREVIATIONS

mt DNA	mitochondrial deoxyribonucleic acid
mt RNA	mitochondrial ribonucleic acid
r RNA	ribosomal ribonucleic acid
t RNA	transfer ribonucleic acid
m RNA	messenger ribonucleic acid
EBr	Ethidium bromide
SDS	Sodium dodecyl sulphate
UV	Ultraviolet
TET	Triethyltin
VEN	Venturicidin
OL	Oligomycin
BA	Bonkre ^g kic acid
HEX	Hexachlorophene [^]
DBCT	Dibutylchloromethyltin
CHI	Cycloheximide
CAP	Chloramphenicol
ERY	Erythromycin
PAR	Paromomycin
SPI	Spiramycin
mik	Mikamycin
ts	Temperature sensitive
poly (A)	poly Adenylic acid
MES	Methane ethane sulphonate
HEPES	N - 2 - Hydroxyethylpiperazine - N - 2 ¹ - ethanesulphonic acid
TEMED	N, N, N ¹ , N ¹ , tetramethylethylenediamine
S	Svedberg unit
NP40	Nonidet P40
TCA	Trichloroacetic acid
ATP	Adenosine triphosphate
GTP	Guanosine triphosphate
UTP	Uridine triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid

Bouyant should read buoyant throughout

CHAPTER ONE

The more traditional interest in mitochondria as energy conserving organelles has in recent years been rivalled by studies related to membrane biogenesis. An extensive literature has shown that mitochondria are semi autonomous with a system for the expression of their own genome in pleiotropy with nuclear genes and cytoplasmic protein synthesis. The presence of cytoplasmic genes in mitochondria provides a physical basis for the long observed phenomenon of maternal inheritance or "non-Mendelian" genetics, (1) in which the cytoplasmic phenotype is derived from the maternal parent only. This has been demonstrated in fungi, (2) amphibians, (3) and, more recently, in mammals (4, 5). The synthesis of mitochondrial membranes from pre-existing structures, as opposed to *de novo* synthesis, is in accordance with the partial genetic autonomy of these organelles. (6, 7). To this end, mitochondria possess DNA, RNA and a protein synthesis system.

1(1) Mitochondrial DNA

The presence of DNA in mitochondria was first demonstrated in 1963 by electron microscopy, (8) and subsequently by autoradiographic and preparative isolation techniques. (9-11). Since these early observations, mt DNA has been isolated and characterized from a wide variety of eukaryotes. Thus the mitochondria of animals, (12-14) protozoa, (15-17) fungi, (18-22) and higher plants (23-25) have all been shown to contain DNA distinct from that of the nucleus. Contour length measurements in the electron microscope show large differences in size between different organisms. (26). These lengths range from 4.45 to 5.85 μ in animals, 20 μ in *Neurospora crassa*, 10 μ in *Aspergillus nidulans*, to 25 μ in *Saccharomyces cerevisiae* and *S. carlsbergensis*. (19, 22, 27). Protozoan mt DNAs show size differences within the phylum, with *Euglena gracilis* at 1.7 μ and

Tetrahymena pyriformis at 15 μ . (26, 28). Plant mt DNAs have been less studied, and are generally greater than 25 μ in length. (12). The facultative anaerobes *S. cerevisiae* and *S. carlsbergensis* have received a great deal of attention with respect to their mitochondrial nucleic acids and protein synthesis. Lesions in the mitochondrial genome, usually lethal in other organisms, facilitates the use of the well proven techniques of microbial genetics and molecular biology in an effort to understand the expression of extranuclear genes. Yeast mt DNA has thus been extensively investigated; while circular mt DNA molecules have never been isolated in significant amounts from Yeast; they have been observed using osmotically shocked mitochondrial preparations and electron microscopy. (20). Similarly in the related fungus *N. crassa*, 20 μ circles have been observed by the same techniques. (27). The low mole percent G + C in Yeast mt DNA of 17% (29), ~~based on bouyant density in CsCl~~, is a consequence of the unusual base sequence arrangement in this DNA. Optical rotatory dispersion and Circular dichroism spectroscopy of various wild type and mutant mt DNAs reveals stretches of poly (dA). (dT) and poly (dA.dT). poly (dA.dT) sequences. This, and studies of melting and reassociation of denatured mt DNA provides an explanation for the low G + C content. (30-32). Bernardi and his co-workers have studied the distribution of A + T rich stretches by elution of these fragments at low salt molarity from hydroxylapatite columns. (33). Spleen acid nuclease digestion of these fragments has revealed a low (10%) proportion of G + C nucleotides. (34). This data, coupled with pyrimidine tract analysis of the total mt DNA and 10% G + C fragments has shown that up to 50% of the wild type genome may consist of A + T rich "spacers" mixed with G + C rich regions. The latter are presumed to^{be} the sites of genetic information. (35). More recent work has confirmed this view and allowed a more precise localization of A + T and G + C rich regions within the

putative 25 μ circle. (36). The combination of micrococcal nuclease and spleen acid nuclease digestion of mt DNA has been shown to result in specific cleavage at A + T and G + C regions respectively. The fragments obtained were sized by gel filtration and analysed by renaturation and pyrimidine tract analysis. Presumptive coding regions ranged from 25 to 50% G + C with smaller stretches as high as 65%. Of interest in regard to coding capacity of mt DNA is the observation that the G + C content of RNA transcribed from Yeast mt DNA is 28.9% and 35.1% for the two strains used by these authors. (37). Being close to the mean 32% described by Bernardi, (36) this implies that the spacer regions are not transcribed, and that only half of the Yeast mt DNA molecule contains meaningful genetic information. The presence of A + T rich stretches is not restricted to Yeast mt DNA however; denaturation mapping of Neurospora DNA has revealed an asymmetric distribution of spacers similar to Drosophila melanogaster and Xenopus spp. (38-41). In the case of Drosophila, its 1.5 μ spacer represents about 40% of the whole genome (40). The presence of these spacers then, may be considered to be an eukaryotic trait.

Interest in Yeast cytoplasmic genetics was first aroused by the discovery of the petite mutation, phenotypically characterized by small colonies on agar plates, and an inability to grow on non fermentable carbon sources. (42). Further investigations showed a lack of cytochromes a, a₃, b and c₁. Sherman introduced the term rho (ρ) factor in 1963, it being a genetic element changed upon petite mutagenesis. (43). Petites are classified according to their suppressiveness, that is the proportion of petites in diploid progeny formed by crosses to a grande strain. (44). They may have suppressiveness of up to 100% or have a value of zero, in which case they are termed neutral petites. (44). In the former case, mt DNA is still

present, albeit altered in bouyant density and base composition. (45). In the latter, mt DNA is absent, (46) in most but not all cases of zero suppressiveness where mt DNA present may be grossly modified. (47). While the original petites studied were spontaneous at a frequency of about 1%, further studies with the intercalating dyes ethidium bromide and acridines showed higher rates of mutagenesis. (45). In addition to petites; point mutations have been generated by UV irradiation resulting in resistance to several inhibitors of mitochondrial function. The first group of cytoplasmically inherited resistance markers were the inhibitors of mitochondrial protein synthesis Erythromycin, Chloramphenicol, Paromomycin, Spiramycin and Mikamycin. (48-51). These were followed by the oxidative phosphorylation inhibitors Oligomycin (Rutamycin), Venturicidin, and Triethyltin. (52-54). Multifactorial crosses and deletion mapping with petites, have ordered the markers on a circular map. (55). The presence or absence of these markers in petite mutants has helped to determine the structural changes to mt DNA which occur on mutagenesis. In a comprehensive study of several petite mutants, Slonimski and co-workers in France, and Rabinowitz's group in America have followed losses of drug resistant markers in ethidium bromide induced petites. (56-61). Faye *et al* (57) have found that Erythromycin resistance (E^R), and Chloramphenicol resistance (C^R), are lost together, suggesting that these are closely linked. Oligomycin resistance (O^R) is less likely to be lost however, and is therefore further away on the mitochondrial genome. The most common phenotype is the retention of one marker only, pointing to the massive deletion of other genes. That ^{this} represents a loss of polynucleotide sequences is shown by a loss of kinetic complexity and decreased hybridization to labelled grande (wild type) mt DNA. (59-60). The sequences remaining have been analysed in terms of bouyant density. Thus $\rho^{-}C^R O^R E^R$ mt DNA was heavier

than ρ^+ DNA by as much as 4.5 mg/cm^3 , representing a change from 1.683 to 1.687 g/cm^3 . Conversely the petite $\rho^{-C^R E^O}$ was 3 mg/cm^3 lighter than the grande. The E^R gene is therefore richer in G + C sequences than the C^R gene. The increase of bouyant density with E^R petites is compatible with the idea of gene amplification. Mitochondrial DNA is known to code for tRNA and rRNA species whose properties will be discussed later. Various ^3H aminoacyl tRNAs hybridize to mt DNA, a property of use in determining the degree of amplification. $\rho^{-C^R E^O}$ DNA shows a tenfold greater hybridization to ^3H Leucyl tRNA than the grande, while retaining one eighth of the kinetic complexity. This is explainable in terms of deletion of seven eighths of the genome followed by amplification of the remaining eighth. In the case of $\rho^{-C^R E^R}$ however, the enrichment for Leucyl tRNA is higher than expected from kinetic complexity measurements, likewise Tyrosine tRNA, suggesting selective amplification of certain genes. Similar properties have been found with rRNA genes. (57). The above is consistent with the fact that the amount of DNA is the same in petite and grande cells. (62). Studies of petite mt DNA with the electron microscope have provided visual evidence of gene reiteration. (58, 61). Fully denatured DNA, in which not more than 11% renaturation was expected to occur, was observed to renature 60 to 85%. Various unimolecular renatured duplexes were found, and were independent of DNA concentration. The duplex regions were analysed by renaturation kinetics and found to correspond with the kinetic complexity of the whole petite genome. In one petite E41, duplex regions representing conserved genes were positioned in tandem repeats head to head or tail to tail. In addition, a single stranded spacer region twice the duplex length was observed connecting two duplex regions. In general, this repeat-spacer sequence represents the contour length of the small circular DNAs also observed with this petite. (58). Another petite F13, shows tandem inverted repeats

(head to tail) without a spacer, and corresponding to a small circle size of about 1.4 μ . The presence of small circular DNA molecules in petites is a common observation (63, 64) and probably are significant in any consideration of the mechanism of petite induction. While the mechanisms underlying the petite mutation are far from understood, several provocative theories have emerged based on, among others, defective mt DNA polymerases, (65) a master copy of mt DNA in the nucleus, (66) or accumulation of point mutations (12). As Clark-Walker and Miklos have pointed out however, (67) these theories fail to account for their high (1%) spontaneous mutation rate, a frequency too high for frame shifts, transitions or transversions. These authors suggest that excision/insertion events occur between small circular mt DNA molecules originally produced by an excision event. The known recombinant properties of mt DNA, (68, 69) is consistent with multiple recombination events involved in this mechanism of mutagenesis (67). In addition, the presence of non transcribed regions in grande (33-35) and petite DNAs (59, 61) might be of significance in the recombination process. Finally on this point, the action of intercalating dyes may not be a simple intercalative effect when considering petite induction (70). Also mt DNA synthesis is inhibited by Ethidium bromide, secondary effects may involve recombination events. Mahler and Bastos (71, 72) have reported a covalent attachment of Ethidium bromide to mt DNA, and a series of degradative reactions *in vitro* using isolated mitochondria. Study of this reaction *in vivo* may answer some open questions concerning petite induction.

The synthesis of mitochondrial DNA and its dependence on chromosomal DNA replication is of interest when considering the control of mitochondrial proliferation. Early reports of the semi-conservative nature of mt DNA replication in *Neurospora* (73) and rat

liver (74) were inconclusive. Flory and Vinograd however, have shown that HeLa cell mt DNA replicates semi-conservatively (75). Electron microscopy has revealed a novel mode of replication for animal mt DNAs. Tissue cultured mouse cells and isolated chick mitochondria (76, 77) possess closed circular duplex molecules hybridized to a short (3%) single stranded DNA, E DNA which displaces the light strand of the duplex to form a D or displacement loop. A second mechanism unique to *Tetrahymena pyriformis* is the formation of a duplex "eye" in the linear duplex DNA of this organism. In this model, the replicating forks forming the eye expand along the length of the duplex until two daughter duplexes are formed (78, 79). *In vitro* DNA biosynthesis studies with isolated Yeast mitochondria have revealed aspects of this process not found in other organisms (80-82). Tritiated nucleotide triphosphates were incorporated into small (5 - 8 S) fragments as analysed on isokinetic $CsCl$ gradients. Isopyknic $CsCl$ density gradients showed that these fragments were skewed towards the heavier G + C rich position. By implication then, Yeast mt DNA synthesized *in vitro* is progressively degraded with the appearance of G + C rich stretches sometimes as heavy as nuclear DNA (82). The presence of an endogenous mitochondrial nuclease implied by these observations may be involved in recombination events between A + T rich spacers in accordance with Prunell and Bernardi (36).

The timing of Yeast mt DNA replication and its dependence on nuclear DNA replication has been the subject for several elegant experiments. Sena *et al*, (83) have pulse labelled logarithmic phase cells, previously labelled with ^{15}N , with ^{14}N . These were separated according to size and age in the zonal rotor and their total DNA analysed in the analytical ultracentrifuge. Hybrid $^{14}N/^{15}N$ bands gave an estimate of the amount of $\frac{n}{d}$ DNA synthesis, while mt DNA synthesis was estimated by the amount of ^{14}N in the mitochondrial

peak. The results showed that δ DNA synthesis is discontinuous throughout the cell cycle while mt DNA levels remain essentially constant. This separation between the two replicating species has been demonstrated biochemically with pulse labelled cells. Cycloheximide, (CHX) was found by Grossman *et al* (84) to inhibit δ DNA replication while allowing mt DNA synthesis to continue. The main effect of this antibiotic is on cytoplasmic ribosomes which suggests that a constant pool of nuclear coded proteins is required for δ DNA but not mt DNA synthesis. Amino acid starvation as well as the α Yeast mating pheromone had similar effects (85). Also a temperature sensitive mutant inhibited δ DNA synthesis but without the disadvantages of using antibiotics which may affect other cellular processes (86). Finally Banks has reported that Yeast cells made permeable to deoxyribonucleotide triphosphates synthesize mt DNA exclusively (87). In conclusion it appears that the two replicating systems can be separated temporally demonstrating a lack of rigid control of mt DNA replication by nuclear genes.

1(ii) Mitochondrial RNA

The three classes of RNA necessary for gene expression have been purified from mitochondria, ribosomal, transfer and putative messenger RNA fractions are present and exhibit different properties from their nuclear counterparts.

The action of Chloramphenicol and Erythromycin on mitochondrial protein synthesis ~~inhibitory~~ with no effect on cytoplasmic ribosomes implies that mitochondria have their own class of ribosomes. Such mitoribosomes have been analysed on sucrose gradients and assigned S values, thus animal ribosomes have values of about 55 S consisting of 40 and 30 S subunits, (88) Yeast at 73 S comprising 50 and 38 S subunits, (14) and Tetrahymena at 80 S (89). Yeast mitoribosomes contain equimolar amounts of two rRNA species. Using a combination

of sedimentation, gel electrophoretic and electron microscopic techniques, the molecular weights of these species have been determined as 0.7×10^6 and 1.3×10^6 daltons respectively (90). Gel electrophoresis of mt RNA gives different molecular weights from those determined by sedimentation studies as well as being dependent on temperature and ionic strength. This points to a loosely folded structure present *in vitro* and possibly *in vivo* (26, 88). With the possible exception of a 3 S RNA found in hamster mitochondria, (91) no equivalent of the 5 S bacterial RNA has been demonstrated, possibly because of "masking" by 4 S tRNAs on gels and gradients.

It is now clear that rRNA is encoded by mt DNA. Hybridization studies with labelled rRNA bound to mt DNA in excess show one copy of each rRNA subunit is present per genome. This conclusion has been drawn in Yeast from the low hybridization saturation values, 4.0% for *Candida utilis*, (92) and about 2.3% for *S. cerevisiae* and *S. carlsbergensis* (93, 94). Similar findings have been reported for HeLa cells (95). In Yeast, the two cistrons lie far apart on the mt DNA. This has been inferred in two ways: firstly, petites are able to transcribe sequences remaining after primary deletion (96). Studies with various mutants have shown loss of the 16 S rRNA with retention of the 23 S subunit suggesting that the cistrons are separated. Secondly, the construction of a physical map of mt DNA from *S. carlsbergensis* using specific fragments generated by restriction endonucleases (97) has assigned positions of rRNA cistrons with respect to the circular genome (98). This situation contrasts with that of *Neurospora* (99) and HeLa cells (95), where the two cistrons lie together, in the case of the former, specifying a 32 S precursor rRNA. The coding origin of mitochondrial ribosomal proteins appears to be the nucleus. Labelling cells in the presence of the cytoplasmic

protein synthesis. Inhibitor cycloheximide results in no ribosomal protein synthesis, while handling in the mitochondrial protein synthesis inhibitor chloramphenicol has no effect (100). The inhibition of a nuclear inherited temperature sensitive mutant affecting ribosome function supports but does not rigorously prove this point (101). The coding sites of ribosomal proteins is of importance when considering the molecular basis of resistance to antibiotics protein synthesis inhibitors. In bacteria, resistance to tetracycline is associated with a protein in the large ribosomal subunit (102). Using the same techniques of gel electrophoresis and cell culture discrimination, Orvell has failed to find differences in the ribosomal proteins of yeast mitochondria between sensitive and resistant strains (103). Burns *et al.*, (104) have put forward the idea that drug resistance mutations are the result of altered membrane permeability as a consequence of a tight association between mitochondria and the inner mitochondrial membrane. The arguments employed are not compelling however, and are analysed in depth by Orvell (105). A third alternative, which appears to be correct, is that ATPase gene sequences in the rRNA are responsible for these mutations. Hybridization of large and small subunit rRNAs to petites in which specific New markers are retained are in agreement with this. Xytronein and chloramphenicol lie within the 23 S rRNA cistron, and paromomycin lies within the 16 S cistron (106, 106). The unusual site of resistance may be a reflection of the evolutionary need to conserve rRNA sequences in a vital state simply unlike nuclear DNA sequences where for only one copy of each polynucleotide. In conclusion, the role of mitochondrial components is largely well understood and their function has been correlated with the ribosomal proteins only through to some extent by conventional techniques while the role of mitochondrial components in overall cellular function

protein synthesis inhibitor cycloheximide results in no ribosomal proteins synthesized, while labelling in the mitochondrial protein synthesis inhibitor chloramphenicol has no effect (100). The isolation of a nuclear inherited temperature sensitive mutant affecting ribosome function supports but does not rigorously prove this point (101). The coding site of ribosomal proteins is of importance when considering the molecular basis of resistance to antibiotic protein synthesis inhibitors. In bacteria, resistance to Erythromycin is associated with a protein in the large ribosomal subunit (102). Using the same techniques of gel electrophoresis and CM cellulose chromatography, Grivell has failed to find differences in the ribosomal proteins of Yeast mitochondria between sensitive and resistant strains (103). Bunn *et al*, (104) have put forward the idea that drug resistance mutations are the result of altered membrane permeability as a consequence of a tight association between mitoribosomes and the inner mitochondrial membrane. The arguments employed are not compelling however, and are analysed in depth by Grivell (105). A third alternative, which appears to be correct, is that altered base sequences in the rRNA are responsible for these mutations. Hybridization of large and small subunit rRNAs to petites in which specific drug markers are retained are in agreement with this. Erythromycin and Chloramphenicol lie within the 23 S rRNA cistron, and Paromomycin lies within the 16 S cistron (98, 106). The unusual site of resistance may be a reflection of the evolutionary need to conserve rRNA cistrons in a viable state since, unlike nuclear rDNA cistrons, there is only one copy of each per genome. In conclusion, the coding origin of mitoribosomal components is fairly well understood but caution must be exercised with the ribosomal proteins; only about 20 have been examined by conventional techniques while recent two dimensional gel analysis reveals 107 different

protein species in animal mitoribosomes, (107). Clearly much work remains to be done with these structures.

The occurrence of mitochondrion specific transfer RNAs has been demonstrated for several years. Differences between mitochondrial and cytoplasmic tRNAs are based on the following criteria: Differential response to their corresponding aminoacyl tRNA synthetases, (108) different chromatographic properties, (109) and levels of hybridization to nuclear and mitochondrial DNA. The latter data for Yeast have indicated the possibility of at least 20 to 30 tRNAs per mitochondrial genome (110). Direct observation of ferritin coupled tRNAs in *Xenopus laevis* and HeLa cells have pointed to only 12 cistrons per genome (111, 112). This restricted set of tRNAs may be the basis of the selective uptake of amino acids into HeLa mitochondrial proteins reported by Attardi (113). Fifteen tRNAs specifically hybridize Yeast mt DNA (114, 115). Of interest is the presence of Formyl methionyl tRNA, confirming the similarity between mitochondrial and bacterial protein synthesis. Reversed phase chromatography (RPC) of Yeast tRNAs has revealed several isoaccepting species, some of which hybridize to mt DNA (116-118). Thus far, two to four Arginyl, three Valyl and two Glutamyl tRNAs show this property. Further, the partial ordering of tRNA cistrons with respect to drug resistance markers has been achieved using deletion mapping techniques as for rRNAs (118, 119). This elegant work, coupled with previous data on rRNA (98) is presented as a genetic map in Figure 1:1.

1(111) Mitochondrial Protein Synthesis

Several lines of evidence indicate that most of the mitochondrial membrane proteins are synthesized on cytoribosomes. The most convincing evidence for this is the presence of proteins in petites; by necessity, those mitochondrial proteins remaining after mutagenesis are synthesized in the cytoplasm. Thus the citric acid cycle enzymes in the matrix,

Order of tRNA
cistrons:

his
[leu
[lys]
gln
asp
tyr
ala
phe
ile
val
pro

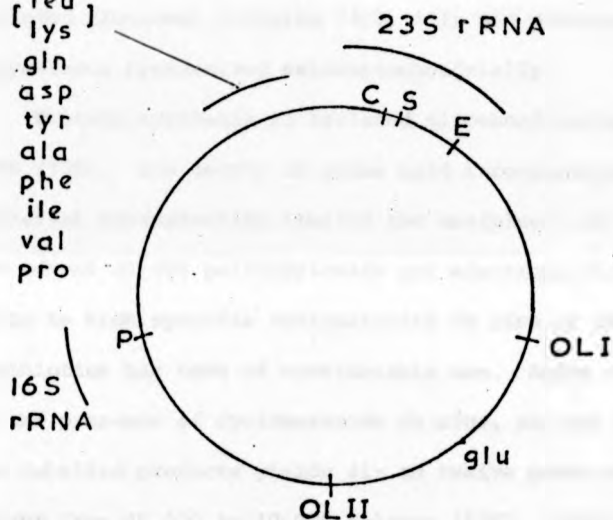


FIGURE 1:1

Order of t RNA and r RNA cistrons in relation to drug
resistance markers

The position of each RNA gene was determined by hybridization of labelled RNA to petites containing various deletions of drug resistance markers (98, 106, 118, 119).

(120) mitochondrial elongation factors, (121) and RNA polymerase, (122) are present unaltered in petites. Freeze etching studies have failed to detect any gross differences in morphology between grande and petite mitochondria, suggesting that most ~~membranes~~ ^{mitochondrial membrane components} are synthesized outside the mitochondrion (123). Labelling studies with inhibitors of cytoplasmic and mitochondrial protein synthesis have included ribosomal proteins (100, 101) and mitochondrial DNA and RNA polymerases synthesized extramitochondrially.

Protein synthesis in isolated mitochondria has been known since 1958 (125). Low levels of amino acid incorporation and problems of bacterial contamination limited the usefulness of this technique. The advent of SDS polyacrylamide gel electrophoresis and labelling cells to high specific radioactivity *in vivo* in the presence of antibiotics has been of considerable use. Amino acid incorporation in the presence of Cycloheximide *in vivo*, and gel electrophoresis of the labelled products yields six to twelve peaks ranging in molecular weight from 45,000 to 10,000 daltons (126). More recent techniques for protein analysis using gradient slab electrophoresis and $^{35}\text{SO}_4$ labelled mitochondria, has extended this number to at least twenty (127). Mitotic segregation in diploids and Meiotic 4:0 segregation, both criteria of cytoplasmic inheritance show most of these proteins to be encoded by mt DNA in Yeast. This number is consistent with approximate calculations of its coding capacity based on the known size of the molecule (26).

It is now clear that mitochondrial protein synthesis is involved in the assembly of the inner membrane enzyme complexes, namely ATP Synthetase and the Cytochromes, except Cytochrome c which is synthesized in the cytoplasm (128).

ATP synthetase (ATPase).

The extensive work of Tzagoloff and his co-workers (129) has

shown that both mitochondrial and nuclear genes specify this enzyme complex in Yeast. Triton extracted and gradient purified ATPase consists of ten subunits as analysed on conventional SDS gel systems. Five of these comprise the F_1^1 ATPase or the catalytic moiety of the enzyme connected via a sixth, OSCP, to four membrane subunits (130). That F_1^1 is synthesized on cytoribosomes was shown by immunoprecipitation of *in vivo* labelled mitochondria and coelectrophoresis with authentic F_1^1 ATPase. Biosynthesis of OSCP was assayed by the stimulation of binding of F_1^1 to depleted membranes.. Both systems were sensitive to Cycloheximide and hence cytoplasmic (129). The four membrane polypeptides, F_0^0 (5, 6, 8, 9 in Tzagoloff's nomenclature) are not produced in the presence of Chloramphenicol, and are mitochondrially synthesized. The most extensively studied mitochondrial product to date is subunit 9 of the ATPase complex. Amino acid analyses show an unusually high proportion of hydrophobic residues as well as high solubility in organic solvents. It migrates in gels of Triton extracts with an apparent molecular weight of 45,000, treatment with acid, base or Chloroform/Methanol prior to electrophoresis convert it to a molecular weight of 7,500. This disaggregation behaviour may be a consequence of interaction with membrane lipids as implied by ^{32}P labelling of phospholipids associated with subunit 9 (131). Alternatively glycoproteins may be involved in this phenomenon. Bosmann has demonstrated the synthesis of glycoproteins *in vitro* by rat liver and cerebral cortex mitochondria (132). Four of these have been detected on gels, one at a molecular weight of 8,000, any comparison with known products of mitochondrial protein synthesis, however, would have to await more rigorous techniques such as peptide mapping despite the similarities between mammalian and Yeast mitochondrial products (126).

Evidence from Tzagoloff's laboratory and studies of strains resistant to antibiotics involved in energy linked functions implicate

shown that both mitochondrial and nuclear genes specify this enzyme complex in Yeast. Triton extracted and gradient purified ATPase consists of ten subunits as analysed on conventional SDS gel systems. Five of these comprise the F_1^1 ATPase or the catalytic moiety of the enzyme connected via a sixth, OSCP, to four membrane subunits (130). That F_1^1 is synthesized on cytoribosomes was shown by immunoprecipitation of *in vivo* labelled mitochondria and coelectrophoresis with authentic F_1^1 ATPase. Biosynthesis of OSCP was assayed by the stimulation of binding of F_1^1 to depleted membranes. Both systems were sensitive to Cycloheximide and hence cytoplasmic (129). The four membrane polypeptides, F_0^0 , (5, 6, 8, 9 in Tzagoloff's nomenclature) are not produced in the presence of Chloramphenicol, and are mitochondrially synthesized. The most extensively studied mitochondrial product to date is subunit 9 of the ATPase complex. Amino acid analyses show an unusually high proportion of hydrophobic residues as well as high solubility in organic solvents. It migrates in gels of Triton extracts with an apparent molecular weight of 45,000; treatment with acid, base or Chloroform/Methanol prior to electrophoresis convert it to a molecular weight of 7,500. This disaggregation behaviour may be a consequence of interaction with membrane lipids as implied by ^{32}P labelling of phospholipids associated with subunit 9 (131). Alternatively glycoproteins may be involved in this phenomenon. Bosmann has demonstrated the synthesis of glycoproteins *in vitro* by rat liver and cerebral cortex mitochondria (132). Four of these have been detected on gels, one at a molecular weight of 8,000; any comparison with known products of mitochondrial protein synthesis, however, would have to await more rigorous techniques such as peptide mapping despite the similarities between mammalian and Yeast mitochondrial products (126).

Evidence from Tzagoloff's laboratory and studies of strains resistant to antibiotics involved in energy linked functions implicate

the four membrane subunits in the resistance phenomenon (129, 133). Several linkage groups, cytoplasmically inherited, and showing resistance to Oligomycin, Venturicidin and Triethyltin point to there being more than one polypeptide involved (134). The usefulness of these mutations in gene mapping will be discussed later.

Cytochrome oxidase.

Biogenetic studies on this enzyme have been essentially the same as those described for ATPase. A variety of fractionation procedures in several laboratories has shown the complex to consist of seven subunits. Antibiotic inhibition studies show the three largest, I, II and III, to be synthesized on mitoribosomes (135). While not exhibiting the same solubility in organic solvents as the ATPase subunits, they are still relatively hydrophobic. The presence of oxygen is essential to either their synthesis or integration into the inner membrane (136), a process that can be blocked by nuclear mutations, pet mutants, affecting mitochondrial function (137-139). The control of enzyme assembly by the availability of cytoplasmically synthesized subunits has also been demonstrated with ATPase (129) and may therefore reflect a general phenomenon of integration of Catalytic subunits into anchor points buried in the membrane. The problem of assigning functions to each subunit of these enzymes has been highlighted by the recent work of Chan and Mahler (140). They have prepared a highly active preparation of cytochrome oxidase completely devoid of the three high molecular weight polypeptides. Clearly it is possible to assign the genetic origin of these proteins although it is possible that they have little relevance to the catalytic activity of their associated enzyme.

Cytochrome b

Most work on this cytochrome has been performed with *Neurospora*; Gel filtration in the presence of bile salts reveals a 30,000 molecular

weight polypeptide associated with the haem group (141). Cycloheximide inhibition studies shows that this is synthesized on mitoribosomes. In Yeast, a genetic analysis of nuclear and cytoplasmic mutants deficient in one or more enzymic activities showed some mutants with a loss of cytochrome b activity; assayed as UQ cytochrome c, reductase (139). Thus this cytochrome is probably encoded by mt DNA in Yeast but, like the other membrane enzymes, is dependant on nuclear genes for its integration into the membrane.

Cytochrome c₁.

The single apoprotein of cytochrome c₁ of molecular weight 27,000 daltons is present in petites and is therefore synthesized on cytoribosomes (142). The binding of a haem group is under mitochondrial control however, leading Ross *et al* to suggest that an associated membrane polypeptide must be synthesized before this occurs (135). The tight coupling of cytochrome b to c₁ *in vivo* makes the former a likely candidate.

1(iv) Mapping of Mitochondrial Genes

Drug resistance mutations in relation to the ATP synthetase complex have been mentioned briefly (p.14). In this section, the use of these, and other genetic manipulations, in mapping mitochondrial genes is discussed.

To date, the most useful data regarding the arrangement of genes on Yeast mt DNA has been derived from linkage analysis of mutations conferring resistance to a variety of antibiotics. Mutations associated with ribosomes, Erythromycin, Paromomycin, Chloramphenicol, Spiramycin (48-51), cytochromes bc₁, mikamycin (143), and ATP synthetase, Oligomycin, Venturicidin, Triethyltin (52-54), have been mapped by several procedures. Markers linked closely to w, a locus determining the polarity of recombination, can be mapped by

conventional recombination analysis: examples are ERY^R , CAP^R , SPI^R . Markers further away from this locus, PAR , OL_I , OL_{II} , OL_{III} , mik , have been mapped by analysing the frequency of retention of markers on petite mutagenesis; this, when a strain carrying several markers is mutagenized with EBr to form petites, markers that are closely linked will tend to remain together or be lost together. An analysis of pairs of markers remaining in this way can provide a rough deletion map. In this way, known Drug^R loci have been ordered on a circular genetic map (Figure 1:2). The OL^R mutants shown were isolated in this laboratory, but several similar mutants have been described by others which are allelic to these (143).

Although the petite deletion mapping technique has proved useful in providing reference points for further genetic analysis, the limiting factor in fine structure analysis has been the availability of further mutants lying within the segments of mt DNA defined by Drug^R mutations. To overcome this problem, further mutants were sought after, the most promising being mit^- and temperature sensitive mutants. mit^- mutants are characterized by a malfunction of the energy conservation system which does not lead to the ρ^- state. Unlike Drug^R mutations, these presumably arise from deletions of mt DNA rather than point mutations (138, 139, 144). The mutants studied by Tzagoloff and co-workers (138, 139) were screened for protein synthesis by a microassay procedure, and examined for the presence of cytochromes and ATP synthetase by spectral and enzymatic assays respectively. In order to assign the relative positions of mit^- mutants on the genome, the following crosses were used: $mit^- \times \rho^-$, $mit^- \times mit^-$ and $mit^- \times Drug^R$ (145). The positions of the four linkage groups OXI 1, OXI 2, OXI 3 and COB are shown in figure 1:2. Another technique has been the isolation of mutants conferring heat or cold sensitivity to mitochondrial functions. The

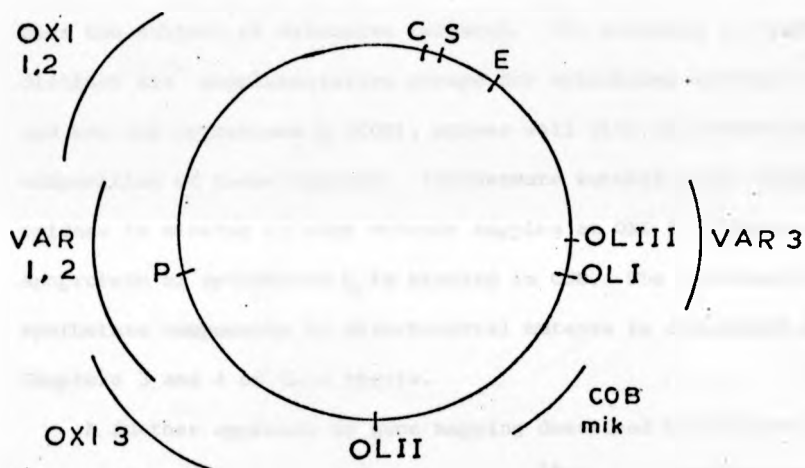


FIGURE 1:2

The Relative Positions of mit^- and VAR mutations on the Mitochondrial Genome

The genetic loci OXI 1, 2, 3 and COB were mapped by single factor crosses between themselves and drug resistance markers as well as by deletion mapping as for figure 1:1 (138, 139, 145). mik is the resistance locus to mikamycin described by Linnane *et al* (143) and probably represents an alteration in cytochrome *b*. VAR 1, 2 and 3 loci were mapped by recombination with OL_I, OL_{II} and P (147).

cold sensitive mutant described by Trembath *et al* (146) appears to be allelic to the well defined locus OL_I (52). The ts mutants isolated by Dr. W. E. Lancashire in this laboratory are shown in Figure 1:3. All, with the exception of D6/N302 show possible allelism with known mitochondrial markers. D6/N302 may prove to be a novel genetic locus and is worthy of further study.

The biochemical basis of the mutational changes described has been the subject of extensive research. The presence of three distinct mit⁻ complementation groups for cytochrome oxidase (OXI 1,2,3) and one for cytochrome b (COB), agrees well with the known subunit composition of these enzymes: furthermore subunit I of cytochrome oxidase is missing in some mutants mapping at OXI 3, likewise the apoprotein of cytochrome b is missing in COB. The relation of ATP synthetase components to mitochondrial mutants is discussed in Chapters 3 and 4 of this thesis.

A further approach to gene mapping described by Douglas and Butow (127) involves direct analysis of $^{35}SO_4$ labelled mitochondrial proteins synthesized in the presence of Cycloheximide. While screening laboratory strains, these authors found "polymorphisms" in the distribution of proteins on slab gels. The absence of bands at 45,000 and 25,000 daltons, VAR 3 and VAR 2 respectively, and the faster mobility of VAR 1 at 40,000 was used as a marker for genetic analysis. After establishing the cytoplasmic mode of their inheritance, their position relative to known markers on mt DNA was established by conventional recombination analysis (147). VAR 1 and VAR 2 always appear together, and map near Paromomycin (Figure 1:2), but are unrelated to the OXI loci. It is possible that VAR 1 and VAR 2 represent the products of a single precursor molecule with unknown function. VAR 3 maps very closely to OL_I/OL_{III} and may therefore be allelic. This is discussed further in Chapter 4.

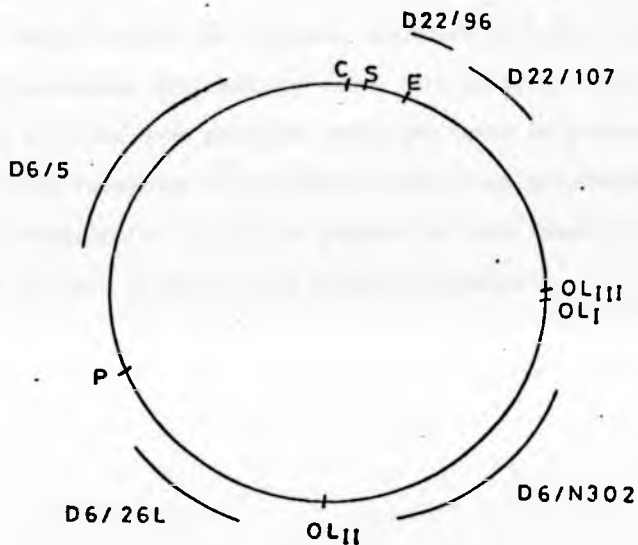


FIGURE 1:3

Relation of Temperature Sensitive Mutants to Mitochondrial Markers

ts mutants were isolated by Dr. W. E. Lancashire in this laboratory and mapped by deletion mapping with petites retaining or losing drug resistance markers.

The methodology of biochemical and genetic analysis of mitochondrial genes is improving rapidly. The lower limit of twenty proteins synthesized on mitoribosomes (127) presents a great challenge in assigning their genetic origin, function and organization. The refinement of genetic dissection, for example in the production of more useful mutants or physical isolation of genes using restriction endonucleases will help to solve this problem. Likewise, the analysis of known gene products using the tools of protein chemistry will provide knowledge of the genetic origin of mitochondrial membrane components. It is the purpose of this thesis to illustrate some of the ways in which this might be possible.

CHAPTER 2

1. INTRODUCTION

In the previous chapter, cytoplasmic mutants resistant to inhibitors of mitochondrial metabolism were discussed in relation to gene mapping. The loci OL_I , OL_{II} , OL_{III} , for example have been shown to be associated with mitochondrial DNA. However resistance to other inhibitors with similar functions, Triethyltin (TET), Venturicidin, (VEN), and Rhodamine 6G (R6G), is not genetically linked to this DNA. A study of the petites formed on Ethidium bromide mutagenesis of $TET^R VEN^R R6G^R$ strains was made. Several petites showed retention of these markers on back crosses to a sensitive tester strain, they also showed a suppressiveness of zero. Petites with and without the resistance phenotype were examined for the presence of mt DNA by a variety of techniques. No DNA exhibiting the properties of mt DNA was found, supporting the view that these mutations lie on a separate DNA molecule. The relation of this class of mutations to a closed circular DNA fraction in Yeast is discussed.

2. METHODS and MATERIALS

2(1) Yeast Strains and Genetic Analysis

The strain D22/72 a, ade , VEN^R , TET^R , $R6G^R$, BA^R , HEX^R , CHI^R was used throughout. 1799^R , BA^R , HEX^R , refer to resistance to 1799 (bis [hexafluoroacetyl] acetone), bonkreikic acid, and hexachlorophene respectively. This strain exhibits normal sensitivity to Oligomycin, Chloramphenicol, and Erythromycin.

Ethidium bromide (EBr) induced petites were produced by sub-culturing twice in 1% Yeast extract, 1% Peptone, 2% Glucose, sodium-potassium phosphate pH 6.25, plus 10 μ g/ml EBr. Retention or loss of resistance determinants was analysed by the marker rescue technique

of back crossing to the sensitive grande D41, α , his as described by Lancashire and Griffiths (148). The resultant diploids were plated onto solid glycerol medium, 1% Yeast extract, 1% Peptone, 3% Glycerol, 2% Agar, sodium potassium phosphate pH 6.25, containing Triethyltin sulphate (5, 10, 20 $\mu\text{g/ml}$), Venturicidin, (.5, 1, 2 $\mu\text{g/ml}$) and Rhodamine 6G (25, 50, 100 $\mu\text{g/ml}$). Growth was scored after two days.

Suppressivity of petites was determined by the percentage of petite zygotes in synchronous zygotes formed in crosses between petites and D41 (149, 150).

Anaerobic growth of strains was carried out in a cabinet flushed with nitrogen which had been passed through 10 litres of Bakers yeast in 2% Glucose, 5g/l Tween 80, 20 mg/l Ergosterol, followed by Fieser's solution and water. Growth was for 36 hours at 30°C in 1% Yeast extract, 1% Peptone, 2% Glucose, sodium potassium phosphate pH 6.25. Genetic markers were scored as described above.

2(ii) Growth of Cells and Isolation of DNA

Batch cultures, 10 or 15 litres, were grown in 18 l round bottom flasks with forced aeration and magnetic stirring. Growth was at 30°C overnight until the transition phase had been reached. Culture media contained 0.5% Yeast extract, 0.5% Peptone, 2% Glucose, .3% KH_2PO_4 , and 0.1 g/l Adenine sulphate.

Cells were harvested by centrifugation at 2,000 g for 10 minutes in the MSE Mistral, and washed twice with water.

For the isolation of all DNA species, cells were broken and mitochondria isolated by a modification of the procedure of Clark-Walker (151) and O'Connor *et al* (152). After washing cells once in ESE (0.5 M Sorbitol, 0.05 M EDTA pH 7.0 + 0.5 mg/ml EBr), cells were broken in the Braun shaker with an equal volume of glass beads for 30 to 45 seconds with CO_2 cooling. Cellular debris and nuclei were

removed by two successive spins at 4,000 rpm in the Sorvall GSA rotor. Mitochondria were pelleted in the SS-34 rotor at 13,500 rpm for 20 minutes. All operations were performed at 0-4°C and under reduced light to minimize damage to DNA by photoactivated EBr. Mitochondrial lysates for EBr-CsCl density gradient centrifugation were prepared by lysis of the mitochondrial pellets in 2% Sarkosyl. Each gradient contained 1.35 mls EBr solution (1.75 mg/ml in 0.1 M sodium phosphate pH 7.0), 4.1 g CsCl, 0.3 mls 0.1 M EDTA pH 7.0, 0.8 mls H₂O and 2 mls Sarkosyl lysate. Centrifugation was performed in the Beckman L5-65 or MSE superspeed ultracentrifuge, at 50,000 rpm for 42 hours at 10°C in polypropylene tubes, using the 50Ti or 8 x 14 rotor. DNA bands were visualized by illumination with a UV lamp at 360 nm. Photographs were taken through an orange (Wratten no. 23) filter. Negatives were scanned with a Joyce-Loebl microdensitometer. For further analysis, bands were withdrawn by side puncture, as described by Clark-Walker (151), and extracted at 4°C with isoamylalcohol (H₂O saturated) to remove EBr. CsCl was removed by dialysis against H₂O and the dialysate lyophilized and stored at -20°C until further use.

For analysis of mt DNA using DAPI, (4¹ - 6 - diamidino - 2 - phenylindole), whole cell extracts were prepared from sphaeroplast lysates as described by Petes and Williamson (153), or from Eaton press "crushates" (154). Centrifugation conditions were as described (153). DNA bands were visualized under UV light and photographed through a light blue filter. Negatives were scanned as above.

2(111) Analytical Density Gradient Centrifugation

For bouyant density analyses, samples were run in the Spinco model E analytical ultracentrifuge as described by Szybalski and Szybalski (155). *Micrococcus lysodeikticus* DNA was included as an internal standard.

2(iv) Pulse Labelling of Cells with ^3H -Adenine in the Presence of CHI, and Analysis of Labelled DNA

A modification of the method of Weislogel (156) was used: Cultures were grown overnight in 10 mls of 1% Glucose, 1% Yeast extract, 1% Peptone. Cells were harvested, washed once in sterile water and regrown in the same medium for 1 hour. For labelling in the presence of CHI, the antibiotic was added to 200 $\mu\text{g/ml}$ and the culture incubated for a further 15 minutes. Cells were labelled for 3 hours with 100 $\mu\text{Ci/ml}$ ^3H -Adenine. Label was chased by the addition of 1 mg/ml Adenine for 15 minutes. After harvesting and washing twice with water, cells were suspended in 4 mls preincubation buffer (10 mM DTP, 25 mM EDTA, pH 6.4). Incubation was for 30 minutes at 33°C. At the end of this period, cells were suspended in 4 mls of 1.8 M Sorbitol, and Glusulase added to 1%. Incubation was at 33°C for 30 minutes. The resulting sphaeroplasts were washed twice with 1.8 M Sorbitol prior to lysis. Lysis was effected with 1.3% Sarkosyl in 0.1 x SSC (SSC is 0.15 M NaCl, 0.015 M Na citrate pH 7.0). The mixture was kept on ice for at least 15 minutes. 1.4 mls lysate was added to a Beckman cellulose nitrate tube containing 4.2 mls of saturated CsCl solution (in 10 mM Tris Cl, 0.1 M EDTA pH 8.0). Samples were overlaid with paraffin oil and centrifuged at 44,000 rpm for 42 hours at 15°C in the 50 Ti rotor. Five drop fractions were collected from the bottom of the tube, and NaOH added to each to a final concentration of 0.5 M. After incubation overnight at 30°C, 0.1 ml of 1 mg/ml BSA was added to each tube, followed by 0.5 mls of ice cold TCA. Precipitates were filtered onto Whatman GF/A filters and counted in Triton Toluene Butyl BPD scintillation cocktail in a Packard 2425 scintillation counter.

2(v) Staining of Cells with DAPI and Fluorescence Microscopy

The postvital staining procedure of Williamson and Fennell was used (157). Cultures were mixed with 2 volumes of ethanol, washed once with water after 30 minutes, and suspended in 0.1 to 0.5 µg/ml DAPI. Fluorescence microscopy was performed as described (157).

2(vi) Digestion of DNA with Restriction Endonucleases EcoRI and Hind III

25 µl DNA solution (~ 40 µg/ml) was mixed with 5 µl RE buffer (100 mM Tris Cl, 50 mM NaCl, 10 mM Mg SO₄ pH 7.5) and 10 µl restriction enzyme diluted 1:10 (Hind III) or 1:50 (EcoRI) in RE + 10% Glycerol. Digestions were performed at 37°C for 2 hours (Hind III) or 1 hour (EcoRI). The reaction was terminated by the addition of SDS to 2% in a solution containing 30% Glycerol and 0.001% Bromophenol blue. The mixture was heated to 60°C for 10 minutes prior to electrophoresis.

2(vii) Analysis of DNA and Restriction Digests on 1.4% Agarose Gels

Flat bed Agarose slab gels (1.4%) were poured in a homemade apparatus. Sample wells were formed with cylindrical slot formers. The Tris/Borate electrophoresis buffer was that described by Tirmis *et al* (158), containing 1 µg/ml EBr. Electrophoresis was performed for 3 hours at 15 mA. DNA bands were visualized immediately by illumination at 360 nm and photographed through a yellow filter with an exposure of 1 second. Negatives were scanned with a Gilford linear transport accessory mounted to a Zeiss spectrophotometer.

2(viii) Materials Used

Yeast extract, Peptone and Agar were from Difco Ltd., USA. Triethyltin sulphate, Venturicidin, and Rhodamine 6G were supplied

by Dr. W. E. Lancashire. Ethidium bromide was purchased from Sigma or Calbiochem Ltd. DAPI was a kind gift of Dr. O. Dann, Erlangen, Germany. ^3H -Adenine, 26 Ci/mmoles was from the Radiochemical centre, Amersham. Restriction enzymes were kindly donated by Dr. A. H. Scragg, MRE, Portlaoine. Sarkosyl (sodium lauryl sarkosinate) was from Schwarz-Mann, USA. Glusulase was from Endo laboratories, USA. With the exception of CsCl and salts for growth media, all reagents were analytical grade where possible.

3. RESULTS and DISCUSSION

3(i)

The genetics of resistance to Triethyltin, Venturicidin and related inhibitors has been extensively studied in this laboratory (148, 159, 160). These mutants fall into two phenotypic groups, Class 1 and Class 2. The former are characterized by cross resistance to a wide variety of inhibitors and show Mendelian inheritance, that is 2:2 segregation in Meiosis. Class 2 mutants show 4:0 segregation on Meiosis as well as mitotic segregation, and are therefore cytoplasmic. Further, they are cross resistant to a limited number of related inhibitors implying that these share a common site of action (148, 159). That the site of action is part of the ATP synthetase complex has been demonstrated by reconstitution experiments: Purified components of the enzyme F_1 , OSCP, and the membrane fraction F_0 , were obtained from sensitive and (Venturicidin) resistant strains, and the enzyme reconstituted. In this way, resistance was found to be associated with the membrane fraction as is found for Oligomycin (159). Similar findings have been made with Triethyltin (148). Other inhibitors cross resistant to TET and VEN are specifically associated with mitochondrial membrane function; the ADP translocase inhibitors Bonkreikic acid and Hexachlorophene are such examples. Rhodamine 6G prevents oxidative metabolism when

cells are grown on a non fermentable substrate. The inhibitory action of this is not clear however. Resistance to the uncoupler 1799 is another example of the close association of these genetic determinants with mitochondrial membrane structure. A detailed genetic study of such mutants is useful for several reasons; Firstly, the location of an inhibitor binding site on a specific protein can help establish the latter's physiological role, in this case in relation to oxidative phosphorylation and ion transport. Secondly, the location of specific genes specifying resistance determinants may provide an understanding of the biogenesis of mitochondrial membranes, and the role of nuclear and cytoplasmic genes in this process.

Recombination analysis of spontaneous VEN^R mutants reveals two linkage groups associated with this phenotype. One shows linkage to OL_I on the mt DNA and has been named OL_{III} (160). This locus is useful for distinguishing between the two Oligomycin loci OL_I and OL_{II} , and implies that one of the Venturicidin binding sites on the ATP synthetase complex is common with that of Oligomycin.

When $VEN^R TET^R$ determinants were crossed to known mitochondrial markers, 45% recombination was observed. The upper limit for recombination between mt DNA molecules is 25%. This data shows that $VEN^R TET^R$ mutants are unlinked to the mitochondrial genome. Further work with TET^R mutants alone showed similar behaviour (160). The existence of a separate linkage group VI (TI), indicates that these determinants are carried on a DNA molecule other than mt DNA. A close association with mt DNA is still evident however: loss of the determinant always leads to the ρ^- state whether by EBr mutagenesis or spontaneously (150). These mutants are not unique in being cytoplasmic and yet not encoded by mt DNA. The killer phenotype described by Woods and Bevan (161) fulfils these criteria. The molecular basis of the growth inhibition of killer sensitive by

killer strains at low pH is thought to be the presence of a double stranded RNA virus (162). Mutations affecting the utilization of Ureidosuccinic acid, ure 3 (163), also shows the genetic properties of a gene unlinked to mt DNA. Of interest in relation to the $VEN^R TET^R$ mutants is the mutant described by Rotman (164). The intercalating drug Primaquin (8 - [4 amino - 1 - methylbutylamino] 6 methoxy quinoline), prevents growth when functional mitochondria are essential, but unlike Ethidium bromide, is not mutagenic. The expression of the resistance phenotype is controlled by a nuclear (pri) and cytoplasmic factor (FP). This was inferred from crosses between sensitive and resistant strains because of the following features: Mitotic segregation was observed in diploids (cytoplasmic), no meiotic segregation of sensitive diploids and Mendelian segregation of meiotic segregants (nuclear). This behaviour is very similar, if not identical to the $VEN^R TET^R$ strains described below.

The strain D22/72 was used in this study. Genetic characterization of the petites derived from it was carried out by Dr. G. Carignani in this laboratory. As well as possessing resistance to Triethyltin and Venturicidin, this strain shows resistance to Rhodamine 6G, 1799, Hexachlorophene, ^gBonkreik acid and Cycloheximide. _A Petites were induced by EBr mutagenesis as described in Methods. Table 2:1 shows representative examples of these with their phenotype as determined by crossing to a drug sensitive tester strain and the sensitivity of the resultant diploids to various drug concentrations. Suppressivity, obtained as described in Methods, is also shown. The strains used for the biochemical studies are marked +. Like Primaquin resistance, these mutants show cytoplasmic and nuclear characteristics. Mitotic segregation of markers is apparent when $VEN^R TET^R R6G^R$ strains are crossed to sensitive ones, thereby establishing a cytoplasmic

STRAIN	GENOTYPE	PHENOTYPE OF DIPLOID	% SUPPRESSIVENESS
D22/69 PARENTAL	$\rho^+ v^R$	$VEN^R TET^R R6G^R 1799^R$	
D22/69/D1	$\rho^o v^R$	$VEN^R TET^R R6G^R 1799^R$	0
D22/69/D3	$\rho^o v^o$	$VEN^S TET^S R6G^S 1799^S$	0
D22/69/D4	$\rho^o v^R$	$VEN^R TET^R R6G^R 1799^R$	0
D22/69/D8	$\rho^o v^o$	$VEN^S TET^S R6G^S 1799^S$	0
+ D22/72 PARENTAL	$\rho^+ v^R$	$VEN^R TET^R R6G^R 1799^R (HEX, BA)^R$	
D22/72/D10	$\rho^o v^R$	$VEN^R TET^R R6G^R$	0
D22/72/D11	$\rho^o v^S$	$VEN^S TET^S R6G^S$	0
+ D22/72/D12	$\rho^o v^S$	$VEN^S TET^S R6G^S$	0
+ D22/72/D13	$\rho^o v^R$	$VEN^R TET^R R6G^R$	0
D22/72/D14	$\rho^o v^R$	$VEN^R TET^R R6G^R$	0
D22/72/D15	$\rho^o v^R$	$VEN^R TET^R R6G^R$	0
D22/72/D16	$\rho^o v^R$	$VEN^R TET^R R6G^R$	0
D22/72/D17	$\rho^o v^R$	$VEN^R TET^S R6G^R$	0

TABLE 2:1

Petites Induced by Ethidium bromide mutagenesis of parental strains D22/69 and D22/79. Retention or loss of resistance markers was determined by the ability of diploids formed on crosses between petites and the grande D41, α , \arg to grow on drug plates. Suppressivity was determined by the percentage of petites in synchronous zygotes formed in crosses between strains and D41.

mode of inheritance. Because of the low viability of spores (~10%), meiotic segregation was analysed by random spore analysis (160). At high inhibitor concentrations, 2:2 segregation was observed among meiotic segregants, thereby implicating a nuclear component. When sensitive diploids were sporulated, 2:2 segregation was observed at both high and low drug concentrations. These results suggest that both nuclear and cytoplasmic factors are present in resistant segregants, and that only the nuclear component remains in the sensitive ones. Further genetic analysis of these petites involved following the segregation of more than one genetic marker, i.e. VEN^R , TET^R and $R6G^R$. The ρ^o petite D12 $VEN^o TET^o R6G^o$ shows no segregation of either type (meiotic or mitotic) in which case both genetic determinants have been deleted. On the other hand, D11, $VEN^o TET^o R6G^o$ and D17 $VEN^R TET^o R6G^R$ show both types of segregation, and hence have both factors. Of further interest is the fact that diploids formed from crosses between D11 and a drug^s haploid show some degree of independent segregation. The possibility of recombination events between markers is demonstrated, but has not been investigated further.

In order to understand the molecular basis of these resistances, two ρ^o petites were selected, one showing cross resistance to all inhibitors VEN, TET, R6G etc., and the other with all markers deleted. (D22/72 D13 and D12 in Table 2:1). The presence of determinants in a strain showing zero suppressivity provides a means of locating genetic determinants other than mt DNA, since the latter is normally absent in the ρ^o state (46). The latter correlation does not always hold however, (47) so that in order to establish the complete absence of mt DNA in these strains, a careful analysis of their nucleic acids was necessary. In this work, several techniques were used to show the complete absence of a DNA species exhibiting the properties of yeast mt DNA.

3(ii) Analysis of DNA in Equilibrium density gradients

DNA from the mitochondrial fractions of D22/72, D13 and D12 was analysed on CsCl-EBr density gradients as described in Methods. Figure 2:1 shows a scan of the fluorescent bands excited by irradiation at 360 nm. Only the grande D22/72 (A) has a DNA band of mitochondrial density, while all three have nuclear DNA, and a circular DNA fraction designated o DNA to be described. The area of the preparative gradients corresponding to nuclear and mitochondrial DNA was withdrawn by side puncture, and the samples analysed in the analytical ultracentrifuge. This procedure gives better resolution of DNA species differing slightly in buoyant density than centrifugation in the presence of Ethidium bromide. Figure 2:2 shows a scan of the resulting separation. Despite overloading, the petite D13 DNA (A) has no peak of mt DNA corresponding to the grande D22/72 (B) at a density of 1.683 g/ml. The peak at 1.731 g/ml is marker DNA. Further independent confirmation of this was achieved using CsCl-DAPI gradients. The fluorescent Berenil analogue DAPI binds strongly to (A + T) rich stretches of DNA. As described in Chapter 1, Yeast mt DNA has an unusually high proportion of these stretches, so that enhanced separation of this from n DNA is possible on CsCl gradients due to less Cs^+ binding sites being available and thereby lowering the buoyant density. The dramatic increase in separation of mt and n DNA on these gradients is apparent in 2:3. Like the other gradients described, no mt DNA was detectable in the petites.

In order to exclude the possibility that mt DNA was present but altered to the buoyant density of nuclear DNA, the following experiment was performed: The inhibitory effect of Cycloheximide (CHI) on n DNA replication has been observed in cultures pulse labelled with 3H - Adenine (84). After several hours labelling, the

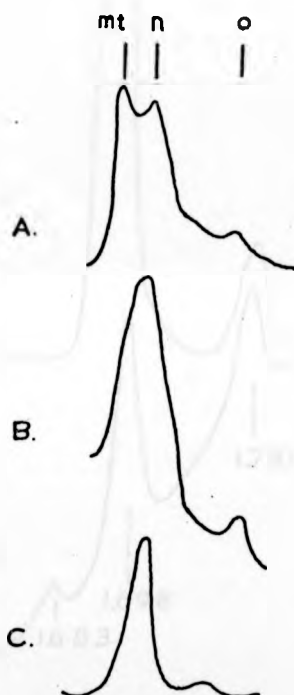


FIGURE 2:1

CsCl-EBr Density Gradient Centrifugation of Mitochondrial Lysates

Mitochondria isolated in the presence of EBr were lysed in 2% Sarkosyl as in methods. Lysates were spun to equilibrium with CsCl to 1.56 g/ml and ~500 µg/ml EBr. Centrifugation was for 42 hours at 10 C and 48,000 rpm in the Beckman 50 Ti rotor. Bands were excited by UV irradiation at 360 nm and photographed. Negatives were scanned with a Joyce Loebl microdensitometer. A. D22/72 ρ⁺. B. D13 ρ⁺ VEN⁺. C. D12 ρ⁺ VEN⁺. mt, n, o, mitochondrial, nuclear, omicron DNA.

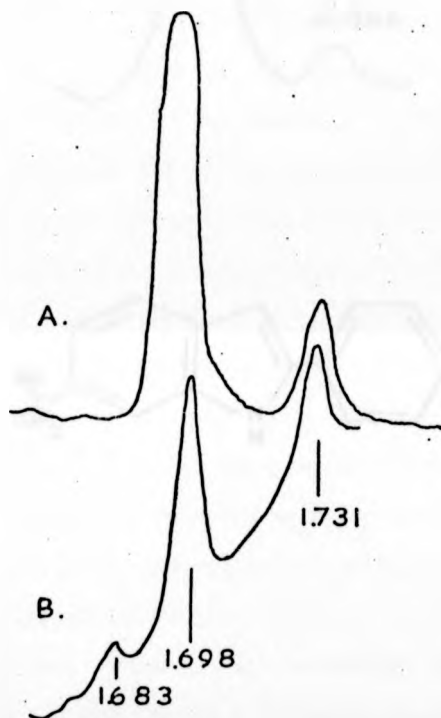


FIGURE 2:2

Analytical CsCl Density Gradient Centrifugation of DNA

Gradients were prepared and formed in the Spinco model E ultracentrifuge. *Micrococcus lysodeikticus* DNA, $\rho = 1.731$, was included as bouyant density standard. *M.C.* DNA from D22/72. *P.A.* DNA from D13. mt DNA, $\rho = 1.683$; n DNA, $\rho = 1.698$

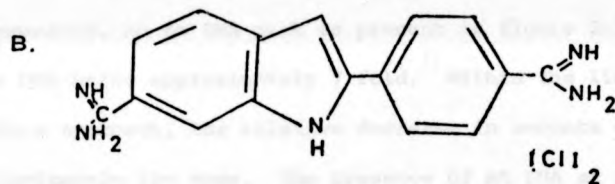
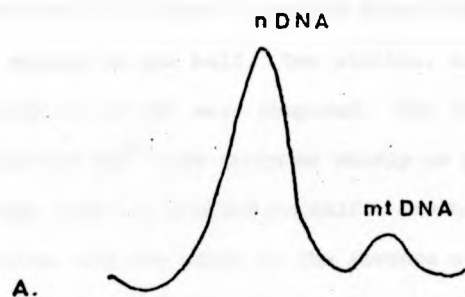


FIGURE 2:3

Preparative CsCl-DAPI Density Gradient Centrifugation

Whole cell extracts were prepared by the Eaton press method (154). Centrifugation was performed with CsCl at 1.56 g/ml and 200 µg/ml DAPI. Bands were visualized with UV light after centrifugation in the 50 Ti rotor at 48,000 rpm for 45 hours. Bands were photographed through a blue filter and scanned.

A. Gradient profile of D22/72 whole cell DNA.

B. Structural formula of DAPI.

proportion of nuclear to mt DNA drops markedly from about a ten fold excess to one half. Two strains, each grown in the presence and absence of CHI were compared. The grande D22/72 VEN^R and the petite D13 VEN^R were grown as nearly as possible to the same phase, and the cultures divided in half. One half was labelled in the presence, and the other in the absence of CHI. DNA was analysed as described in Methods. The gradient profiles are shown for D22/72 and D13 in Figures 2:4 and 2:5 respectively. Increased synthesis of mt DNA (~frac 19) at the expense of n DNA (~frac 16) is clearly shown, representing an approximately 8.6 fold decrease in n DNA. As expected, no mt DNA peak is present in figure 2:5, the decrease in n DNA being approximately 7 fold. Within the limits of error of this approach, the relative decrease in amounts of n DNA are approximately the same. The presence of mt DNA at nuclear density would presumably be detected as a decrease in the relative drop in n DNA synthesis in D13 relative to D22/72; if such a DNA species was truly mitochondrial, it would not decrease in the presence of CHI, but could increase. In the absence of a major difference in the relative labelling of n DNA peaks between grande and petite, it is concluded that there is no mt DNA of nuclear density within the limits of detection by this method. This is confirmed by Agarose gel electrophoresis of whole DNA extracts in which no minor bands are present in the petite. (Result not shown.)

3(iii) Cytological Detection of Extrachromosomal DNA

As well as providing a convenient means of separating nuclear and mitochondrial DNA, the compound DAPI can be used to stain DNA *in situ*. The grande and petites were therefore stained as described in Methods and observed under a microscope with incident UV illumination. Nuclei and mitochondria can be clearly detected by the blue fluorescence observed on UV excitation of stained specimens.

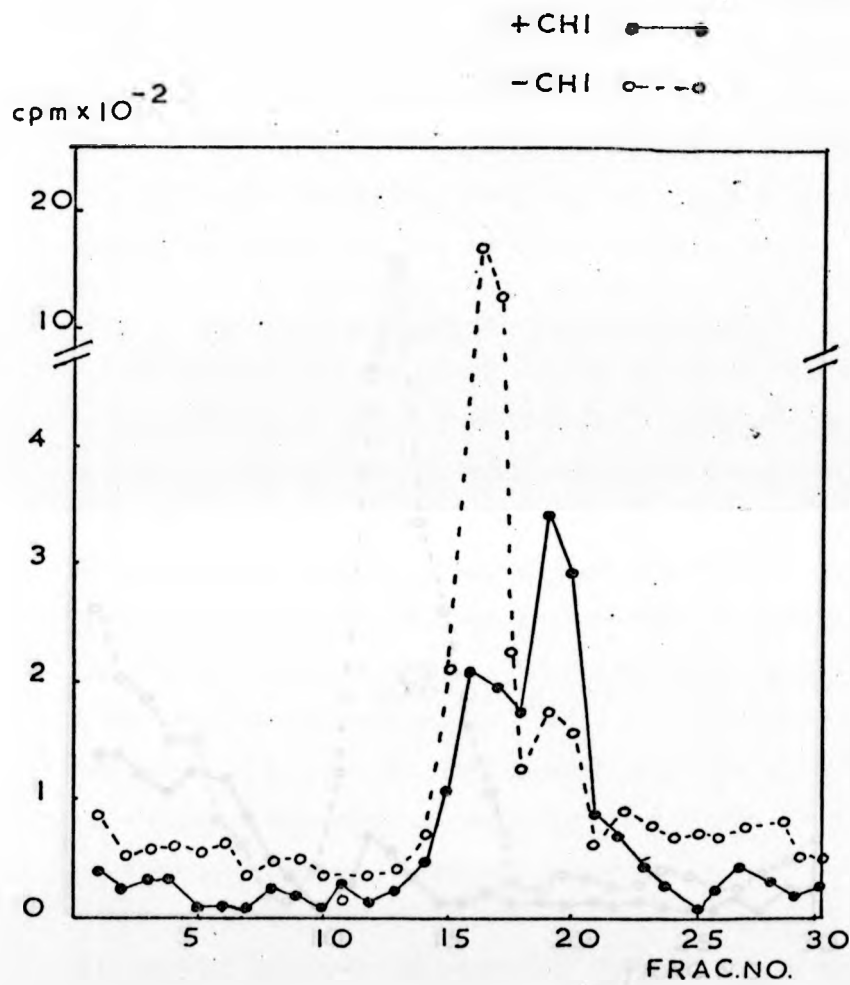


FIGURE 2:4

Analysis of ³H-Adenine labelled DNA on CsCl density gradients

D22/72 was labelled in the presence and absence of CHI for 3 hours as described in methods. Centrifugation was performed in the 50 Ti rotor for 41 hours at 20°C and 45,000 rpm. 5 drop fractions were collected from the bottom and the RNA hydrolysed with NaOH. Cold TCA precipitates were collected on filters and counted.

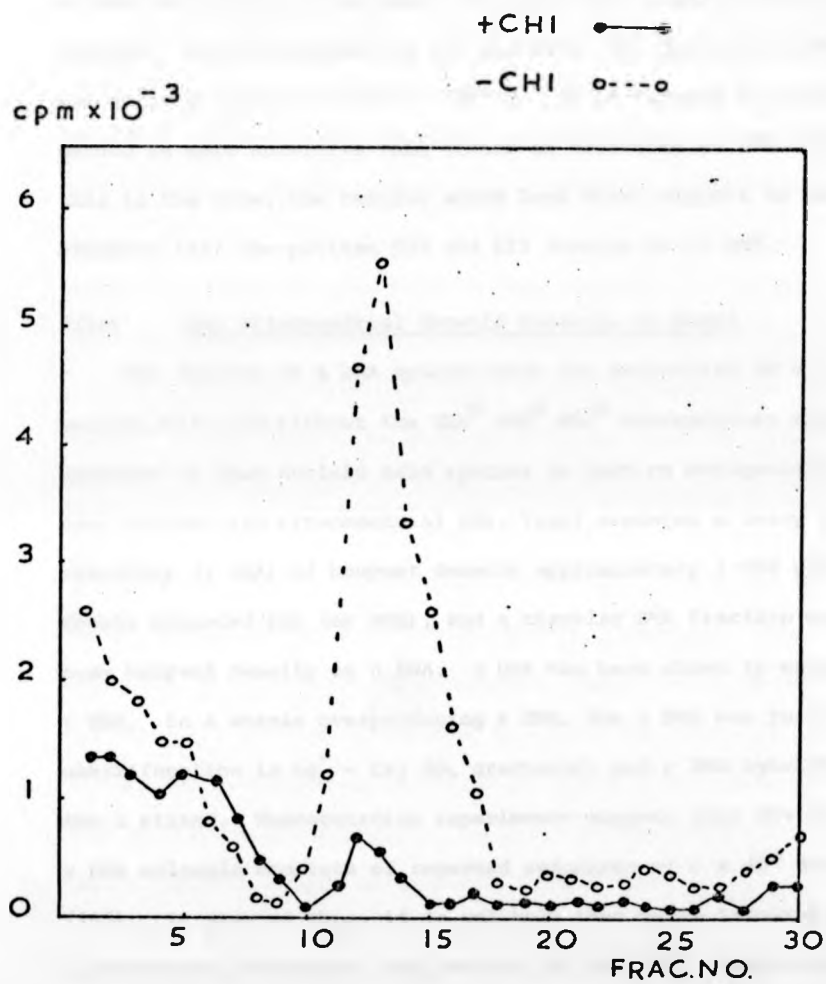


FIGURE 2:5

Labelling of ρ^- DNA with ^3H -Adenine and Analysis on CsCl Density Gradients

The procedure described in the legend to figure 2:4 was carried out with the strain D22/72 D13 VEN^R.

In strains lacking cytoplasmic DNA, only the single nucleus is visible. This was found for D13 and D12. Mitochondrial fluorescence was clearly visible in the ρ^+ D22/72. It is claimed that this method is more sensitive than others in detecting mt DNA (157). If this is the case, the results above lend final support to the evidence that the petites D13 and D12 contain no mt DNA.

3(iv) Non Mitochondrial Genetic Elements in Yeast

The absence of a DNA species with the properties of mt DNA in petites with and without the $VEN^R TET^R R6G^R$ determinants raises the question of what nucleic acid species is lost on mutagenesis. Apart from nuclear and mitochondrial DNA, Yeast contains a heavy nuclear satellite (γ DNA) of bouyant density approximately 1.702 g/ml, double stranded RNA (ds RNA), and a circular DNA fraction with the same bouyant density as n DNA. γ DNA has been shown to encode nuclear r RNA. In a strain overproducing r DNA, the γ DNA was purified by centrifugation in $Ag^+ - Cs_2 SO_4$ gradients, and r RNA hybridized to the L strand. Reassociation experiments suggest that 80% of the γ DNA molecule consists of repeated sequences of 6×10^6 daltons (165). In view of this, it is unlikely that genes involved in mitochondrial biogenesis are carried on this DNA. Fractionation of total RNA on cellulose columns yields about 5% ds RNA (Results not shown). The killer phenotype has been associated with a specific ds RNA, and a one to one correspondence between it and a band on gels demonstrated (162). Other ds RNA species occur in Yeast as detected on acrylamide gel electrophoresis of purified ds RNA. No difference was detected between VEN^R and VEN^O strains; further, the involvement of a nucleic acid type normally associated with viruses in mitochondrial biogenesis appears highly unlikely.

From a consideration of the above data, the association of $VEN^R TET^R R6G^R$ determinants with a closed circular DNA fraction

remains a distinct possibility in the absence of any other known cytoplasmic nucleic acids. Circular DNA with nuclear bouyant density has been isolated in several laboratories (20, 150, 151, 153, 166-171). CsCl density gradient centrifugation in the presence of EBr results in the separation of circular from linear molecules due to an unwinding of the former by EBr and greater binding of Cs⁺ ions. Yeast mitochondrial lysates have been analysed in this way, and the circular DNA examined under the electron microscope. The average contour length observed by several authors is about 2 μ m (151, 153, 166-169), with oligomeric forms of n x 2 μ . That these circles are not artifacts produced by the mechanical disruption of cells is clearly demonstrated by the work of Petes and Williamson (153). These authors have observed double branched 2 μ circles, similar to those described by Cairns for the E-coli chromosome, which have the features of replicative intermediates. Replication is under the control of a nuclear temperature sensitive DNA propagation mutant CDC 8, the proportion of replicative intermediates increasing at non permissive temperatures. The nuclear involvement in circular DNA metabolism raises the question of its cellular location: most published preparations have utilized a mitochondrial fraction for the purification of this DNA (150, 151, 166-171), suggesting an association with these organelles. Clark-Walker has purified mitochondria on Sucrose gradients and found circular DNA (omicron DNA in his nomenclature), associated with the lighter of two bands (151). The other denser fraction contained DNA of mitochondrial density, while the former did not. In view of this, Clark-Walker has suggested that omicron DNA (o DNA) may be associated with peroxysomes which band on Sucrose gradients at the position of the lighter band. Evidence for the association of o DNA with the nucleus is based on its similar properties to n DNA. Its nuclear bouyant

density and loss on incubation of cells with CHI are cited as examples (170). Also, in the majority of strains examined, loss of mt DNA on EBr mutagenesis does not lead to loss of o DNA. Nevertheless, in a study of o DNA in purified nuclei and cytoplasm, Clark-Walker was unable to find significant numbers of molecules in the former. If o DNA represents amplified segments of chromosomal DNA "informosomes", which are transported into the cytoplasm, 2 μ circular DNA should be present in the nucleus (169). In conclusion, no clear evidence on the cellular location is available, but it seems unlikely that it is located in the nucleus.

To date, no physiological function has been clearly assigned to o DNA. Guerineau *et al* (171) have examined several strains carrying resistance to Oligomycin, Venturicidin, Chloramphenicol, Cycloheximide and Triethyltin. Although these mutants are unlinked to mt DNA, they are totally different from the VEN^R, TET^R R6G^R mutants described here. They show complex Mendelian genetics, linkage to the centromere, and as such behave as Class 1 mutants. On the other hand, they exhibit loss of markers on vegetative multiplication, an episomal characteristic. Resistance is not expressed at the level of ATP synthetase for OL^R, VEN^R, TET^R mutants, or at the purified ribosomal level for CHI^R mutants. The resistance phenomenon is probably due therefore to a permeability barrier. This is not the case for the VEN^R, TET^R, R6G^R mutants used in this study. As well as resistance being expressed at the purified ATPase level, the disruption of mitochondrial membranes has no effect on the resistance levels. The parental D22/72 and VEN^R petite D13 were grown anaerobically as described in Methods. Identical cultures were grown aerobically, and both crossed to the D41 tester strain and tested for loss of resistance as described. No loss of resistance in 100 colonies of grande or petite was

observed. Anaerobic growth is known to disrupt mitochondrial membranes (172), so that in this case, the mechanism of resistance is not a permeability barrier. The resistances described by Guerineau *et al* are related to the presence or absence of o DNA. Oligomycin resistance is lost spontaneously with the concomitant loss of this DNA. The other resistances can be lost with EBr, but without loss of o DNA. A possible explanation for this is that an alteration in membrane structure brought about by the mutations is a primary event leading to loss of o DNA as a result; altered binding sites, replicators etc., could be envisaged. What is clear is that the close association between the mutants and o DNA claimed by these authors should be clarified, particularly in relation to known mitochondrial genes.

3(v) Restriction Endonuclease Analysis of o DNA from VEN^R and VEN^O Strains

In an effort to relate VEN^R, TET^R, R6G^R mutants to o DNA, D13 VEN^R, and D12 VEN^O, were examined for the presence or absence of o DNA. Figure 2:1 shows that both these strains have this DNA species. The amounts varied in different experiments, and it was impossible to quantitate o DNA in each petite. In order to detect base sequence changes, (additions, deletions) between o DNAs from VEN^R and VEN^O petites restriction endonuclease analysis was performed. These enzymes have the property of cleaving DNAs at defined base sequences so that the pattern of fragments so obtained is unique for a particular DNA species (97). Under the digestion conditions used, the enzyme Hind III did not cleave any o DNA preparations. Hind III recognizes the sequence A⁺AGCTT, which therefore may not be present in these strains. EcoRI however showed different patterns on gels between VEN^R and VEN^O o DNAs (Figure 2:6). 1. is D13 VEN^R. Peaks a and b correspond to uncleaved o DNA as determined by running

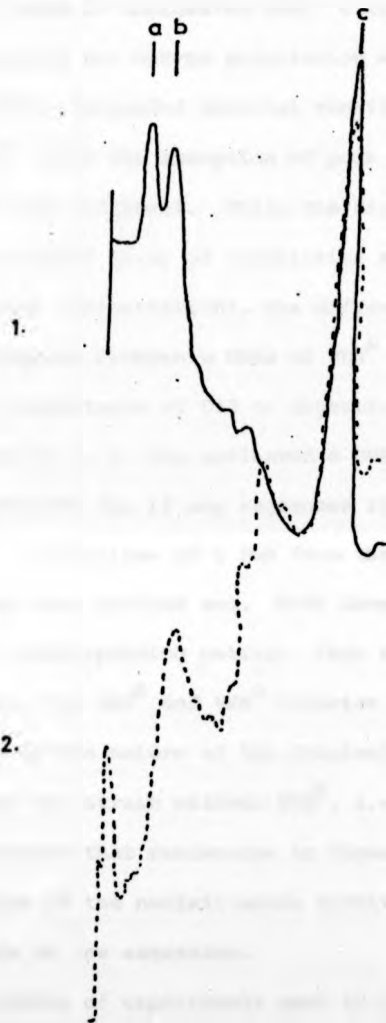


FIGURE 2:6

Agarose Gel Electrophoresis of EcoRI digests of ϕ DNA

ϕ DNA was digested for 1 hour with EcoRI restriction endonuclease. Flat bed 1.4% Agarose slab gels were run in the presence of 1 μ g/ml EBr. Electrophoresis was for 3 hours at 15 mA. DNA bands were photographed with incident UV illumination, and the negatives scanned. The top of the gel (-ve) is to the left.

1. D13 VEN^R ϕ DNA. 2. D12 VEN^O ϕ DNA.

a, b - unrestricted ϕ DNA. c. endonuclease products.

parallel gels of undigested DNA. c represents exonuclease activity contaminating the enzyme preparation which results in a relatively large peak of degraded material running near the dye front. 2. is D12 VEN^o. With the exception of peak c, the digestion pattern is clearly very different. While the digestion has not resulted in a clearly defined array of restriction fragments (possibly due to the exonuclease contamination), the difference in profiles is suggestive of differences between o DNAs of VEN^R and VEN^o petites. The comparative resistance of D13 to digestion by EcoRI, indicated by the retention of a, b, the uncleaved o DNA fraction, suggests that its o DNA contains few if any sequences recognized by the enzyme, namely G[↓]AATTC. Digestions of o DNA from the grande D22 VEN^S and D22/72 VEN^R were also carried out. Both show identical cleavage patterns to their corresponding petites, that is the VEN^R strains are identical, the VEN^S and VEN^o likewise. This result is significant in terms of the nature of the original base sequence changes. The fact that the strain without VEN^R, i.e. VEN^o is the same as VEN^S would suggest that resistance to these inhibitors involves gross alteration of the nucleic acids involved, possibly even *de novo* synthesis of new sequences.

A number of experiments need to be performed in order to clarify this situation. Firstly, the availability of a large number of sequence specific endonucleases could be useful in providing unambiguous cleavage patterns of o DNA. While the experiments described in this section are suggestive of major sequence changes in o DNA, they do not give any quantitative data on these. Secondly, the technique of molecular hybridization can be used to detect sequence homologies between different DNAs. Hybridization of labelled o DNA from VEN^R strains to o DNA in excess from VEN^o, or vice versa, is a possibility. Attempts were made to label o DNA

with $^{32}\text{PO}_4$ to high specific activity. The low levels (1-5%) of this DNA compared to other Yeast nucleic acids resulted in material of very low activity. Recent methods for the radioiodination of DNA could overcome this problem (173).

3(vi) Conclusion

The work described here has shown that cytoplasmic genetic determinants unlinked to mt DNA may be present in petites shown to lack this molecular species. These mutants exhibit the properties of an episomal system, possibly associated with mt DNA. Evidence for this comes from the following properties of these mutations:

- a) They confer multiple resistances to drugs affecting a common target, the mitochondrial membrane.
- b) Resistance can be lost on treatment with EBr.
- c) The association of $\text{VEN}^R \text{TET}^R$ with mitochondrial genes, e.g. OL_1 , and the common binding site of inhibitors on mitochondrial and non mitochondrial (episomal?) products has been suggested by genetic studies (160).
- d) A significant number of spontaneous petites have also lost resistance determinants (149) implying an association with the ρ factor.
- e) The presence or absence of these mutations in a strain carrying a mitochondrial mutation will cause a strong increase or decrease of transmission of the mitochondrial gene to the zygotic progeny. This could be explained by a model involving incorporation of the VEN^R determinant into mt DNA at various stages during mitosis and zygote formation, a characteristic of episomes (174).
- f) Preliminary analysis with EcoRI endonuclease has suggested that ρ DNA might fulfil this function; this DNA is extranuclear and associated with a mitochondrial fraction. Yields of DNA are greater from cells grown to the transition phase than from mid logarithmic or stationary phase. A more quantitative study of this throughout the growth cycle would be useful. Variations during the growth cycle of synchronous

cultures could be a reflection of the integration or release of an episome from a larger chromosome.

Genetic studies also show that a nuclear determinant is involved in the resistance phenomenon. This could conceivably act as a regulator of the cytoplasmic factors, but as yet, no evidence is available.

At present, the data available on resistance to TET, VEN and R6G is not sufficient to construct a model of this phenomenon; the presence of these genes however should provide an understanding of totally novel nucleocytoplasmic and episomal interactions which may be applicable to higher organisms.

CHAPTER 3

1. INTRODUCTION

A spontaneous petite containing the $VEN^R OL^R$ locus was obtained from a strain DL191 carrying the five drug resistance markers ERY, CAP, PAR, ($VEN OL_I$), OL_{II} .

In order to study the expression of mitochondrial genes *in vitro*, mt DNA was isolated from DL191, transcribed by E.coli RNA polymerase, and the resulting RNA translated in an E.coli ribosomal system. Proteins synthesized *in vitro* were compared with mitochondrial proteins synthesized in isolated mitochondria by Sephadex G-100 gel exclusion chromatography. A large peak co-eluting with cytochrome c was found in both systems. Gel electrophoresis of this revealed a single peak of approximately 10,000 daltons molecular weight. The results were similar to those described for *Neurospora crassa*, and suggest that transcription and translation is proceeding with fidelity.

Total RNA was isolated from DL191, and the spontaneous petite $VEN^R OL^R$, and used to programme cell free translation systems from E.coli and *Xenopus* oocytes. In the former case, endogenous messenger activity was stimulated without the production of mt RNA directed products, in the latter, no products were detected using antisera to cytochrome oxidase.

When RNA fractionated into a poly (A) rich fraction on oligo dT cellulose was used in a system derived from Wheat germ, discrete polypeptides were synthesized. The electrophoretic profile of $VEN^R OL_I^R$ programmed products was much simpler, confirming a deletion of major portions of the mitochondrial genome in this petite.

RNA fractions used in this study were analysed by pulse labelling with $^{32}PO_4$ or 3H -Adenine. The $VEN^R OL_I^R$ petite RNA appeared to have a small poly (A) sequence at its 3' end, characteristic of eukaryote m RNAs. The significance of these findings in relation to

gene mapping is discussed.

Transcription and translation of omicron DNA *in vitro*, as described for mt DNA, resulted in the synthesis of two products of molecular weights 45,000 and 22,500 daltons respectively. The relation of these to mitochondrial membrane proteins is unknown.

2. METHODS AND MATERIALS

2(i) Construction of Strains and Isolation of Petites

The diploid strain DL191, ERY^R, CAP^R, PAR^R, OL^R, VEN^R, OL^R_{II} was constructed by Dr. W. E. Lancashire as shown in Table 3:1. The locus OL_I VEN displays the same phenotype as OL_I, and is therefore useful in separating OL_I from OL_{II}. Spontaneous petites were plated onto drug plates: a single mutant retaining the OL_I marker only was used in this study. The genetic methodology has been described (175).

2(ii) Growth of Cells and Isolation of DNA

Growth conditions were those described in Chapter 2, except that 1% Glucose and no Adenⁿine sulphate were used for DL191. For RNA extraction, cultures were grown to mid^{mid} logarithmic phase.

mt DNA was isolated by the Polylysine Kieselg^ular method of Finkelstein *et al* (176), or by CsCl - EBr centrifugation of whole cell extracts as described before (153, 154). DNA from CsCl gradients was recentrifuged to remove as much nuclear DNA as possible. After removal with a Pasteur pipette, the DNA was extracted at 4°C with isoamyl alcohol to remove EBr, and dialysed against 20 mM Tris Cl pH 7.5. If concentration was necessary, dialysis bags containing the DNA solution were placed in solid sucrose, and the latter removed by dialysis as before.

o DNA was prepared from mitochondrial fractions as described in Chapter 2.

D22/A15 OL^R x IL8-8C CAP^RERY^R

DIPLOID

DS197 ERY^RCAP^ROL^R

HAPLOID

D22/60 VEN^R x PS197-1B ERY^RCAP^ROL^R_α,his
(OL_I)

DIPLOID DL160 VEN^ROL^R

HAPLOID

KL60-2C x DL160-1C VEN^ROL^R
CAP^RERY^RPAR^R

DIPLOID DL191 CAP^RERY^RVEN^RPAR^ROLI^R

HAPLOID

SPONTANEOUS PETITES

PLATE ONTO GRANDE LAWN (DRUG^S)

SCORE FOR GROWTH ON: OLIGOMYCIN

VENTURICIDIN

ERYTHROMYCIN

CHLORAMPHENICOL

PAROMOMYCIN

TABLE 3:1

Construction of the strain DL191 containing 5 drug resistance markers. Petites retaining markers singly or in combination were selected on drug plates using the marker rescue technique described in the text.

2(iii) Transcription of mitochondrial and omicron DNA

Transcription conditions for mt DNA were those described by Scragg and Thomas (177). Commercial RNA polymerase, 250 U/ml, from *E. coli* was used. Electrophoretic analysis confirmed that the preparation contained α , β , β^1 and σ subunits (178).

The high KCl concentration (0.25 M) used in mt DNA transcription was reduced to 0.15 M for o DNA transcription. For determining the kinetics of RNA synthesis, ^3H -UTP, 16.9 mCi/mmole , was used. Aliquots of 15 μl were taken every 15 minutes, precipitated with ice cold TCA (10% in 100 mM sodium pyrophosphate), filtered and counted in Triton scintillation fluid in a Packard 2425 counter.

RNA synthesized *in vitro* was precipitated with 1 mg/ml carrier t RNA from *E. coli* on addition of ethanol at -20°C . Precipitates were collected, washed once in 70% ethanol containing 50 mM KCl, and dissolved in water. All solutions and glassware were sterilized by autoclaving or Millipore filtration where appropriate.

2(iv) Translation of *in vitro* transcripts in S-30 system from *E. coli*

S-30s (30,000 g supernatants) were prepared from *Escherichia coli* MRE 600, RNA ase⁻, exactly as described by Hartley *et al* (179). RNA from 250 μl transcription mixtures programmed with 5 μg DNA was used in a single 0.5 ml assay. Transcription assays minus DNA were processed as described in 2(iii) and used as controls for the translation assays. These contained, for controls, 20 μl ^{14}C -Leucine, 340 mCi/mmole ; assays with mt RNA contained 10 μl ^3H -Leucine, 58 Ci/mmole . After incubation for 40 minutes at 37°C , the two reaction mixtures were pooled, mixed with 1 mg/ml cytochrome *c* as internal molecular weight marker, and made 1% in SDS.

For o DNA directed translation, ^{35}S -Methionine, 270 Ci/mmole , was used as label. Incorporation of label was determined by spotting

5 μ l aliquots onto Whatman 3 mm or GF/A paper and boiling in 10% TCA plus 0.5 mg/ml Methionine for 5 minutes. Filters were washed with ethanol, ethanol ether 2:1, and ether. After air drying, filters were counted as before. A blank filter was used to subtract background counts from all assays.

For gel electrophoresis, assay mixtures were made 10% in TCA plus 0.5 mg/ml Methionine, boiled for 10 minutes and kept on ice for 3 hours. Precipitates were collected by centrifugation and taken up in electrophoresis sample buffer (2% SDS, 10% glycerol, 1% Mercaptoethanol, 0.05 M Acetate/Borate buffer pH 8.5, 0.001% bromophenol blue (BPB).) Complete solubilization often required the addition of Tris base to about 0.5 M. Samples were boiled for 5 minutes and stored at -20°C prior to electrophoresis.

2(v) Protein Synthesis by Isolated Mitochondria in the Presence of Cycloheximide

Mitochondrial S-40s (40,000 g supernatants) were prepared according to Kuntzels procedure for *Neurospora crassa* (180). Pre-incubated extracts were incubated with ^{35}S -Methionine, 25 μCi , 180 Ci/mmole , for 30 minutes at 37°C . Reactions were terminated with 0.1 ml of 10% SDS, and cytochrome c, 1 mg/ml, added as internal molecular weight marker.

2(vi) Analysis of *in vitro* products on Sephadex G-100 Columns

Reaction mixtures, treated as described in 2(iv) and (v), were layered onto Sephadex G-100 columns (2 x 50 cm) equilibrated with 50 mM Tris Cl, 1 mM DTT, 0.5% SDS pH 7.5. Elution was performed with the same buffer. 40 drop fractions were collected in an LKB Ultrarac fraction collector with a flow rate of about 0.2 ml/minute. 0.5 ml Aliquots of each fraction were counted in 10 ml Triton scintillation cocktail. The position of cytochrome c was measured by reading absorptions of red fractions at 550 nm in a Pye Unicam

SP1800 spectrophotometer. Peak fractions were prepared for electrophoresis by dialysis against 2000 volumes of water at 4°C followed by lyophilization and dissolution in electrophoresis sample buffer.

2(vii) Preparation of Mitochondria for RNA Extraction

Yeast strains were grown to mid logarithmic phase as described in 2(ii). After washing in sterile water, cells were suspended in sterile BSA buffer (0.5 M Sorbitol, 20 mM Tris Cl, 4 mM EDTA, 1 mg/ml BSA, pH 7.5). Suspensions were shaken with an equal volume of glass beads, (0.45 mm) in the Braun shaker for 45 seconds. Glass beads were washed with several volumes of BSA buffer, pooled, and the suspension centrifuged twice at 4,000 rpm for 10 minutes in the Sorvall GSA rotor to remove cellular debris and nuclei. Mitochondria were pelleted by centrifugation at 13,500 rpm for 20 minutes in the Sorvall SS-34 or Beckman JA-20 rotor. Pellets were treated in either of two ways. a) Mitochondria were washed 3 times in 40 mls of BSA buffer by gentle resuspension in a homogenizer and respinning at 13,500 rpm. b) Discontinuous sucrose gradients were formed by layering 13 mls each of 70%, 50%, 30% and 20% sucrose (in 10 mM Tris Cl, 4 mM EDTA pH 7.2) in 70 ml cellulose nitrate tubes for the MSE 3 x 70 ml swing out rotor. Samples were layered onto the precooled gradients and centrifugation performed in the MSE Superspeed ultracentrifuge at this temperature at 23,000 rpm for 2.5 hours. Mitochondria were visible as two layers which were removed with a Pasteur pipette. After addition of BSA buffer to 40 mls, mitochondria were pelleted and washed once as in (a).

2(viii) Extraction of RNA from Purified Mitochondria

Three methods of RNA isolation were used:

- a) The Phenol/Triisopropyl Napthalene Sulphonate method of Kirby and Parish (181).

b) The Phenol/Chloroform/Isoamyl Alcohol method of Penman (182).

c) The Phenol/Chloroform method of Mayer *et al* (183).

For (a), the mitochondrial pellet was suspended in 10 mls of solution A (10 mM Tris Cl, 50 mM NaCl, 1% Triisopropyl Napthalene Sulphonate, 6% 4-Amino-Salicylate, 6% 2-Butanol pH 7.5). An equal volume of redistilled Phenol containing /100 mls, 20 mls H₂O, 0.1 g 8-Hydroxy Quinoline, and 14 mls of m-Cresol. Extraction was at room temperature as described (181). RNA was precipitated on addition of 2 volumes 95% Ethanol containing 0.2 M sodium acetate at -20°C.

For (b), the procedure of (182) was followed except that extraction was performed at 20°C, not 60°C. RNA was precipitated as in (a).

In (c), the procedure of (183) was followed exactly.

In order to remove contaminating DNA, all RNA samples were dissolved in 50 mM MES, 2 mM MgCl₂ pH 7.0, and DNA ase added to 50 µg/ml. Digestion was for 20 minutes at 0°C. RNA was precipitated with 90% ethanol containing 0.2 M sodium acetate, washed twice with this solution, and stored in suspension at -20°C. To minimize RNA ase contamination, all solutions and glassware were autoclaved.

2(ix) Fractionation of RNA on oligo dT Cellulose

In order to purify RNAs with short (≤ 20) poly Adenylic acid (poly (A)) sequences at their 3' end, the procedure of Rosen and Edelman was used with modification. For analysis of ³²P₄ labelled RNA, 3 ml columns of oligo dT cellulose were used. RNA was dissolved in 2 mls of binding buffer (0.5 M KCl, 10 mM Tris Cl pH 7.5) and applied to the column at 4°C. Unbound RNA was washed through with 10 mls of the same buffer at 4°C, and 10 drop fractions collected. To elute poly (A) containing RNA, the column was warmed to room

temperature and eluted with 10 mls of 10 mM Tris Cl, 0.2% SDS pH 7.5. Fractions (10 drop) were collected, and 200 μ l aliquots of each counted in 10 mls of Triton scintillation cocktail. Radioactivity was expressed as counts/ml of fraction eluted for unbound and bound RNA.

For the large scale RNA preparations used for *in vitro* protein synthesis, a batchwise extraction procedure was adopted. 4 mls RNA in binding buffer (about 100 E₂₆₀ units) was mixed with 3 mls oligo dT cellulose in a centrifuge tube, and stood on ice for 2 minutes. Unbound RNA was removed by centrifugation at 2,500 rpm for 5 minutes in a BTL bench centrifuge. The oligo dT cellulose pellet was washed 4 times with 5 mls of binding buffer, and resuspended in 5 mls of H₂O as eluant. RNA was recovered by precipitation with 90% Ethanol, or, in the case of poly (A) RNA, by lyophilization.

2(x) Pulse Labelling of RNA with ³²PO₄ and ³H-Adenine

250 ml cultures were grown to mid logarithmic phase as described in 2(ii), and supplemented with 6 mCi of ³²P orthophosphate 91 C₁/mg P₁, or 0.5 μ C₁ ³H-Adenine (26 C₁/mmole). Growth was contained for 25 minutes with ³²PO₄ and 2.5 hours with ³H-Adenine. Cell fractionation, isolation of mitochondria and RNA purification were as described previously, (2(vii) and (viii)). ³²PO₄ labelled RNA was analysed on oligo dT cellulose columns as described previously in 2(ix). ³H-Adenine labelled RNA was fractionated by the batch extraction procedure prior to poly (A) tract analysis.

2(xi) Analysis of ³ poly (A) Terminus by RNAase digestion

The procedure of Perlman *et al* (185) was used. 15,000 cpm ³H-Adenine labelled RNA, fractionated on oligo dT cellulose, were dissolved in MSB buffer (10 mM Tris Cl, 100 mM NaCl, 10 mM MgCl₂ pH 7.4) and digested with 50 μ g/ml DNAase at 37°C for 1 minute. Sodium chloride and sodium citrate were added to 0.3 M, and the RNA

digested with TI Ribonuclease (50 units/ml) and Pancreatic Ribonuclease (20 µg/ml) for 30 minutes at 37°C. Reactions were terminated with 5 µl of diethylpyrocarbonate, and RNAase resistant RNA precipitated with 90% Ethanol and 0.5 mg/ml tRNA (E.coli) as carrier. Precipitates were dissolved in 2% SDS, 10% Glycerol, 0.001% BPB prior to electrophoresis on 10% SDS polyacrylamide gels.

2(xii) Translation of mt RNA in E.coli S-30s

E.coli S-30s were prepared as described in 2(iv). Total mt RNA, (500 µg), prepared by method (a) of section 2(viii), was used in 250 µl assays with 20 µCi of ³⁵S-Methionine. MS2 viral RNA (20 µg) was used to test the activity of each S-30 preparation. The Magnesium concentration was adjusted to give maximal stimulation of hot TCA insoluble counts over background levels. These optima were 2.5 mM for mt RNA, and 11 mM for MS2. Protein synthesis assays and preparation of samples for electrophoresis were carried out as described in 2(iv).

2(xiii) Injection of mt RNA into Xenopus oocytes

Total mt RNA was extracted from gradient purified mitochondria by the Phenol/Chloroform/Isoamyl alcohol method of 2(viii). RNA was dissolved in 0.1 M KCl at concentrations of 1 and 5 mg/ml. 60 oocytes from *Xenopus laevis* were injected with 50 nl of 0.1 M KCl as controls, followed by two series of 30 eggs injected with 50 nl of 1 and 5 mg/ml RNA respectively. Each series was bathed in labelling medium plus 50 µCi ³⁵S-Methionine. Oocytes were incubated overnight at 30°C and frozen at -20°C until use. For measurement of protein synthesis, 2 eggs were homogenized in 100 µl of electrophoresis sample buffer in a microfuge tube. Cell debris was removed by spinning at 3,000 g for 10 minutes. The supernatant was removed by side puncture with a syringe to avoid the floating lipid pellicle. 5 µl aliquots were

spotted onto filter paper, processed and counted as before.

For reaction to Cytochrome oxidase antibody, 10 oocytes were homogenized in 100 μ l of 1% Nonidet P40 (NP40) in PBS (0.15 M NaCl, 4 mM KCl, 2 mM KH_2PO_4 , 8 mM Na_2HPO_4), and the supernatant extracted as before. 20 μ l antibody to cytochrome oxidase raised in rabbits was added, followed by incubation at 37°C for 30 minutes. 200 μ l Goat anti Rabbit antiserum was added, and the mixture incubated a further 45 minutes. Antiglas were precipitated overnight at 4°C. Precipitates were collected by centrifugation at 9,000 g for 10 minutes, washed four times in PBS, and taken up in 100 μ l electrophoresis sample buffer. 5 μ l aliquots were counted as before.

2(xiv) Translation of mt RNA in a Wheat Germ cell free system

Wheat germ extracts were prepared from General Mills raw wheat germ according to Marcu and Dadock (1977). Fidelity of translation was tested with 5 μ g of Vesicular Stomatitis viral RNA, (VSV RNA).

100 μ l assay mixtures were constructed as follows:

20 μ l cocktail consisting of (in 500 μ l):

50 μ l HEPES pH 8.4 0.466 M

100 μ l ATP 25 mM

25 μ l Mg Acetate 88 mM

25 μ l KCl 2.1 M

50 μ l GTP 1 mM

250 μ l H_2O

4 μ l Creatine phosphate/Creatine phosphokinase

(0.16 M/0.2 mg/ml)

10 μ l 0.5 mM 19 amino acids - Methionine

10 μ l S-Methionine (250 μCi /ml in 0.14 mM DTT)

40 μ l Wheat germ S-30

14 μ l RNA in H_2O

RNA was used in the quantities indicated in legends to figures.

Incubation was for 1 hour at 30°C. Reaction mixtures were counted as described in 2(iv), and samples treated for electrophoresis.

2(xv) Gel Electrophoresis Techniques

(a) Products of *in vitro* protein synthesis were analysed on disc or slab gels as indicated in legends to figures.

For analysis of G-100 column fractions and o DNA translation products, 10% SDS polyacrylamide disc gels were used with 0.05 M Acetate/Borate buffer^p pH 8.5 as electrophoresis buffer. Gels were run at 8 mA per gel for five hours.

For all other experiments, the discontinuous buffer system described by Douglas and Eutow (127) was used in either 15% disc gels or 10-15% gradient slab gels. The latter was constructed in a vertical plate apparatus by pumping 10% acrylamide solution into a mixing chamber containing 15% Acrylamide which was then pumped into the gel apparatus. Electrophoresis was carried out at 4°C for 5 to 6 hours at 35 mA, or 10 mA per disc gel. Gels were fixed and stained in Methanol, Acetic acid, Water, 5:1:5, containing 0.1% Coomassie brilliant blue, or 1% Amido black, for 30 minutes. Destaining was effected by agitating gels overnight in 7% Acetic acid with a skein of wool to absorb dye.

For measurement of radioactivity, disc gels were frozen at -70°C and sliced into 1 mm sections in the Mickle gel slicer, incubated with 0.5 mls of 30% H₂O₂ for several hours, and counted in 10 mls Triton scintillation cocktail. Slab gels were dried onto filter paper by heating over a steam bath and evacuating between two silicone rubber sheets. Steam was applied for 20 minutes and the gel allowed to cool for a further 30 minutes. Gels were exposed to Kodak RP/R2 or XOMat H medical X ray film and exposed for times indicated in the legends to figures. Films were processed with DX and FX developer and fixer according to manufacturer's instructions. Positive prints were

obtained by photography of autoradiograms illuminated with a light box.

Molecular weights were calculated from a standard curve of mobility versus the logarithm of the molecular weight, the following standard proteins were used:

BSA (68,000), Pyruvate kinase (57,000), Glutamate dehydrogenase (53,000), Alcohol dehydrogenase (41,000), Glyceraldehyde phosphate dehydrogenase (36,000), Trypsin (23,000), Lysozyme (13,930), Cytochrome c (11,370). Mobilities were calculated relative to cytochrome c.

(b) Acrylamide/Agarose Composite Gels

RNA was analysed on 2.2% Acrylamide, 0.5% Agarose composite disc gels according to Peacock and Dingman (188). For radioactivity analysis, gels were sliced into 2 mm sections in the Mickle gel slicer and processed as in (a). For optical density scanning, gels were washed three times with H₂O over a period of 30 minutes, and scanned at 260 nm in a Gilford gel scanner. For molecular size standards, total yeast RNA, E.coli t RNA (4S) were run in parallel gels and scanned as above.

2(xvi) Materials Used

Growth media and general reagents have been described in Chapter 2.

All radiochemicals were purchased from the radiochemical centre, Amersham with specific activities as indicated in Methods.

RNA polymerase and rA₄ were from Boeringher, Mannheim, Acrylamide, electrophoretically pure and N,N,N¹,N¹, Tetramethylene bis acrylamide (recrystallized from Acetone) Yeast RNA, t RNA and NP40 were from BDH Ltd., Poole. MS2 RNA and goat antirabbit antiserum were from Miles Yeda Ltd., Slough. Oligo dT cellulose (Type VII) was from PL biochemicals, Milwaukee. Xenopus oocytes were kindly supplied and

injected by Dr. H. Woodland, Warwick University. Antisera to cytochrome oxidase were prepared by Dr. E. J. Griffiths in this laboratory. VSV RNA and General Mills wheat germ were kind gifts of Dr. A. Gutierrez-Hartman, Dallas. Kieselguhr was Fisher "Hyflo supercel". Polylysine, Type VI, RNAases and Molecular weight standards were supplied by Sigma Ltd., St. Louis.

3. RESULTS AND DISCUSSION

3(i) The Use of Cell Free Systems in Mitochondrial Biogenesis Studies

Until recently, data on mitochondrial biogenesis has been almost exclusively derived from studies of isolated mitochondria, whole cells *in vivo*, or the physicochemical properties of mitochondrial membrane complexes. These findings have been reviewed in Chapter 1 and certain aspects dealt with experimentally in Chapter 4. Another approach, which has been applied with considerable success to many prokaryote and eukaryote systems, is the use of cell free transcription and translation systems. Such systems can take the form of a DNA directed system in which purified DNA is transcribed with RNA polymerase *in vitro*, and the products used to direct protein synthesis with a crude or purified cell free ribosomal system. These processes may be separated temporally by using separate *in vitro* assays, or coupled directly in a single system. Alternatively, RNA transcribed *in vivo* is purified and used to direct protein synthesis in a system normally derived from a different organism. Examples of these systems are reviewed in (189).

Cell free systems have several applications in studying the expression of mitochondrial genes: a) Analysis of products synthesized under the direction of mt DNA and comparison with proteins known to be synthesized on mitoribosomes could directly determine the coding origin of mitochondrial proteins. To date, the

demonstration of all m RNA translated on mitoribosomes being encoded by mt DNA has not been made, and it is possible that some mt RNAs are imported from the nucleus (26).

b) The structural properties of mt RNA have an intrinsic interest, particularly in relation to the similarities or differences between prokaryotes and mitochondria. Such properties are reflected in the type of ribosomal system capable of translating mitochondrial messages. c) The biological amplification of genes found in petites opens up new possibilities for gene expression *in vitro*. Amplification of r RNA and t RNA genes has been discussed in Chapter 1. The presence of a mitochondrial RNA polymerase, even in petites lacking mt DNA (190), supports the finding that RNA is still transcribed in the ρ^- state. Assuming that mt DNA is transcribed into m RNA fractions, evidence for which is reviewed later, it is likely that m RNA sequences are also amplified at the expense of other genes. Such systems could be used for gene mapping; if proteins synthesized in cell free systems directed by grande RNA are compared with those coded by petite DNA amplified in certain regions, those sequences lost from the grande mt DNA should be reflected in a loss of specific protein products. Comparison of several petites deleted in different regions would lead to a "translational map" of products synthesized *in vitro*. The possibilities of such a mapping technique form the basis of this chapter.

While the problems involved in determining the optimal conditions for gene expression *in vitro* are immense, for example in ensuring initiation and termination of messages, several promising systems have been described. Kuntzel and Blossy have described a system in which *Neurospora crassa* mt DNA is transcribed by *E. coli* RNA polymerase and translated in a ribosomal system from this organism (180). Gel electrophoresis of products revealed high molecular weight aggregates which bore no relation to mitochondrial proteins synthesized *in vivo*.

If reaction mixtures were made 0.1% in SDS and analysed on G-100 columns however, aggregation was prevented, and a peak of molecular weight 12,000 daltons coeluting with authentic mitochondrial proteins. Further work has shown this peak to contain hydrophobic proteins with an average molecular weight on gel electrophoresis of 8,000 daltons (191). Studies with yeast mt DNA (177) mt RNA translated in oocytes (192) or E.coli S-30s (193) indicate that such systems can synthesize proteins with antigenic and electrophoretic properties similar to mitochondrial membrane components. Padmanaban *et al* (194) have provided the most impressive demonstration of cell free translation of mt RNA however. RNA purified from isolated mitochondria was used in conjunction with an E.coli S-30 system to produce polypeptides precipitable with cytochrome oxidase antisera, and showing similar mobilities to the three mitochondrially coded subunits of this enzyme. These findings are open to the criticism that when carrier enzyme is used to precipitate *in vitro* products, non specific binding of label to this may occur, and artifactually label cytochrome oxidase as reflected in the subsequent gel profile. Alternative identification techniques, such as peptide mapping, are needed to clarify this.

3(ii) Construction and Use of Strain DL191 in Gene Mapping

The 5 marker strain DL191 was used throughout this study; its construction and genotype is shown in table 3:1. The spontaneous petite retaining the VEN OL_I locus only was selected as described in methods. The maximum region of the genome that could be amplified is shown in figure 3:1. With reference to figures 1:1 and 1:2, this petite lacks several t RNAs, both r RNAs as well as the gene products of OL_{II}, OXI 1, 2 and 3, and VAR 1 and 2. The possibility that other regions are selectively amplified has not been eliminated, but this seems unlikely.

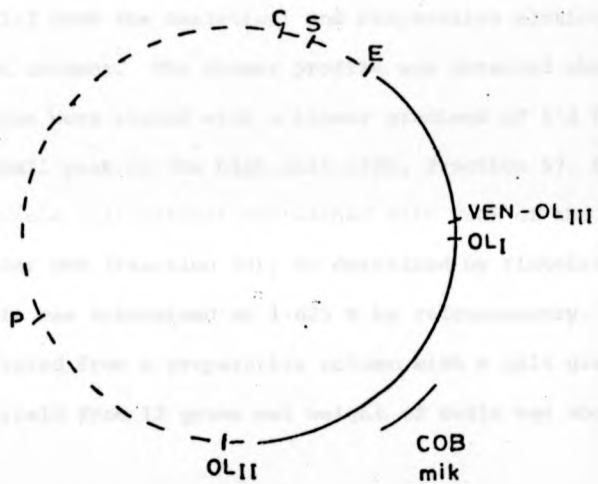


FIGURE 3:1

Region of Deletion of mt DNA in the OL VEN Gene Amplified Petite

The dashed lines indicate the *minimum* region of deletion in a spontaneous petite derived from DI.191 carrying the 5 drug resistance markers shown.

3(iii) Transcription and Translation of mt DNA *in vitro*

Preliminary experiments used in developing a system suitable for gene mapping involved the use of purified mt DNA from DL191. DNA was isolated as described in methods. Of the two procedures described, CsCl centrifugation of whole cell extracts was used mostly as it had the advantage of high yields (up to 100 µg). The PLK method had the advantage of speed, (1 day) and is cheaper. Figures 3:2 and 3:3 show the Analytical and Preparative elution profiles from PLK columns. The former profile was obtained when whole cell extracts were eluted with a linear gradient of 1.4 to 3 M NaCl. The small peak on the high salt side, fraction 57, is mt DNA. The bulk of the whole cell extract was washed with NaCl of the elution molarity of nuclear DNA (Fraction 54), as described by Finkelstein *et al* (176). This was determined as 1.825 M by refractometry. The mt DNA was eluted from a preparative column with a salt gradient as before. The yield from 12 grams wet weight of cells was about 20 µg mt DNA.

Transcription was performed under the same conditions as those of Scragg and Thomas (177). The high KCl concentration necessary for maximum RNA synthesis is possibly a reflection of the unusual base composition of Yeast mt DNA which may alter the binding properties of RNA polymerase. Assay conditions were those described by Hartley *et al* (179) except that the Magnesium concentration was 4 mM and Leucine, ³H or ¹⁴C was used as the labelled amino acid.

In order to analyse *in vitro* translation products, SDS was added to 1% and the disaggregated proteins analysed on Sephadex G-100 columns as described by Küntzel (180). The approach was used because the products directed by yeast mt DNA could be easily distinguished from the high endogenous^o incorporation of the S-30s. To facilitate this further, a double label technique was used in which endogenous synthesis was detected by ¹⁴C Leucine incorporation and endogenous^x by

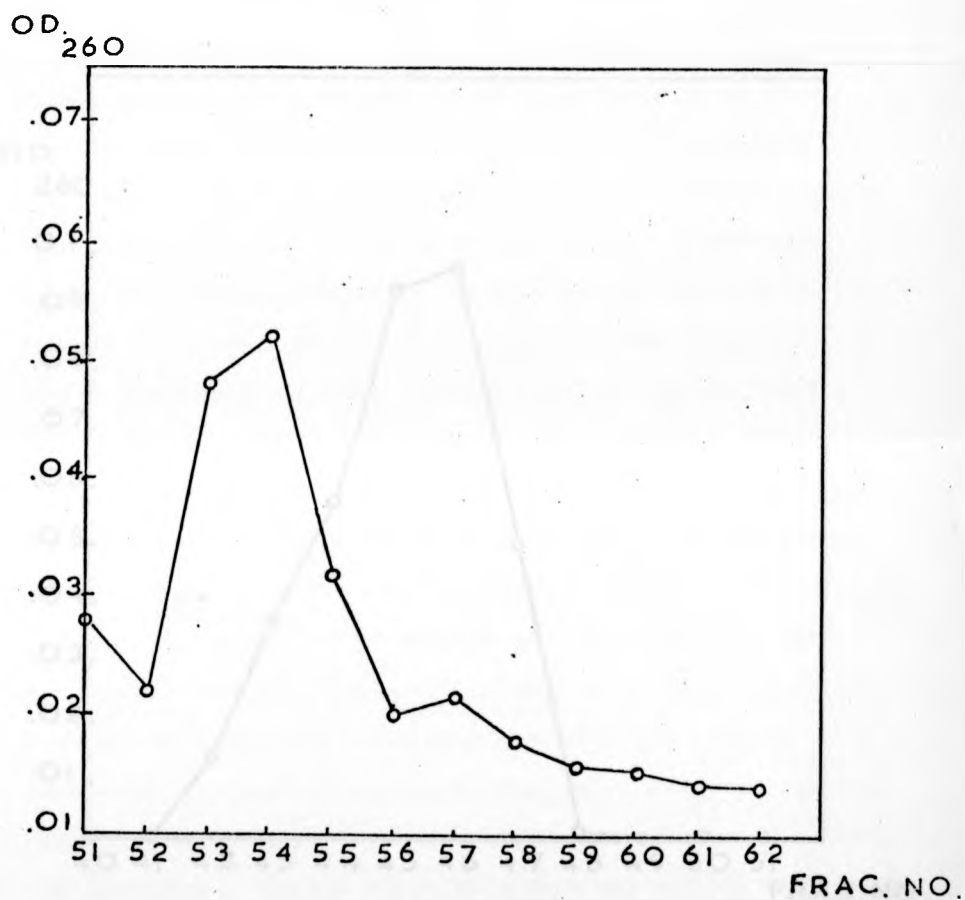


FIGURE 3:2

Analytical PLK Chromatography of Yeast Nucleic Acids

Whole cell extracts were prepared and an aliquot chromatographed on polylysine kieselguhr as described by Finkelstein *et al* (176). Elution was performed with a linear gradient of 1.4 to 3 M NaCl. Fractions were read at 260 nm in a Zeiss spectrophotometer.

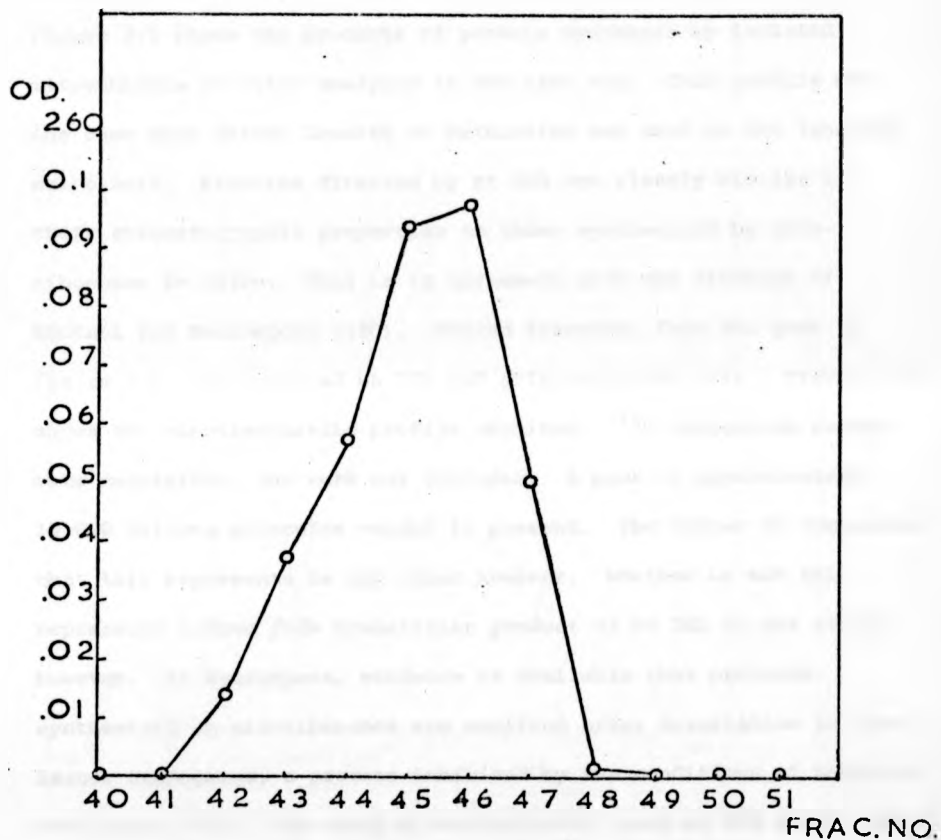


FIGURE 3:3

Preparative PLK Chromatography of mt DNA

Whole cell extracts were processed as described in methods. n DNA was washed out with the NaCl molarity required for its elution as determined in figure 3:2. mt DNA was eluted from PLK columns with a linear gradient of 1.4 to 3 M NaCl.

³H Leucine. Figure 3:4 shows a G-100 profile of products directed by mt DNA *in vitro*. Background incorporation is easily detected where peaks coincide, but a major peak with the same elution properties of cytochrome c is seen centred around fraction 26. Figure 3:5 shows the products of protein synthesis by isolated mitochondria *in vitro* analysed in the same way. This profile was the same when either Leucine or Methionine was used as the labelled amino acid. Proteins directed by mt DNA are clearly similar in their chromatographic properties to those synthesized by mitochondria *in vitro*. This is in agreement with the findings of Küntzel for Neurospora (180). Pooled fractions from the peak in figure 3:4 were analysed on 10% SDS polyacrylamide gels. Figure 3:6 shows the electrophoretic profile obtained. ¹⁴C endogenous counts were negligible, and were not included. A peak of approximately 10,000 daltons molecular weight is present. The number of components that this represents is not clear however. Whether or not this represents a *bona fide* translation product of mt DNA is not clear however. In Neurospora, evidence is available that proteins synthesized on mitoribosomes are modified after translation to form larger aggregates, a process inhibited by the conditions of analysis used here (191). The results obtained with Yeast mt DNA would suggest that the same situation occurs in yeast mitochondria. It is conceivable that small polypeptides represent primary gene products, and that under normal physiological conditions, these can form the known components of the mitochondrial inner membrane. If this is the case, it would appear that transcription and translation are proceeding with fidelity in this system.

In order to be of use in gene mapping, several peaks on gel or column profiles are necessary to correlate loss of proteins with loss of DNA sequences. To overcome the problems of high background

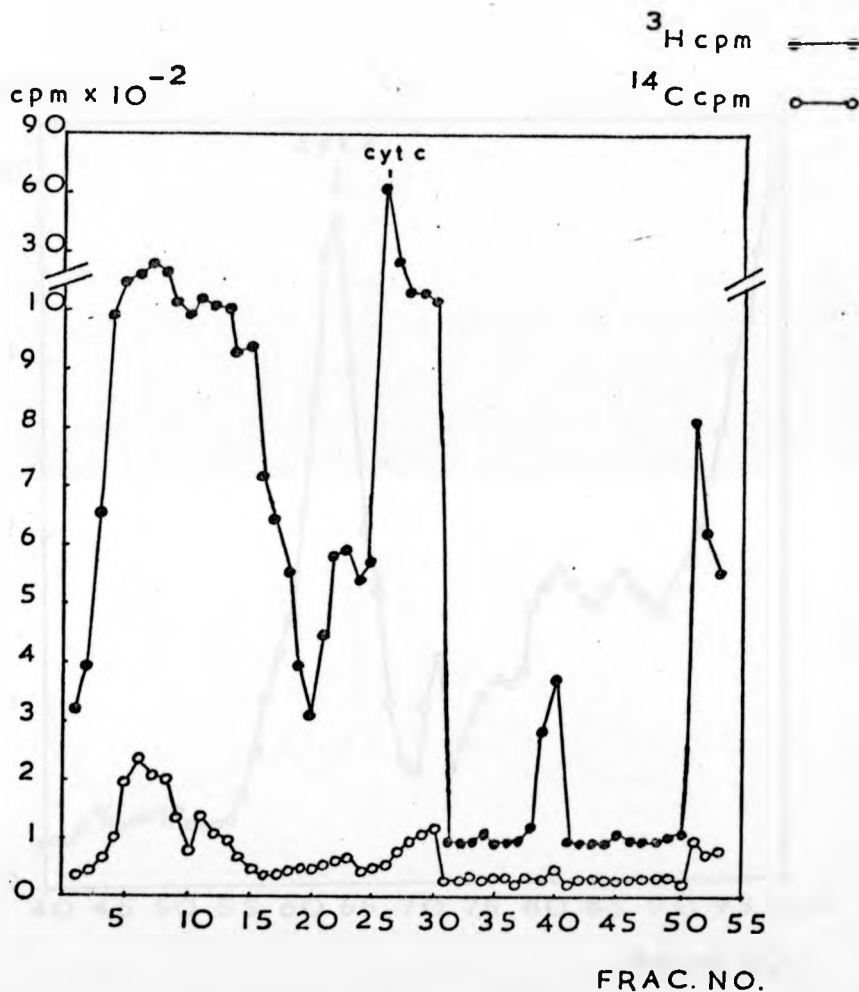


FIGURE 3.4

G-100 chromatography of mt DNA directed translation products

mt DNA from DL191 was transcribed by E.coli RNA polymerase and the products used to direct translation in E.coli S-30s *in vitro*. Controls were labelled with ^{14}C -Leucine, RNA samples with ^3H -Leucine. After incubation at 37°C for 45 minutes, both were mixed, made 1% in SDS, and analysed on a Sephadex G-100 column. 40 drop fractions were collected and counted as in methods. Cytochrome c was included as an internal molecular weight marker.

^{35}S cpm $\times 10^{-3}$

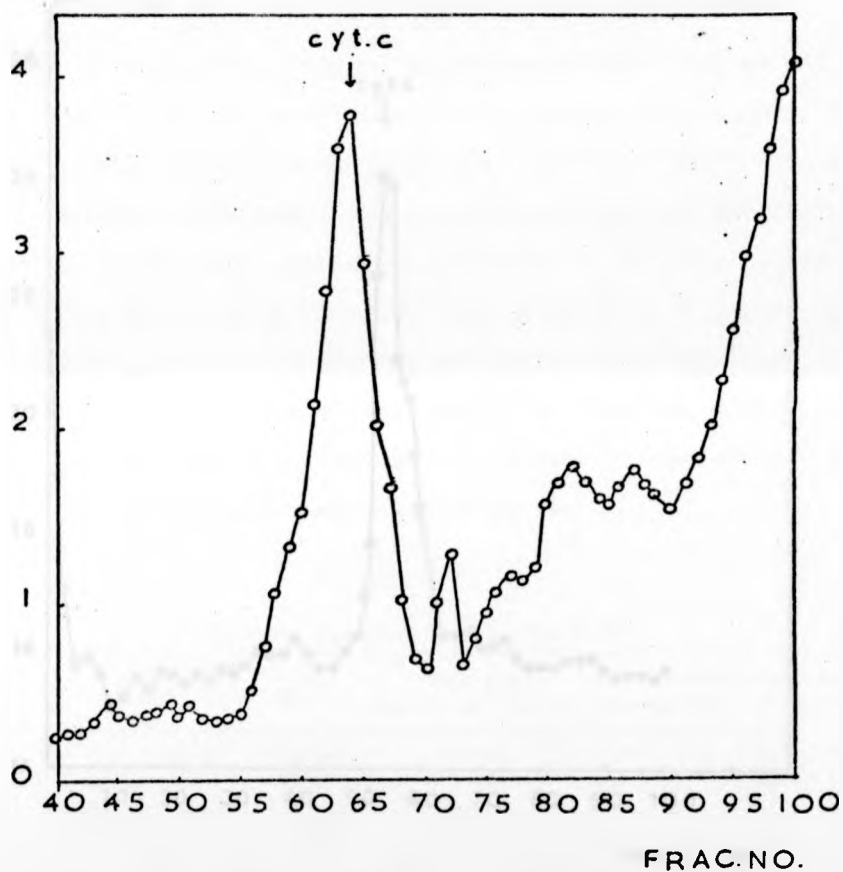


FIGURE 3:5

Analysis of Proteins synthesized by isolated mitochondria
by G-100 gel exclusion chromatography

Mitochondria were labelled with ^{35}S -Methionine as described in methods, made 1% in SDS, and analysed as in the legend to figure 3:4.

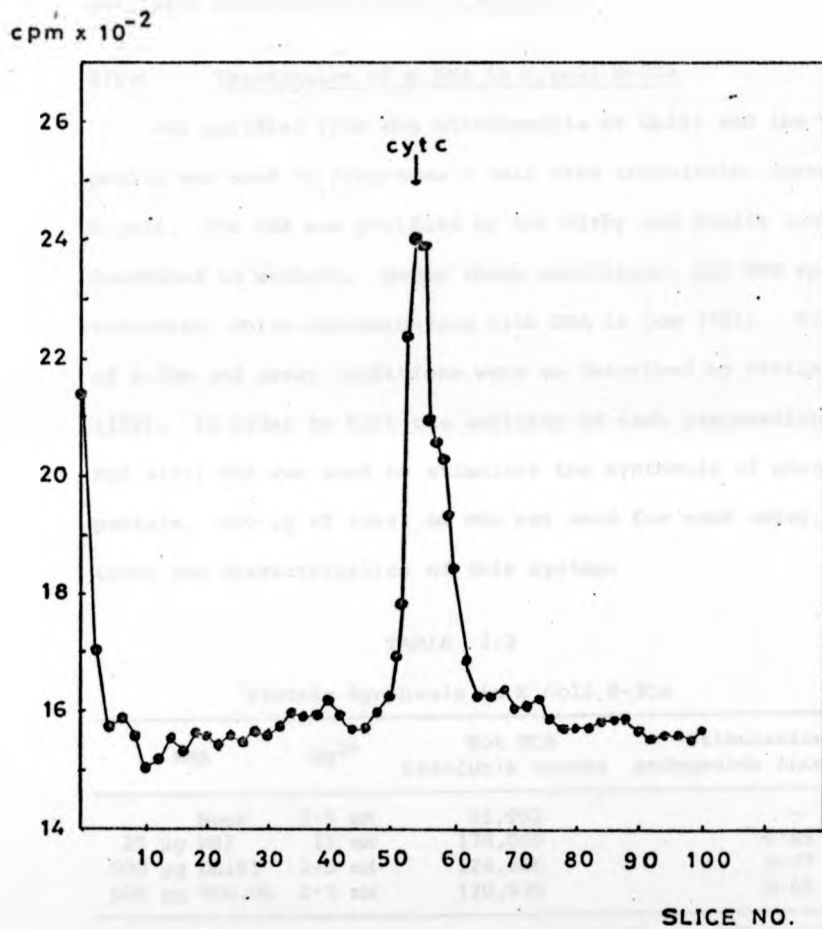


FIGURE 3:6

Gel Electrophoresis of G-100 column fractions from
mt DNA directed translation assays

The major peak shown in figure 3:4 was pooled, dialysed against H₂O and lyophilized. Electrophoresis was performed in 10% SDS polyacrylamide disc gels. Cytochrome c was included as an internal molecular weight standard. 1 mm slices were cut, and radioactivity analysed as in methods.

incorporation which necessitated the chromatographic approach to products analysis, alternative cell free systems were investigated utilizing purified mt RNA as template.

3(iv) Translation of m RNA in E.coli S-30s

RNA purified from the mitochondria of DL191 and the VEN, OL petite was used to programme a cell free translation system from E.coli. The RNA was purified by the Kirby and Parish technique as described in methods. Under these conditions, all RNA species are extracted, while contamination with DNA is low (181). Preparation of S-30s and assay conditions were as described by Hartley *et al* (179). In order to test the activity of each preparation, 25 µg of MS2 viral RNA was used to stimulate the synthesis of phage coat protein. 500 µg of total mt RNA was used for each assay. Table 3:2 shows the characteristics of this system:

TABLE 3:2

Protein Synthesis in E.coli S-30s

RNA	Mg ²⁺	Hot TCA insoluble counts	Stimulation over endogenous incorporation
None	2.5 mM	25,992	-
25 µg MS2	11 mM	178,080	6.85
500 µg DL191	2.5 mM	124,056	4.77
500 µg VEN,OL	2.5 mM	120,939	4.65

250 µl reaction mixtures containing 20 µCi ³⁵S-Methionine were incubated at 37 C for 45 minutes and treated as in methods. Counts refer to total assays, and are corrected for background.

Samples were analysed on 10-15% gradient slab gels and radioactive proteins detected by autoradiography. Figure 3:7 shows an autoradiogram of proteins synthesized in the presence and absence of DL191 RNA. All the bands detected were found in samples with and without RNA indicating that no new polypeptides were being directed by mt RNA in this system. Furthermore, the addition of exogenous RNA appears to stimulate endogenous incorporation by the amounts shown in

FIGURE 3:7

Translation of mt RNA in E.coli S-30s

E.coli S-30s were prepared as in methods and incubated with 500 µg total DL191 mt RNA and 20 µCi ³⁵S-Methionine. After incubation at 37°C for 45 minutes, proteins were precipitated with 10% TCA + 0.5 mg/ml Methionine, boiled for 10 minutes and kept on ice. After dissolution in SDS buffer, samples were analysed on a 10-15% gradient slab gel. After five hours at 30 mA, the gel was fixed in 50% TCA, washed in 7% Acetic acid, and dried. Autoradiography was for 2 days with RP/RZ X-ray film.

- A. ~~NO~~ RNA.
- B. + mt RNA.

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Table 3:2. This effect has been observed with studies of globin messenger translation under certain conditions, and illustrates the fact that TCA precipitable counts alone cannot be used as an indication of messenger activity (195). Using a similar approach to gene mapping, namely the translation of yeast mt RNA from a gene amplified petite *in vitro*, Halbreich *et al* (196) have also used a system derived from E.coli. Total mt RNA, from a petite carrying oligomycin resistance genes, was translated in a purified ribosomal system. This system is different from the one described above, which could reflect in the different magnesium optimum, 12.5 mM, described by these authors. Electrophoretic analysis revealed broad peaks from 12 to 30,000 daltons seen in both endogenous and exogenous profiles. In order to demonstrate synthesis of polypeptides directed by mt RNA, a "difference spectrum" was constructed between the two profiles. The presence of a large background makes interpretation of this data uncertain, since it is not absolutely clear that addition of mt RNA has not stimulated endogenous incorporation as the system described above. No further identification of "products" was made, so that the fidelity of translation remains an open question.

3(v) Injection of RNA into Xenopus oocytes

The system in which foreign macromolecules are microinjected into viable oocytes has proved invaluable as a tool for the study of animal development, and, to a lesser extent, the activity of messenger RNA (197). Eggitt and Scragg (192) have reported that total mt RNA injected into Xenopus oocytes can direct the synthesis of proteins precipitable with antisera to whole mitochondrial membranes. DL191 mt RNA, prepared by the method of Penman (182), was injected into oocytes and incubated overnight in the presence of ³⁵S-Methionine. Oocytes were processed as described in methods. Table 3:3 shows the characteristics of this system:

TABLE 3:3

³⁵S-Methionine Incorporation into Oocytes Injected with DL191 mt RNA

RNA	Hot TCA insoluble counts/oocyte	Depression of synthesis below endogenous
None	760,000	-
5 mg/ml DL191	423,000	1.8
1 mg/ml DL191	380,000	2.0

30 oocytes were injected with 50 nl 0.1 M KCl containing RNA as indicated. After incubation with 50 μ Ci ³⁵S-Methionine overnight, oocytes were homogenized in electrophoresis sample buffer and counted as described in methods. Counts are taken to the nearest round figure.

The oocyte system is unusual in that the background is very high and the synthesis of proteins directed by m RNA is reflected in a depression of TCA insoluble counts (198).

Oocytes incubated with and without mt RNA were analysed on 10-15% gradient slab gels as described in methods. Figure 3:8 shows the profiles obtained. No new polypeptides were detectable in samples injected with RNA as indicated by the similarity of all profiles. The overloading artifact near the top of the gel is due to the oocyte yolk protein present in large amounts. In order to detect mitochondrial proteins masked by the high background of oocyte proteins, oocytes were lysed in the non ionic detergent NP40 and reacted with antiserum to cytochrome oxidase. The three high molecular weight subunits of this enzyme are probably encoded by mt DNA (135). Table 3:4 shows the relative amounts of radioactivity precipitated by the double antibody technique described in methods:

TABLE 3:4

Precipitation of Oocyte Proteins with Antisera to Cytochrome Oxidase

RNA	³⁵ S counts in precipitate	% Total counts in oocyte
None	139,000	18.3
5 mg/ml DL191	121,080	28.6
1 mg/ml DL191	145,800	38.3

10 oocytes were homogenized in 100 μ l of 1% NP40 in PBS and reacted with 20 μ l Cytochrome oxidase antisera, followed by 200 μ l

FIGURE 3:88

Products of Protein Synthesis in Xenopus Oocytes

30 eggs were injected with 50 ml of 1 and 5 mg/ml DL191 mt RNA and incubated at 30° C overnight with 50 μ C₁ ³⁵S-Methionine. 2 eggs were homogenized in electrophoresis sample buffer for direct analysis of labelled proteins. 10 oocytes were lysed in 1% NP40 in PBS and reacted with cytochrome oxidase antiserum as described in methods. Both samples were run on 10-15% gradient slab gels and samples with and without RNA compared. Gels A and B represent whole proteins and antisera precipitates of oocytes incubated without RNA. Gels of mt RNA injected oocytes were identical in both cases.

FIGURE 3:98

Products of Protein Synthesis in Xenopus Oocytes

30 eggs were injected with 50 ml of 1 and 5 mg/ml DL191 mt RNA and incubated at 30°C overnight with 50 μ Ci 35 S-Methionine. 2 eggs were homogenized in electrophoresis sample buffer for direct analysis of labelled proteins. 10 oocytes were lysed in 1% NP40 in PBS and reacted with cytochrome oxidase antiserum as described in methods. Both samples were run on 10-15% gradient slab gels and samples with and without RNA compared. Gels A and B represent whole proteins and antisera precipitates of oocytes incubated without RNA. Gels of mt RNA injected oocytes were identical in both cases.

a



b



-

+

Goat AntiRabbit antiserum. After standing at 4°C overnight, precipitates were washed 4 x with PBS and taken up in 100 µl electrophoresis sample buffer.

The high percentage of radioactivity in the samples without RNA indicate non specific precipitation of labelled proteins with this immunoprecipitation technique. Antisera precipitates were run on 10-15% gradient slab gels as before: Figure 3:8 shows the result obtained. Although the precipitates were extensively washed to remove non specifically bound proteins, the number of bands is too high to make identification of mitochondrial proteins possible. This disappointing finding suggests that a) the amount of translatable message in total mt RNA preparations is too small to compete with endogenous oocyte synthesis, b) the mitochondrial message cannot be translated on oocyte cytoplasmic ribosomes, and may need to enter mitochondria for this, and c) the cytochrome oxidase subunits represent a small proportion of total mitochondrial proteins, and may be present in amounts too small to be detected by immunoprecipitation.

The results are in contradiction to those of Eggitt and Scragg who have detected differences between antisera precipitates of oocytes injected with and without RNA. Their criterion of synthesis was the detection of proteins precipitable with antisera to whole mitochondrial membranes, and therefore argument (c) above may be applicable. In the absence of clearly defined proteins directed by mt RNA, this system is unsuitable for gene mapping studies.

3(vi) Isolation of Poly (A) containing mt RNA

A characteristic of eukaryotic messenger RNA is the presence of a poly (A) sequence at the 3' end. The number of poly (A) residues varies from about 200 in higher animals to 50 in yeast cytoplasmic polysomal RNA (199). All known eukaryote m RNAs, with the exception of Histone messenger, have been shown to possess these sequences. The

detection of mitochondrial poly (A) containing RNA has been reported for animal and insect systems (184). In having only about 50 poly (A) residues compared to the 200 of their cytoplasmic counterparts, these are readily distinguishable by gel electrophoresis of RNAase resistant sequences. Lower eukaryotes, such as *Trichoderma viride* and *Saccharomyces*, possess much shorter sequences of approximately 20 to 30 poly (A) residues (184,200). Detection of such short sequences requires special chromatographic conditions, which is why earlier reports have failed to detect poly (A) containing RNA in Yeast mitochondria (192, 201). Affinity chromatography of total mt RNA on poly U Sepharose (200) or oligo dT cellulose at 4°C, (184) is capable of purifying mt RNA fractions active in directing protein synthesis *in vitro* (184, 194). Total mt RNA from DL191 was fractionated on oligo dT cellulose as described by Rosen and Edelman (184). RNA pulse labelled with $^{32}\text{PO}_4$ for 25 minutes was analysed in this way as described in methods. Figure 3:9 shows the elution profiles of rapidly labelled RNA bound and unbound to oligo dT cellulose at 4°C. About 20% of the radioactivity is associated with bound RNA, presumably representing poly (A) containing material. Peak fractions of both species were analysed on 2.2% Acrylamide, 0.5% Agarose composite gels. Figures 3:10 and 3:11 show the electrophoretic profiles of unbound and bound fractions respectively. 28S and 4S refer to yeast large subunit r RNA and E.coli t RNA external mobility standards. Both profiles show a broad distribution of poorly resolved peaks. The poly (A) containing RNA appears to be resolved into at least eight peaks distributed over a large size range, (4-28S). The unbound RNA distribution is skewed towards the higher molecular weight range, presumably representing in part, ribosomal RNA precursors.

The percentage of m RNA in total RNA is about 1-3% (199). The

$^{32}\text{Pcpm} \times 10^{-3}$

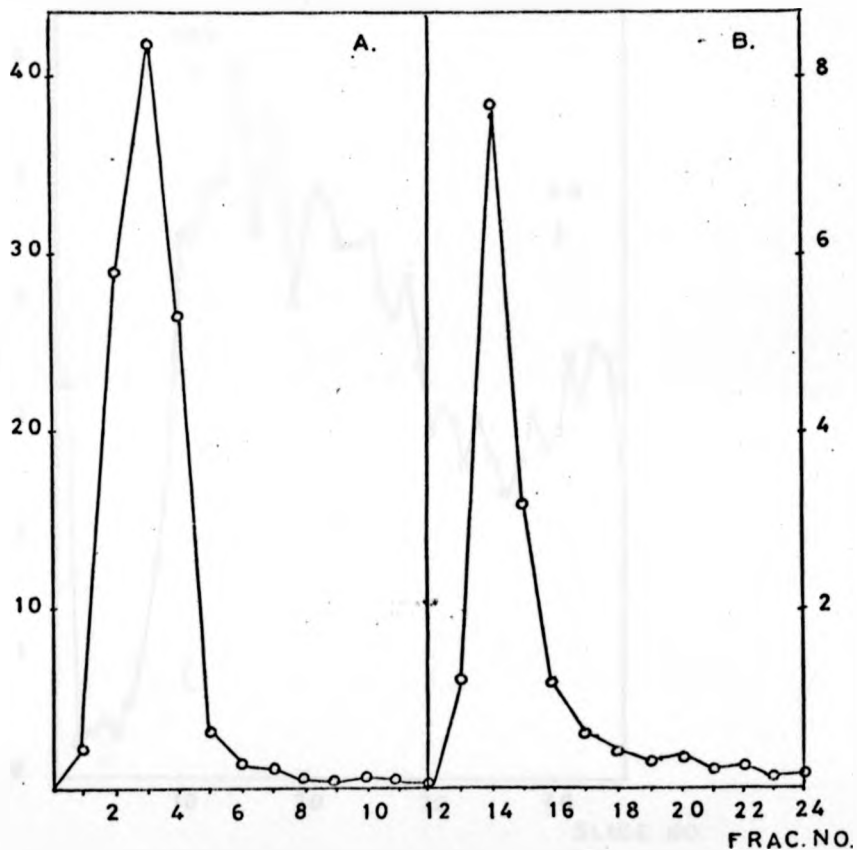


FIGURE 3:9

Analysis of Rapidly Labelled DL191 mt RNA by oligo dT Cellulose Affinity Chromatography

250 ml cultures were pulse labelled with $4 \text{ mCi } ^{32}\text{PO}_4$ for 25 minutes. mt RNA was isolated as described in Methods. RNA lacking poly (A) was eluted from a 3 ml column of oligo dT cellulose with 0.5 M KCl , 10 mM Tris Cl pH 7.5. Poly (A) containing RNA was eluted with 10 mM Tris Cl , $0.2\% \text{ SDS}$ pH 7.5. Fractions were counted as in methods.

A. RNA eluted with 0.5 M KCl buffer. B. Poly (A) containing RNA.

^{32}P cpm $\times 10^{-3}$

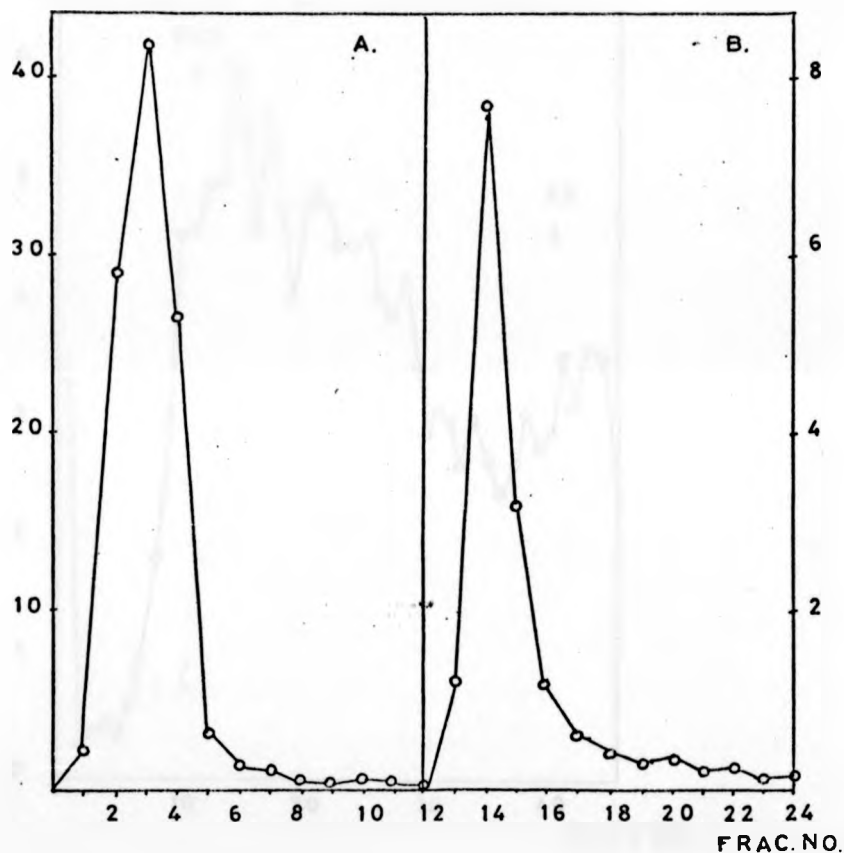


FIGURE 3:9

Analysis of Rapidly Labelled DL191 mt RNA by oligo dT
Cellulose Affinity Chromatography

250 ml cultures were pulse labelled with 4 mCi $^{32}\text{PO}_4$ for 25 minutes. mt RNA was isolated as described in Methods. RNA lacking poly (A) was eluted from a 3 ml column of oligo dT cellulose with 0.5 M KCl, 10 mM Tris Cl pH 7.5. Poly (A) containing RNA was eluted with 10 mM Tris Cl, 0.2% SDS pH 7.5. Fractions were counted as in methods.

A. RNA eluted with 0.5 M KCl buffer. B. Poly (A) containing RNA.

cpm x 10⁻²

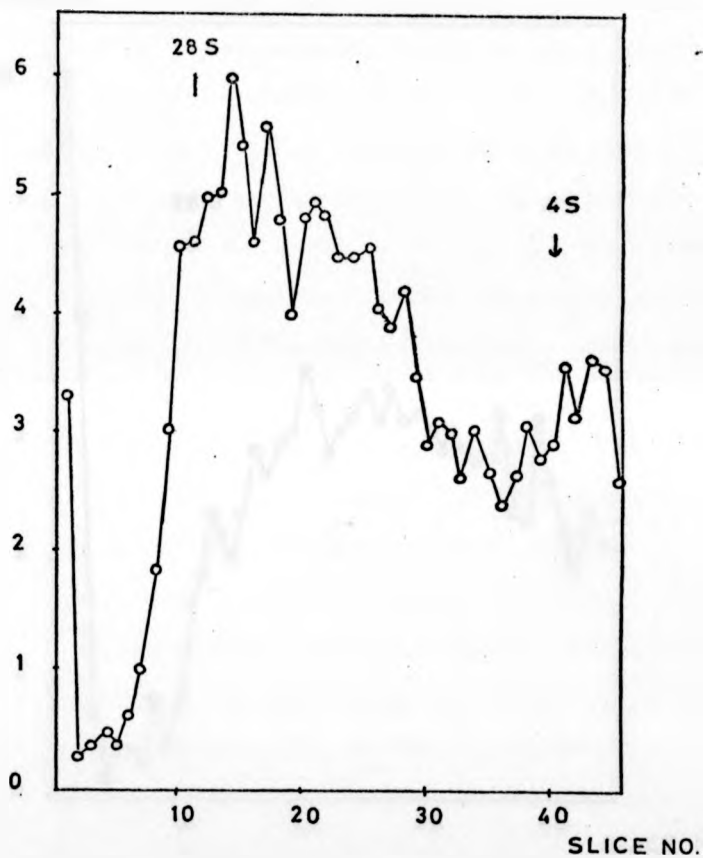


FIGURE 3:10

Gel Electrophoresis of ³²PO₄ labelled RNA lacking poly. (A)

RNA fractions, bound to oligo dT cellulose in high salt, were pooled and analysed in 2.2% Acrylamide, 0.5% Agarose composite gels. Electrophoresis was for 4 hours at 10 mA per gel. Radioactivity was determined in 2 mm slices as described in methods. Yeast r RNA and E.coli t RNA were run in parallel gels and scanned at 260 nm.

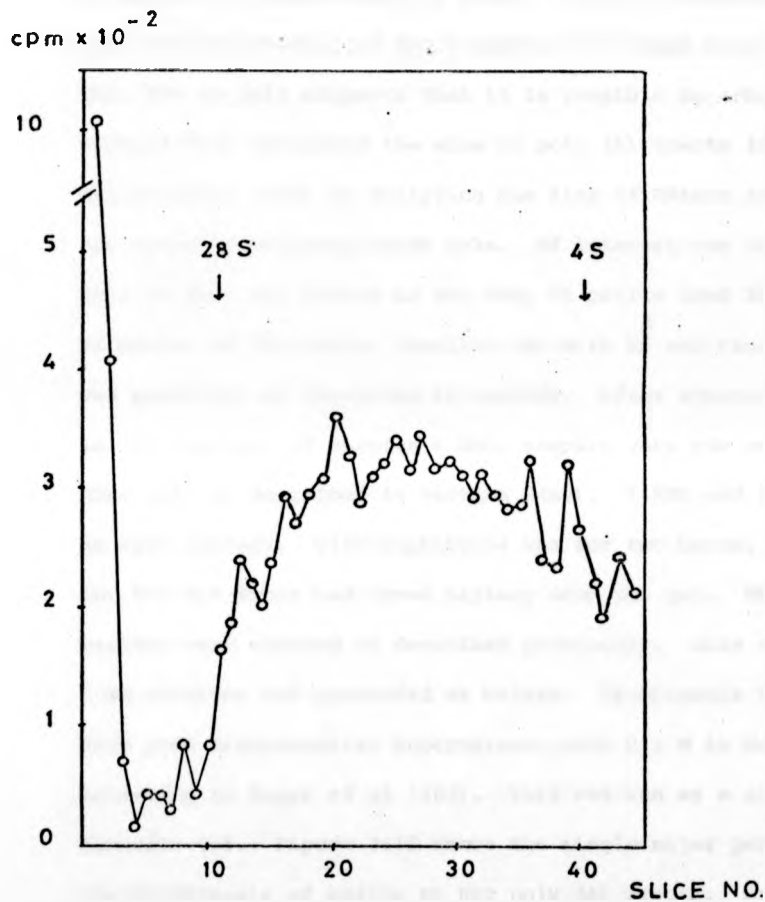


FIGURE 3:11

Gel Electrophoresis of ³²PO₄ poly (A) containing RNA

RNA fractions eluted from oligo dT cellulose columns with 10 mM Tris Cl 0.2% SDS pH 7.5, were pooled and electrophoresed as indicated in the legend to figure 3:10.

above experiments were designed to test the feasibility of purifying m RNA fractions from total mt RNA and thereby improving the efficiency of translation *in vitro*. The high percentage of rapidly labelled RNA bound, and the presence of a broad size distribution of this RNA on gels suggests that it is possible to achieve this. Other workers have estimated the size of poly (A) tracts in grande yeast mitochondria (200) by analysing the size of RNAase resistant RNA on low porosity polyacrylamide gels. Of interest was the presence, if any, of poly (A) tracts in the VEN₁ OL petite used in this study. Digestion of ³H-Adenine labelled RNA with ¹T₁ and Pancreatic RNAases was performed as described in methods. After ethanol precipitation in the presence of carrier t RNA, samples were run on 10% polyacrylamide disc gels as described in section 2(xv). t RNA and rA₄ were included as size markers. Electrophoresis was for two hours, after which time the BPB dye front had moved halfway down the gel. Molecular size markers were scanned as described previously. Gels were sliced into 2 mm sections and processed as before. Cytoplasmic RNA was prepared from post mitochondrial supernatants made 0.1 M in NaCl and treated according to Meyer *et al* (183). This was run as a size marker on a separate gel. Figure 3:12 shows the single major peak obtained on electrophoresis of petite mt RNA poly (A) tracts. On the basis of their mobility relative to the RNA markers, these tracts are very short, being about 10 residues. This result suggests that petite mt RNA, like that grande from which it was derived, contains RNA with the properties of m RNA, and should therefore be capable of directing cell free protein synthesis.

3(vii) *In vitro* Translation of mt messenger RNA in a Wheat Germ Cell free System

Protein synthesis in wheat germ extracts has several advantages over other cell free systems. Several viral and mammalian m RNA

cpm x 10⁻²

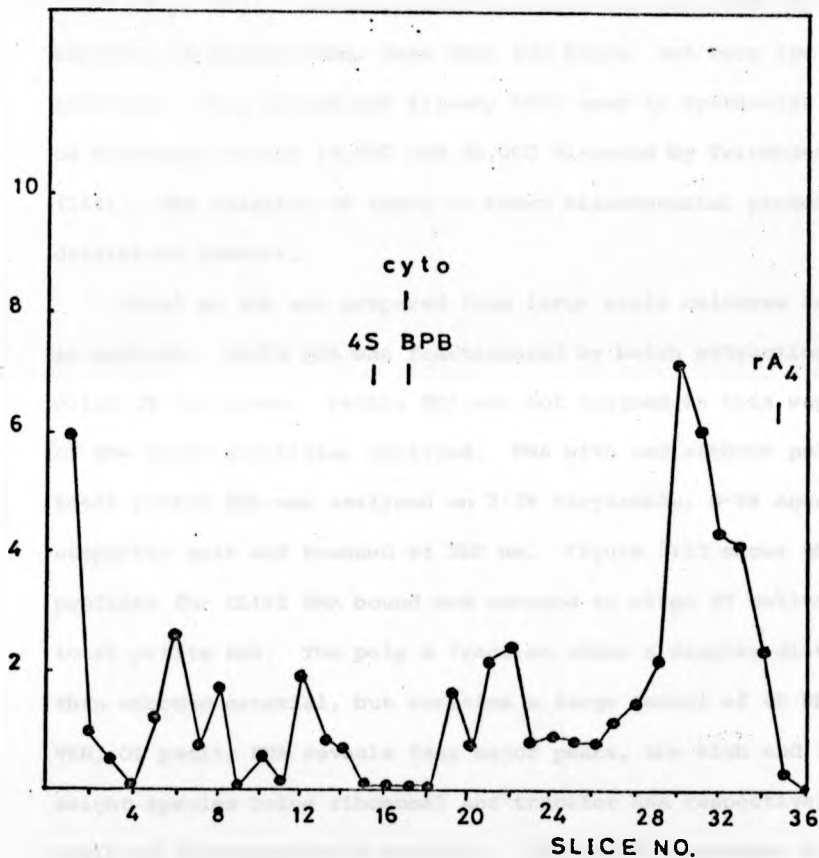


FIGURE 3:12

Electrophoresis of RNAase Resistant ³H-Adenine labelled RNA from a Gene Amplified petite

The VEN, OL petite was labelled for 2.5 hours with 25 μ C₁/ml of ³H-Adenine. mt RNA was extracted and fractionated into a poly (A) rich fraction on oligo dT cellulose. Digestion with Pancreatic and TI RNAases was as described in methods. Electrophoresis was performed in 10% SDS polyacrylamide gels for 1.5 hours at 10 mA. 2 mm slices were cut and counted as before. E.coli t RNA, rA₄ and ³H-Adenine labelled cytoplasmic RNA were run as mobility standards. BPB refers to the bromophenol blue dye front.

fractions have been used to direct the synthesis of authentic polypeptides with high efficiency; furthermore, messenger specificity is not rigorous, so that even RNA from the coliphage Q β can be translated (202). These systems are also characterized by ease and rapidity of preparation, less than two hours, and very low endogenous activity. This system has already been used to synthesize proteins of molecular weight 13,000 and 20,000 directed by *Trichoderma* mt RNA (184). The relation of these to known mitochondrial proteins was not determined however.

Total mt RNA was prepared from large scale cultures as described in methods. DL191 RNA was fractionated by batch extraction onto oligo dT cellulose. Petite RNA was not treated in this way because of the small quantities obtained. RNA with and without poly (A), and total petite RNA was analysed on 2.2% Acrylamide, 0.5% Agarose composite gels and scanned at 260 nm. Figure 3:13 shows absorbance profiles for DL191 RNA bound and unbound to oligo dT cellulose, plus total petite RNA. The poly A fraction shows a simpler distribution than unbound material, but contains a large amount of 4S RNA. Total VEN, OL petite RNA reveals four major peaks, the high and low molecular weight species being ribosomal and transfer RNA respectively on the basis of electrophoretic mobility. The r RNA represents a degree of cytoplasmic contamination since no mitochondrial r RNA is present in this petite. The two other peaks plus several minor ones could represent messenger RNA species.

Wheat germ extracts prepared by the method of Marcu and Dudock (187) were programmed with Vesicular Stomatitis viral RNA (VSV RNA) to assess the fidelity of translation. Table 3:⁵ shows the incorporation of ³⁵S-Methionine obtained with VSV and mt RNA:

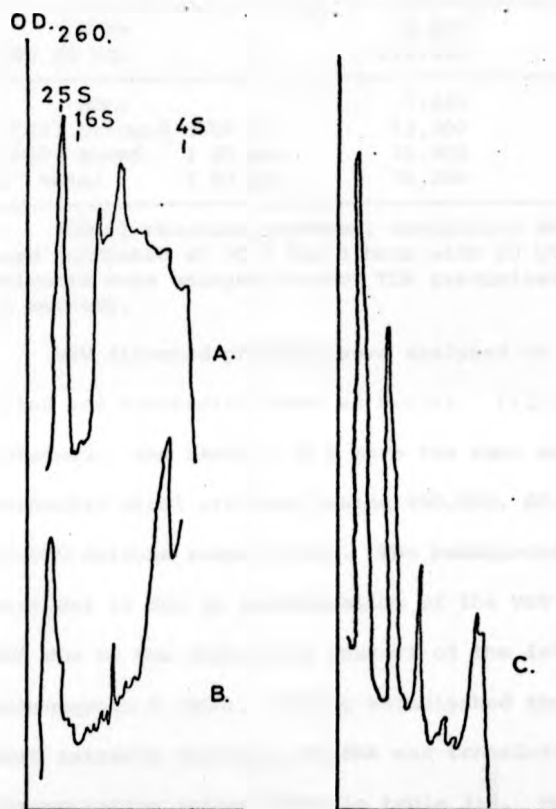


FIGURE 3:13

Gel Electrophoresis of RNA used for *in vitro* translation

Mitochondria were isolated from 15 litre cultures and washed 3 times as in methods. RNA was isolated by the method of Meyer *et al* (183). DL191 RNA was fractionated into poly (A) rich and deficient fractions by batch extraction on oligo dT cellulose. VEN, OL ρ^- RNA was used *in toto*. Aliquots were analysed on 2.2% Acrylamide, 0.5% Agarose composite gels and scanned at 260 nm. Yeast cytoplasmic and E.coli t RNA were run as external standards. A. DL191 poly (A) deficient RNA. B. DL191 poly (A) rich RNA. C. Total petite RNA.

TABLE 3:5

Protein Synthesis in Wheat Germ Extracts

RNA	Hot TCA Insoluble counts/assay	Stimulation over endogenous incorporation
None	4,020	-
VSV (5 µg)	178,420	44.3
None	7,480	-
DL191 Unbound (200 µg)	13,300	1.8
DL191 Bound (20 µg)	25,480	3.4
p ⁻ total (50 µg)	76,200	10.2

100 µl reaction mixtures, containing RNA as indicated in the table, were incubated at 30 °C for 1 hour with 20 µC ³⁵S-Methionine. 5 µl aliquots were assayed for hot TCA precipitable counts as described in methods.

VSV directed products were analysed on 10-15% gradient slab gels, dried and autoradiographed as before. Figure 3:14 shows the profile obtained: the bands A to E have the same molecular weights as the authentic viral proteins, being 190,000, 60,000, 55,000, 50,000 and 30,000 daltons respectively. The background of several minor polypeptides is due to contamination of the VSV RNA preparation with HeLa RNA due to the incomplete shutoff of the latter's synthesis with Actinomycin D (203). Having established the efficiency of the wheat germ extracts employed, mt RNA was translated *in vitro* with the incorporation values shown in table 3:⁵. Magnesium and Potassium were used at concentrations found optimal for VSV translation (2.5 and 60 mM respectively). No optimization of these or other parameters such as pH and RNA concentration was made. Under these conditions, poly A lacking RNA stimulates poorly by a factor of 1.8 less than poly A directed incorporation. Values of poly (A) RNA stimulated protein synthesis normally agreed to within 11% in different assays, while poly (A) deficient values varied by about 25%. The high levels of stimulation by petite mt RNA may be due to a) assay conditions favouring the translation of this particular RNA species preferentially, b) the relative lack of inhibitory non translatable RNA (r RNA, t RNA), or c) the amplification of m RNA sequences and the resulting high

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FIGURE 3:14

Translation of Vesicular Stomatitis Viral RNA in a Wheat Germ System

~5 μg VSV RNA was used to direct protein synthesis in 100 μl reaction mixtures containing a Wheat germ S-30 system, 20 μCi ^{35}S -Methionine, salts and nucleotides as indicated in methods. Incubation was for 1 hour at 30 C, followed by precipitation of proteins with TCA and electrophoretic analysis on 10-15% gradient slab gels as before. Gels were stained to detect molecular weight standards, dried and autoradiographed. Background incorporation was insignificant and is not shown.

$M_r \times 10^{-3}$

190

60
55
50

30



-a

-b

-c
-d

-e

+

proportion of m RNA in the total RNA fraction. From the gel profiles of mt RNA used in this work, b) and c) appear the most likely possibilities.

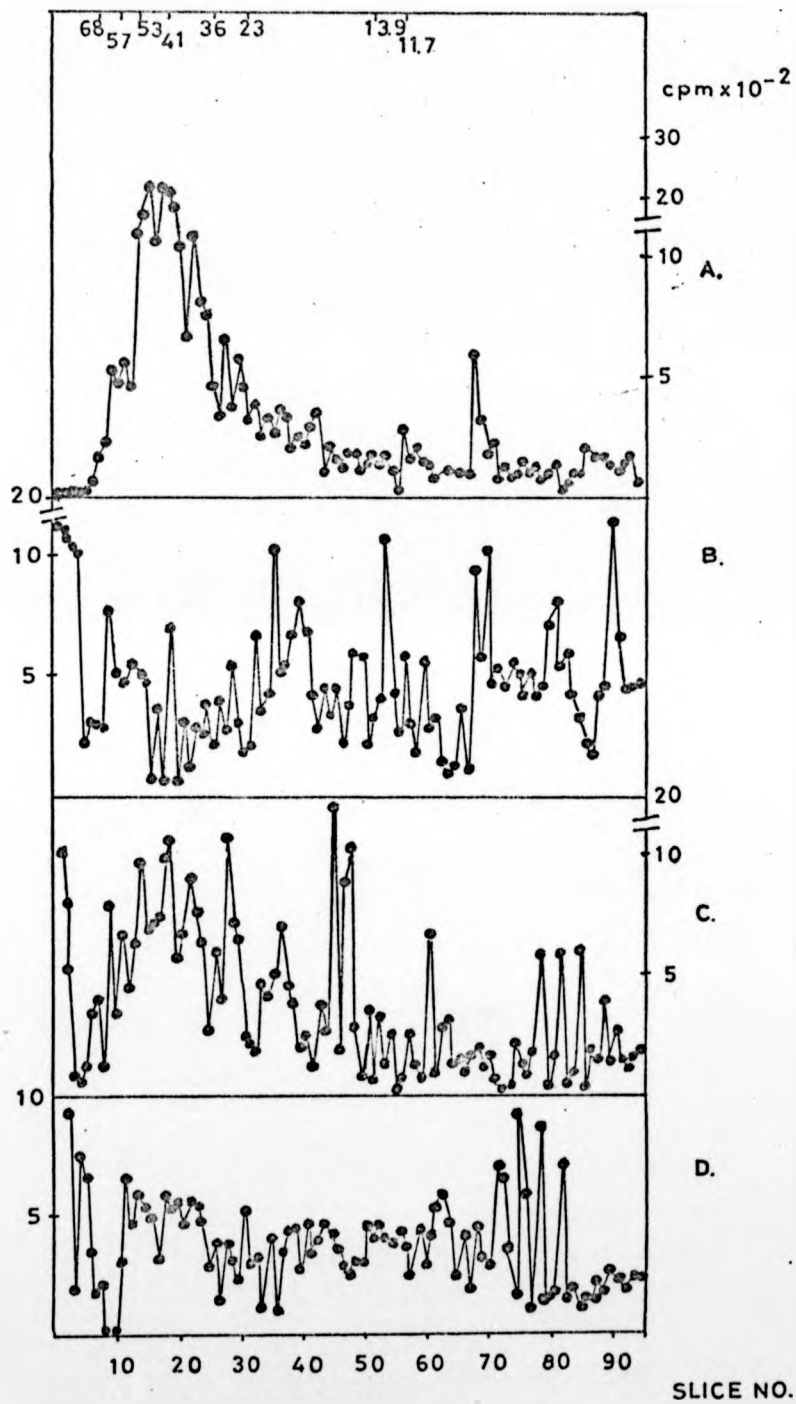
In order to detect small amounts of protein synthesized *in vitro* in the absence of high total counts, reaction mixtures were analysed on 15% disc gels, sliced and counted as in methods. Figure 3:15 shows the results obtained for all three RNA fractions including the control without added RNA. Examination of the *in vitro* products directed by bound and unbound DL191 RNA reveals the synthesis of several major proteins not present in the control minus RNA. Thus unbound RNA has stimulated the synthesis of proteins of 64,000, 24,000, 21,000, 13,000 and less than 10,000 molecular weight which are distinguishable from the endogenous background. In all profiles, the proteins with a molecular weight less than 10,000 have been ignored. Poly (A) containing RNA stimulates the synthesis of at least 10 proteins with a major group in the molecular weight range 23 to 60,000, plus two large peaks at 17 and 16,000 daltons. Of interest is the comparatively simple range of products directed by the petite RNA with peaks ranging from 36 to 53,000 daltons. This suggests that deletion of mitochondrial genes can be demonstrated *in vitro* using the system described above. Although these proteins have not been characterized by other techniques such as antibody precipitation or peptide mapping, the following is noteworthy. The OL_I locus present in DL191 and the petite is phenotypically characterized as a protein of molecular weight 45,000 daltons as described in Chapter 4. Peaks of this molecular weight are present in both grande and petite *in vitro* products as would be expected if the OL_I locus is retained in the latter. In addition to the OL_I locus, the COB region described by Tzagoloff (145) may be present in this petite. The association of COB with a protein of 30,000 molecular weight has been suggested (138, 139). Minor peaks of this size are present in the poly (A)

FIGURE 3:15

In vitro Translation of mt RNA in a Wheat Germ Cell free System

DL191 RNA was fractionated into poly (A) rich and poly (A) deficient fractions as described in methods. Total mt RNA from the petite carrying the VEN OL_T locus was used *in toto*. Wheat germ extracts were used to translate each RNA fraction. 100 μ l assays were prepared as in the text, with 20 μ C, ³⁵S-Methionine as labelled amino acid. Incubation was for 1 hour at 30°C. Proteins were precipitated with 10% TCA + 0.5 mg/ml Methionine. Translation products were analysed on 15% SDS polyacrylamide disc gels with a stacking gel as for slab gels (127). After electrophoresis, gels were sliced into 1 mm sections and counted as before. Molecular weight standards are shown at the top of the diagram.

A. VEN, OL ρ^- RNA. B. DL191 poly (A) deficient RNA.
C. DL191 poly (A) RNA. D. - RNA.



and petite gel profiles.

The wheat germ system described above appears to be the most satisfactory method of translating mitochondrial m RNAs. The broad distribution of proteins synthesized, and the simpler pattern obtained with petite RNA suggests that this system could be of use in gene mapping studies. The following experiments are necessary for a thorough evaluation however: a) conditions of protein synthesis must be optimized for maximum incorporation of labelled amino acid. The addition of spermine is found to stimulate this using other m RNAs (187). In this system however, spermine at 30 µg/ml stimulated endogenous incorporation by about 60%, but had no effect on exogenous messenger stimulation. b) The exact relation of the *in vitro* products to known mitochondrial proteins must be determined for the system to be of use in gene mapping. c) Petites with deletions in other parts of the mt DNA should be used to compare the range of products synthesized with those described here. In addition to gene mapping studies, the mechanisms of mitochondrial gene expression and its control could be elaborated as has been the case with other *in vitro* systems.

3(viii) Transcription and Translation of omicron DNA *in vitro*

In chapter 2, the relation of o DNA to mitochondrial drug resistance markers was discussed. If this DNA species contains genetic information, it should be possible to direct protein synthesis *in vitro* using purified DNA.

The transcription/translation system described for mt DNA was used. Transcription conditions were altered so that the final KCl concentration was 0.15 M. This was chosen because maximal reinitiation occurs with other DNAs of this base composition, including yeast nuclear DNA (177). The kinetics of transcription were studied using 5 µg o DNA in a 250 µl reaction mixture as described in methods.

Figure 3:16 shows the time course of incorporation of ^3H -UTP into cold TCA precipitable counts. Transcription plateaus after about 45 minutes, this time being used for subsequent translation assays. After precipitation with carrier t RNA, RNA transcribed from o DNA was translated in a cell free system from E.coli as for mt DNA.

Table 3:5 shows the characteristics of this system:

TABLE 3:6

Translation of RNA Transcribed from o DNA *in vitro*

RNA	Hot TCA Incorporation counts/250 μl	Stimulation over endogenous
None	80,600	-
o DNA transcript	152,175	1.88

5 μg o DNA was transcribed as described in methods. RNA was precipitated with carrier t RNA (1 mg/ml) and used to programme an *in vitro* ribosomal system derived from E.coli. 5 μl aliquots were assayed for ^{35}S -Methionine incorporation as described, and the remained prepared for electrophoresis.

The relatively low incorporation observed may be due to inefficient transcription of o DNA, or lack of optimization of the conditions necessary for efficient translation. Assays agreed to within 2%. Figure 3:17 shows the gel profile of *in vitro* translation products. The high background and low stimulation levels are reflected in the similarity of almost all the peaks between endogenous and exogenous profiles. Two peaks are seen in the latter however, corresponding to molecular weights of 45,000 and 22,500 daltons respectively. The relation of these to mitochondrial inner membrane proteins is unknown however. It is tempting to speculate that they represent authentic membrane proteins involved in mitochondrial metabolism; further work in improving the assay system and identification of products is clearly necessary.

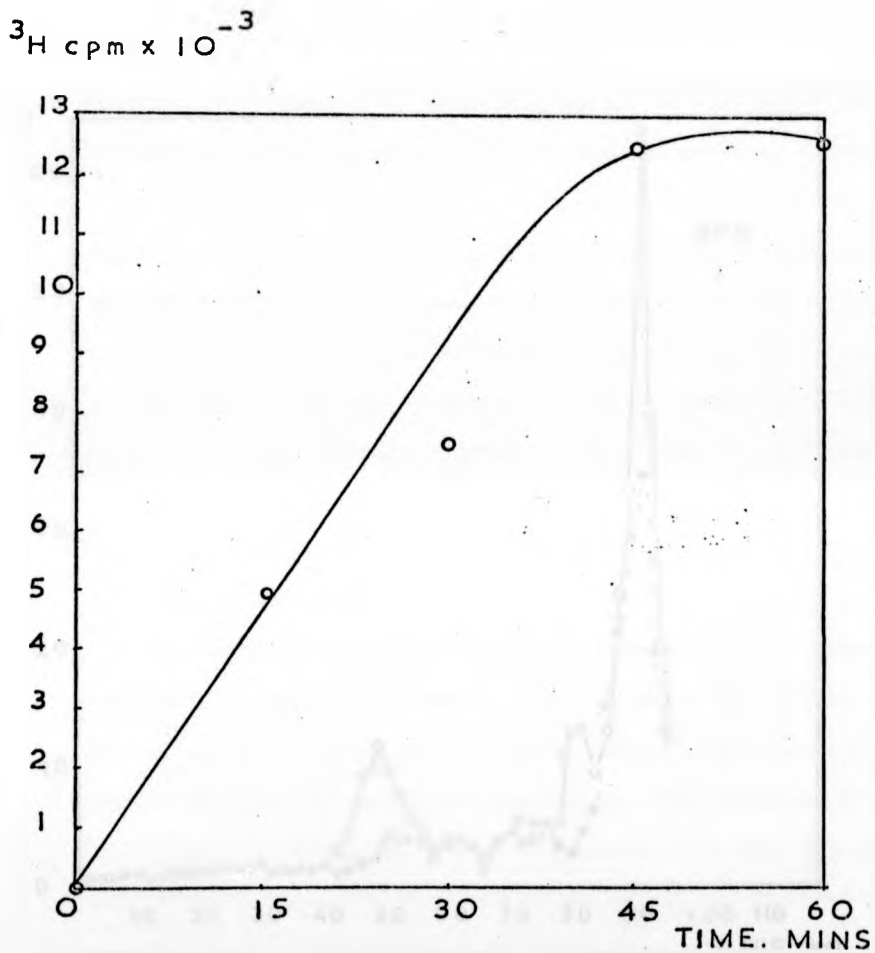


FIGURE 3:16

Transcription of o DNA by E.coli RNA Polymerase *in vitro*

5 μg of o DNA was transcribed in 250 μl reaction mixtures as described in methods. Transcription was assayed with ^3H -UTP as described by Scragg and Thomas (177). 15 μl aliquots taken at 15 minute intervals, were precipitated with 10% TCA + 100 mM sodium pyrophosphate, filtered and counted.

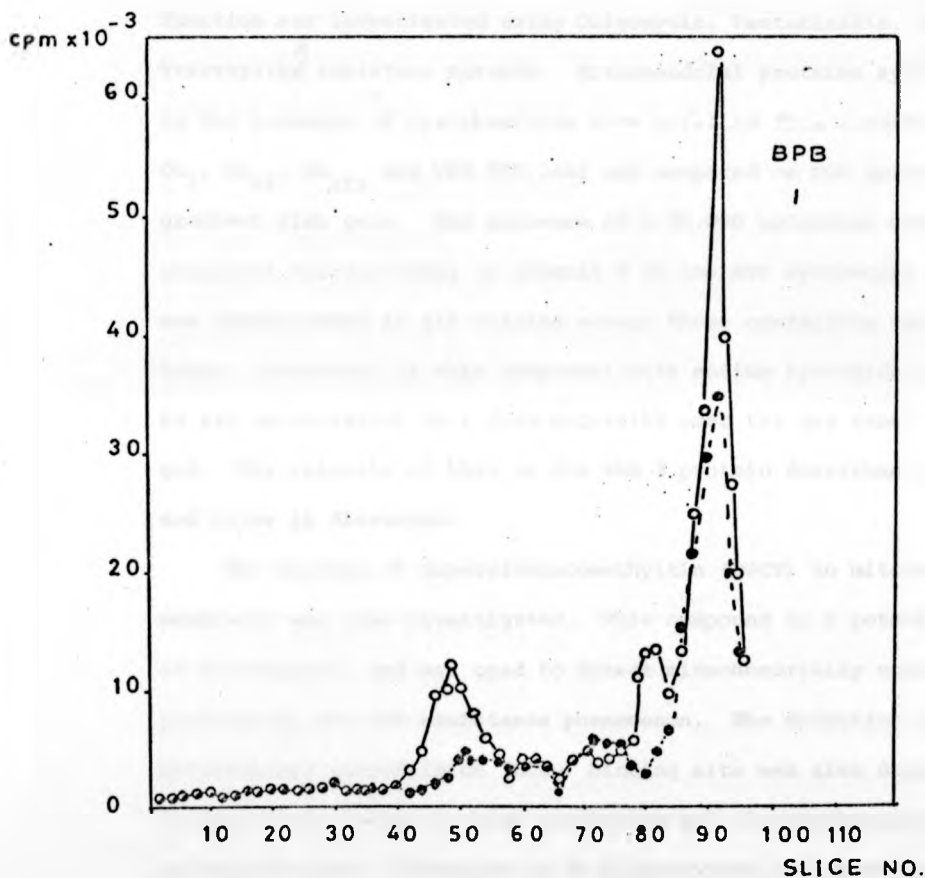


FIGURE 3:17

Translation Products Directed by Transcripts of ϕ DNA *in vitro*

RNA transcribed from ϕ DNA *in vitro* was precipitated with 1 μ g/ml t RNA on addition of 95% ethanol at -20°C . Translation was performed in E.coli S-30 extracts with 20 μC , ^{35}S -Methionine as labelled amino acid. Labelled proteins were precipitated with 10% TCA containing 0.5 mg/ml methionine. Precipitates were dissolved in electrophoresis sample buffer and analysed on 10% SDS polyacrylamide disc gels. Radioactivity was analysed as in methods.

Open circles, + RNA; Closed circles, - RNA.

CHAPTER 4

1. INTRODUCTION

The physical basis of resistance to inhibitors of mitochondrial function was investigated using Oligomycin, Venturicidin, and Triethyltinⁿ resistant mutants. Mitochondrial proteins synthesized in the presence of Cycloheximide were purified from strains containing OL_I, OL_{II}, OL_{III} and VEN TET loci and compared on SDS polyacrylamide gradient slab gels. The presence of a 45,000 molecular weight component corresponding to subunit 9 of the ATP synthetase complex was demonstrated in all strains except those containing the OL_I locus. Treatment of this component with sodium hydroxide resulted in its dissociation to a form migrating near the dye front of the gel. The relation of this to the VAR 3 protein described by Douglas and Butow is discussed.

The binding of dibutylchloromethyltin (DBCT) to mitochondrial membranes was also investigated. This compound is a potent analogue of Triethyltin, and was used to detect mitochondrially coded proteins involved in the TET resistance phenomenon. The detection of a dicyclohexyl carbodiimide (DCCD) binding site was also demonstrated in beef heart ATPase by high resolution gel electrophoretic and autoradiographic techniques as an illustration of a possible approach to the problems of inhibitor binding in general.

Resistance to Oligomycin at the loci OL_I, OL_{II} and OL_{III} is believed to result from a missense mutation in the mt DNA resulting in a polypeptide with altered binding properties. The proteolipid component of the ATP synthetase, subunit 9, was analysed by isoelectric focussing in polyacrylamide gels. Subunits from D22, O₂^L, D22/A15, OL_{II}, D22/A21, OL_I and D22/61, OL_{III} were analysed in this way. Single bands with a pI of approximately 4.75 were observed; D22 and A15 behaved identically, but A21 and D22/61 were displaced towards the acid end of the gel by differing amounts thereby distinguishing

OL_I and OL_{III}. The presence of different mutations within the same protein is discussed in relation to the OL_I and OL_{III} loci.

Oligomycin sensitive ATPase was resolved into at least 30 bands on isoelectric focussing gels; the profiles were too complex to allow direct comparison between wild type and mutant enzymes in order to detect small changes in pI however.

2. METHODS AND MATERIALS

2(i) Strains used

The following strains variously resistant to Oligomycin, Venturicidin and Triethyltin were used; the resistant locus is indicated in parentheses.

D22	(OL ^S VEN ^S TET ^S))	
)	
D22/A15	(OL _{II}))	ref (52, 53)
)	
D22/A21	(OL _I))	
)	
D22/60	(VEN))	
)	(160)
D22/61	(VEN OL _I))	
)	
D22/72	(VEN TET))	(149)

For labelling of mitochondrial proteins, where growth on galactose was desirable, strains were spread onto solid media containing: 1% Galactose, 1% Yeast extract, 1% Peptone, 2% Agar, sodium-potassium phosphate pH 6.25. Revertants from the Gal⁻ state found in D22 strains were scored by growth after 3 days and replated.

2(ii) Labelling of Cells *in vivo* in the Presence of Cycloheximide

The procedure outlined by Douglas and Butow was used (127). 1 ml cultures in 2% galactose, 1% Yeast extract, 1% Peptone, 0.1% KH₂PO₄, 0.12% (NH₄)₂ SO₄ and 0.01% adenine sulphate were grown overnight at 30°C. After addition of 4 mls of the same medium, cultures were regrown for a further hour. Cells were then pelleted in a bench centrifuge and washed twice with sterile water. For labelling in the presence of ³⁵SO₄, 2 mls of the following low sulphate medium

were used with 0.3% Glucose and 0.01% Adenine sulphate.

For the medium the following solutions were sterilized separately:

A.	<u>Rare elements</u>	/litre
	Boric acid	500 mg
	CuSO ₄	40 mg
	KI	100 mg
	ⁿ MnSO ₄	400 mg
	Na ₂ MoO ₄	200 mg
	ZnSO ₄	400 mg
B.	<u>Ferric chloride</u>	200 mg/litre
C.	<u>Vitamins</u>	/200 mls
	Calcium pantothenate	80 mg
	Thiamine HCl	80 mg
	Pyridoxine	80 mg
	Nicotinic acid	20 mg
	Biotin	0.8 mg
D.	<u>Inositol</u>	/litre
	meso Inositol	20 grms.
E.	<u>Mineral Salts</u>	/900 mls
	(NH ₄) ₂ H ₂ PO ₄	60 g
	Mg Cl ₂	1.9 g
	NH ₄ Cl	16.2 g
	KH ₂ PO ₄	10 g
	NaCl	1 g
	CaCl ₂	1 g

Solutions A to E were mixed in the following proportions:

A	1 ml
B	1 ml
C	5 ml
D	1 ml
E	9 mls

Cells were incubated for 21 minutes at 30°C in open beakers with vigorous aeration. Cycloheximide was added to 600 µg/ml, and the cultures incubated a further 4 minutes. Labelling of cells was effected by the addition of carrier free H₂³⁵SO₄ to 200 µC₁/ml followed by incubation for 1 hour. Label was chased by the addition of 2 mls 1% Casamino acids + 1 mg/ml Na₂SO₄. After 10 minutes, cells were pelleted and washed twice with 5 mls of this solution. For isolation of mitochondria, cells were resuspended in 0.4 mls MTE (0.25 M Mannitol, 20 mM Tris SO₄, 1 mM EDTA pH 7.1) and broken according to the microscale procedure of Needleman and Tzagoloff (204). After pelleting in the Sorvall GSA rotor at 9,000 rpm, mitochondria were solubilized in electrophoresis sample buffer and hot TCA precipitable counts estimated as described in Chapter 3.

2(iii) SDS Gel Electrophoresis in 10-15% Gradient slab gels

Gel preparation, running, staining, drying and autoradiography were as described in methods to Chapter 3.

2(iv) Preparation of Triton Extracts Labelled with ³H-DBCT

Labelled extracts were kindly prepared by K. Cain in this laboratory by the following procedure: Mitochondria were prepared by the method described in Chapter 3. Submitochondrial particles (SMPs) were prepared by sonic oscillation of mitochondria suspended in BSA buffer at 20 mg/ml. Sonication was for 4 x 15 seconds at full power in an MSE sonicator. SMPs were pelleted at 35,000 rpm for 30 minutes at 4°C in the 40 rotor of the Beckman L2-50 ultracentrifuge. After resuspension in BSA buffer minus BSA, ³H-DBCT was added to 8 µg/mg protein. After incubation overnight on ice, SMPs were pelleted as before and washed 5 x in BSA buffer. Triton extracts were prepared by suspending SMPs in buffer -BSA and adding Triton X-100 to 0.5%. Extracts were clarified by centrifugation at 35,000 rpm for 1 hour. For electrophoresis, Triton extracts were

mixed with an appropriate volume of SDS sample buffer and boiled for two minutes.

2(v) Preparation of ATPase and Subunit 9

Yeast ATPase and subunit 9 preparations were kindly performed by Dr. M. D. Partis in this laboratory. Beef heart ATPase was prepared by R. Hyams as described in (208). Yeast OS-ATPase was purified on Glycerol gradients as described by Tzagoloff (206). Subunit 9 was prepared by a modification of the procedure of Sierra and Tzagoloff (207) as described by Partis (208).

2(vi) Double Label Counting of Slab Gels

Proteins labelled with $^{35}\text{SO}_4$ were detected by autoradiography. When ^3H counting was required, autoradiograms were placed on carbon paper which lay face down on the dried gel. Relevant bands on the autoradiogram were scored with a pencil. The corresponding band imprinted onto the dried gel was cut out with a scalpel and hydrated in 30% H_2O_2 , dissolved and counted as before on the $^{14}\text{C}/^3\text{H}$ channel of the Packard 2425 counter.

2(vii) Isoelectric Focussing of Membrane Proteins

The procedure of O'Farrell (205) was adopted with slight modification; 130 x 2.5 mm glass tubes were filled with the following solution:

	/10 mls	
Urea	5.5 g	
28.38% Acrylamide	1.62% bisacrylamide	1.33 mls
10% NP40		2.0 mls
20% Triton X-100		1.0 mls
H_2O		0.970 mls
LKB Ampholytes 3-10		0.5 mls

Gels were polymerized by the addition of 10 μl 10% Ammonium

persulphate and 10 μ l TEMED. Changes of overlay buffer and preelectrophoresis were as described by O'Farrell.

Samples were prepared as follows: ATPase preparations containing 0.5% Triton were made 9.5 M in Urea by addition of crystals. An equal volume of Urea sample buffer A (205) was added. 50 μ l of sample was applied to the gel and overlaid with 10 μ l sample overlay K (205). About 50 μ g of protein was used per sample. Chloroform methanol extracts containing subunit 9, were boiled in the presence of 2% SDS until the organic phase had disappeared. Solid urea was added to 9.5 M and solutions A and K added as above. Electrofocussing was for 16 hours at 300 V followed by 1 hour at 400 V. Gels were extruded and stained for 2 hours in 45% Methanol, 10% Acetic acid containing 0.01% Coomassie brilliant blue. Destaining was for 1 hour in 45% Methanol 10% Acetic acid followed by 10% Propanol, 10% Acetic acid and 10% Acetic acid for 1 hour each. Gels were scanned at 640 nm in a Gilford gel scanner attached to a Zeiss spectrophotometer.

pH gradients were measured by cutting a blank gel into 5 mm sections, agitating in 2 mls of degassed water for 20 minutes and measuring the pH of each with a Beckman pH meter.

2(viii) Materials Used

Growth media, reagents and materials for gel electrophoresis have been described in Chapters 2 and 3. $H_2^{35}SO_4$ was purchased from New England Nuclear, Boston, or the Radiochemical Centre, Amersham. Urea was Analar grade from Fisons, Loughborough. Ampholines were the 3-10 range from LKB Ltd., Bromma.

3. RESULTS AND DISCUSSION

3(1) Labelling of Mitochondrial Proteins *in vivo* in the Presence of Cycloheximide

In previous Chapters, different approaches to the study of

mitochondrial gene expression were described; the use of mutants in nucleic acid analysis and expression of the genetic material were shown to be possible approaches to the problem. In this Chapter, the physicochemical properties of known mitochondrial proteins are examined with a view to detecting differences between them related to specific drug resistance loci. The Oligomycin resistance loci OL_I and OL_{II} were chosen as well as the strains D22/60 VEN^R and D22/61 $VEN^R OL_I^R$. D22/60 lies close to the OL_I locus so that recombination is extremely low. D22/61 or OL_{III} recombines with OL_I with frequencies of 0.4 to 3.5% (160). Since these values are very low, it is not clear whether or not the strains are allelic to OL_I , nevertheless they provide useful markers separated from OL_{II} .

The biochemical basis of resistance to Oligomycin has been studied by analysing ATPase from sensitive and resistant strains. Decreased sensitivity of the resistant enzyme to the antibiotic suggests that membrane permeability or detoxification mechanisms are not important here (209). The fact that the resistance mutation does not lead to any major loss of function as in mit^- mutants, as well as the inability of conventional gel electrophoresis systems to detect changes in membrane proteins, suggests that this is a result of a point mutation in the mt DNA. Such amino acid substitutions would result in a change of conformation of the inhibitor site or sites, resulting in the decreased binding and efficacy of the inhibitor. In an attempt to improve the resolution of present techniques, Douglas and Butow have developed the labelling and high-resolution techniques described in Chapter 4 and methods. Figure 4:1 shows a typical autoradiogram of mitochondrial proteins labelled for 1 hour with $^{35}SO_4$ and analysed on a 10% slab gel. Gels of this acrylamide concentration will not resolve proteins of less than

FIGURE 4:1

Products of Mitochondrial Protein Synthesis *in vivo*

Microscale cultures were grown overnight in YEP Gal medium and labelled in a synthetic low sulphate medium as described in the text. CHI was added to 600 µg/ml followed by 100 µCi/ml $H_2^{35}SO_4$. Labelling was for 1 hour followed by a 10 minute chase with Na_2SO_4 . Mitochondria were prepared by the procedure of Needleman and Tzagoloff (204) and dissolved in electrophoresis sample buffer. The gel here is a 10% slab gel run at 30 mA for 5 hours, stained, dried and autoradiographed for 5 days. VAR 1, 2 and 3 are the proteins involved in the VAR polymorphism, (127), Cyt. oxidase I, II and III are the mitochondrially synthesized subunits of cytochrome oxidase (147).

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cyt. oxidase I

II

III

-

-

-

-var3

-var1

-var2

+



20,000 daltons which explains the sharp cutoff at the bottom of the gel. VAR 3, 1 and 2 refer to the "polymorphisms" described by Douglas and Butow (127). Here, VAR 3 and 2 are present, while in the "mutant" state they are absent. VAR 1 is in its "normal" state while the polymorphic form represents a molecular weight increase of about 2,500 daltons. The molecular weights of VAR 3, 1 and 2 are 45,000, 40,000 and 24,000 daltons respectively. Precipitation of mitochondrial proteins with antiserum to cytochrome oxidase results in the purification of the three high molecular weight subunits I, II and III of this enzyme (147). These are indicated in figure 4:1. It should be noted that subunit I behaves non-ideally in this gel since its apparent molecular weight is 36,000 while other systems determine it as 42,000. The mapping of VAR 1 and 2 has been described in Chapter 1. Of interest is the lack of function ascribed to these proteins since they do not correspond to cytochrome oxidase, b or ATPase subunits. The possibility of these being regulatory rather than catalytic proteins must be considered. VAR 3 is expressed as the absence of a band of 45,000 daltons. Genetic analysis reveals that this locus is very close to or allelic with OL_I , OL_{III} with a recombination frequency of only 1.4% (147). The relation of this protein to the 45,000 dalton form of subunit 9 was investigated. A characteristic property of this ATPase subunit is its sensitivity to dissociation to a 7,800 dalton form on treatment with sodium hydroxide, acid, or chloroform methanol 2:1. Samples of labelled mitochondria were therefore made 0.5 M in NaOH and analysed on 10-15% gradient slab gels. Figure 4:2 shows the results obtained. The heavily loaded material ranging from 45,000 daltons up to the top of the gel has been completely dissociated to a band running at the dye front. This was found for stains D22, D22/61 and D22/72. The absence of a single band at 45,000 daltons, and the presence of

FIGURE 4:2

Reaction of Mitochondrial Proteins with Sodium Hydroxide
Prior to Electrophoresis

Mitochondria, labelled with $^{35}\text{SO}_4$ as described in methods, were made 0.5 M NaOH, dissolved and heated in electrophoresis sample buffers and electrophoresed in 10%-15% gradient slab gels. Gels were stained, dried and autoradiographed for 3 days.

Apart from Trypsin, the molecular weight standards described in methods were used with the following additions:
 β - Galactosidase (130,000), Phosphorylase a (92,000), Fumarase (49,000), κ chain (22,500), and Myoglobin (16,900).

MW x 10⁻³

130
92.5

68
57
53
49

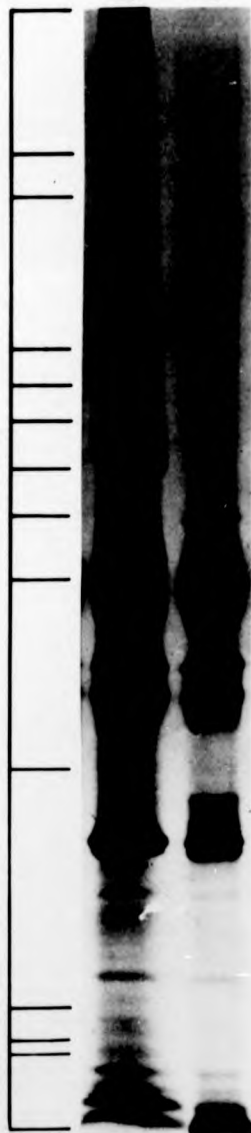
41
36

22.5

16.9
13.9
13.4

D22/6I - NaOH

D22/6I + NaOH



discrete bands of regularly ascending molecular weights in this region, may reflect incomplete solubilization of the membrane proteins in SDS. Despite this, the result shows that VAR 3 is probably phenotypically expressed as an alteration in subunit 9 of the ATPase complex. This has been further confirmed by Tzagoloff using a similar labelling and gel electrophoresis technique in which an OL_I mutant possesses the low molecular weight form of subunit 9 (210). The strains D22, A15, A21, D22/60, D22/61 and D22/72 were examined by the above labelling procedure with the result that only A21 (OL_I) showed a band missing at 45,000 daltons in agreement with Tzagoloff. No other differences were detected between other proteins synthesized *in vivo*. The retention of the aggregate form of subunit 9 in the OL_{III} mutant is of interest since this phenotypically separates it from OL_I. Further studies described later point to the two loci being allelic however, and this apparent contradiction is discussed.

3(ii) The Involvement of Mitochondrially Coded Proteins in Triethyltin Resistance

Triethyltin resistance was discussed in Chapter 2 in relation to extramitochondrial genes carrying the VEN^R TET^R locus. TET resistance is also dependent on membrane components specified by mt DNA however, since resistance is also expressed at the ATP synthetase level; reconstitution experiments described in Chapter 2 point to the F₀ component of this enzyme as such a site. In this context, the following observations are relevant (211): a) Triton extraction under conditions which remove the ATP synthetase complex from the mitochondrial inner membrane removes a high affinity binding site for TET; approximately one third of the radioactivity of labelled TET is unextracted however. This site is also involved in the binding of other inhibitors of mitochondrial function such as

§
A
Bonkrekic acid and Hexachlorophene. This suggests that two TET binding sites are present; one in the ATPase complex, and the other in a membrane complex possibly associated with transport phenomena. The TET mutants described in Chapter 2 are also cross resistant to BA^R and HEX^R mutants, so it is tempting to speculate that unextractable membrane components are encoded by a non-mitochondrial extrachromosomal DNA. b) In studies of labelled TET binding to ATPase, the stoichiometry of 6 nmoles TET to 1 nmole ATPase is observed. The 45,000 form of subunit 9 is a hexamer of the 7,800 form which, assuming a single TET molecule binds to each low molecular weight species, would give the above result. In order to test this experimentally, the TET analogue DBCT, which binds covalently with mitochondria, was bound to SMPs. Preparation of these, labelling with ³H-DBCT and triton extraction were as described in methods. Triton extracts were analysed on 10-15% gradient slab gels and bands corresponding to molecular weights of 45,000, 38,000, 22,000 and 10,000 were cut out and counted. Figure 4:3 shows the stained extract and the counts obtained. Label is clearly associated with the 45,000 and low molecular weight bands which is the behaviour expected on binding to subunit 9. The 38 and 23,000 dalton components were chosen as arbitrary controls and are essentially free of radioactivity. The correspondence of the high molecular weight species with VAR 3 was tested as follows: ³⁵SO₄ labelled mitochondria were mixed with ³H-DBCT labelled triton extracts. The small scale of the ³⁵SO₄ labelling procedure prohibited direct binding of this inhibitor. Electrophoresis was performed as before, and the gel dried and autoradiographed. Figure 4:4 shows the autoradiogram obtained. VAR 3 is present as a faint band in this particular experiment. For double label analysis, the positions of VAR 3, VAR 1 and cytochrome oxidase subunits II and III were marked on the dried

FIGURE 4:3

Gel Electrophoresis of Triton Extracted Membranes Labelled with ^3H -DBCT

Preparation of mitochondria, SMPs, labelling and Triton extraction were as described in methods. Samples were run in parallel slots of a 10-15% gradient slab gel. One slot was stained and the molecular weights of specific bands determined from known molecular weight standards. Unstained bands corresponding to the molecular weights shown in the diagram were cut out and counted in protosol, or digested with 0.5 ml H_2O_2 prior to counting in Triton Toluene scintillation fluid. The following ^3H counts were obtained:

Band	^3H cpm
1	743
2	-
3	63
4	220

$M_r \times 10^{-3}$

45
38
22

~ 10



-1
-2
-3

-4 +

I

FIGURE 4:4

Coelectrophoresis of $^{35}\text{SO}_4$ labelled Mitochondrial Proteins
and ^3H -DBCT Labelled Triton Extracts

$^{35}\text{SO}_4$ labelling and preparation of ^3H -DBCT labelled extracts were as described in methods. Samples were mixed, solubilized in SDS sample buffer and run in 10-15% gradient slab gels. Gels were stained dried and autoradiographed for 3 days. Bands on the X-ray film were located on the dried gel by the carbon paper technique described in methods. After excision, bands were hydrated and solubilized in 0.5 ml H_2O_2 and counted in Triton Toluene scintillation cocktail.

The following ^3H counts were obtained after double label ($^{14}\text{C}/^3\text{H}$) counting:

Band	^3H cpm
1	179
2	16
3	33
4	11
5	12

FIGURE 4:4

Coelectrophoresis of $^{35}\text{SO}_4$ labelled Mitochondrial Proteins
and ^3H -DBCT Labelled Triton Extracts

$^{35}\text{SO}_4$ labelling and preparation of ^3H -DBCT labelled extracts were as described in methods. Samples were mixed, solubilized in SDS sample buffer and run in 10-15% gradient slab gels. Gels were stained dried and autoradiographed for 3 days. Bands on the X-ray film were located on the dried gel by the carbon paper technique described in methods. After excision, bands were hydrated and solubilized in 0.5 ml H_2O_2 and counted in Triton Toluene scintillation cocktail.

The following ^3H counts were obtained after double label ($^{14}\text{C}/^3\text{H}$) counting:

Band	^3H cpm
1	179
2	16
3	33
4	11
5	12

1-
2-
3-
4-
5-



-

+

gel as described in methods. Bands were hydrated and counted with the result shown in figure 4:4. ^3H -DBCT has clearly bound preferentially to the VAR 3 component. The situation is not as simple as this however. Addition of Oligomycin, and Venturicidin to ATPase labelled with TET does not displace the latter from its binding site, suggesting that this is different from those of other related inhibitors (211). While it seems clear that all bind to a common protein site, TET may directly interact with a small molecule bound to subunit 9. The implications and experimental evidence for this are beyond the scope of this thesis however.

In an effort to study the binding sites of other radiolabelled inhibitors by high resolution techniques, beef heart ATPase was reacted with ^{14}C -DCCD in the same way as ^3H -DBCT. Binding of this inhibitor to subunit 9 of the ATPase has been demonstrated (208), but again, an intermediate low molecular weight component may be involved. Figure 4:5 is 10-15% slab gel showing the stained beef heart ATPase alongside an autoradiogram of the ^{14}C -DCCD labelled binding site. The strongly staining low molecular weight protein is clearly associated with DCCD. This approach is potentially very useful in terms of the resolution of membrane proteins: the presence of at least 30 bands stained with Amido black may reflect proteolytic degradation, preparation techniques unable to remove minor contaminants, or *bona fide* ATPase components hitherto undetected. The former possibilities are the most likely at present. Attempts were also made to locate ^3H -DBCT binding sites by fluorography (212), but the PPO impregnation procedure described by Bonner and Laskey removed most of the affinity label from the fixed gel.

3(iii) Analysis of Subunit 9 from Oligomycin Resistant Mutants by Isoelectric Focussing

In section 3(i) the OL_I mutation was shown to be related to the

FIGURE 4:5

Autoradiographic Location of ^{14}C -DCCD on the Beef Heart
ATPase Complex

Beef heart enzyme was labelled with ^{14}C -DCCD as described by Partis (208) to a specific activity of 46,800 cpm/mg protein. 150 μg was run in a 10-15% gradient slab gel, stained with Amido black, dried, and autoradiographed for 6 days. The molecular weights of the known ATPase subunits are shown. DCCD is clearly associated with the 10,000 dalton species.

$M_r \times 10^{-3}$

55
52

30 -
25 -
20 -
19 -

10 12
8



-DCCD

+

-

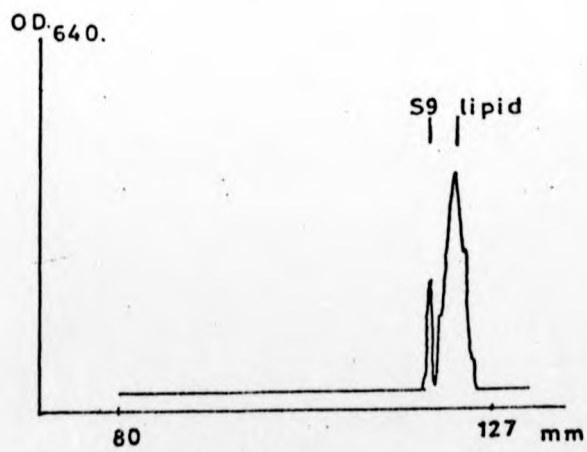
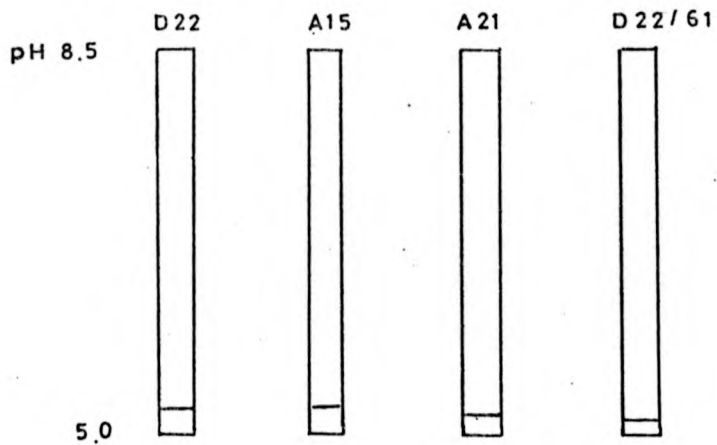
low molecular weight form of subunit 9, and that it and OL_{III} could be phenotypically separated on this basis. Assuming a gross charge difference to be the result of a missense mutation in the mutant mt DNA, subunit 9 from several mutants were compared with the wild type protein by isoelectric focussing. This technique separates proteins on the basis of their isoelectric point, pI, and is capable of extremely high resolution. For the detection of single charge differences in complex protein mixtures, for example ribosomal proteins, the technique is generally used in conjunction with SDS gel electrophoresis as a two dimensional technique (205).

The results of focussing subunit 9 from D22, A15 (OL_{II}), A21 (OL_I) and D22/61 (OL_{III}) are shown in figure 4:6 as a schematic diagram. Proteins migrated as single sharp bands near the acid end of the gel; a small diffuse region staining with Coomassie blue ran slightly ahead of each band: and probably represents lipid. Figure 4:7 shows the pH gradient determined from a parallel gel. The pIs of these proteins therefore lie between pH 4.75 and 5.0. D22 and A15 derived proteins have identical pIs which, on the basis of previous data, is as expected since OL_I maps a significant distance away from OL_{II} (figure 1:2). A21 shows a displacement of about 1 mm towards the acid pH, while D22/61 (OL_{III}) is displaced 3 mm. This finding in the case of A21 is not surprising since subunit 9 was shown to be associated with the OL_I locus, by its altered aggregative properties. The assumption that OL_I and OL_{III} lie on two adjacent cistrons because of the low recombination frequency between them may not be valid. The demonstration of different amino acid substitutions in the same protein is an indication that these particular mutants are cistronic. Further evidence for this comes from the peptide mapping of partial acid hydrolysates of subunit 9. Cleavage of the proteolipid at Aspartate residues was performed in

FIGURE 4:6

Isoelectric Focussing of Subunit 9 from Oligomycin
Resistant Strains

Isoelectric focussing was performed in disc gels using a modification of O'Farrell's procedure as described in methods (205). Samples containing about 30 μ g of subunit 9 were run at 300 V for 16 hours and 400 V for 1 hour. Gels were stained as described in Methods. Gels were scanned at 640 nm, and the result for D22 S9 is shown.



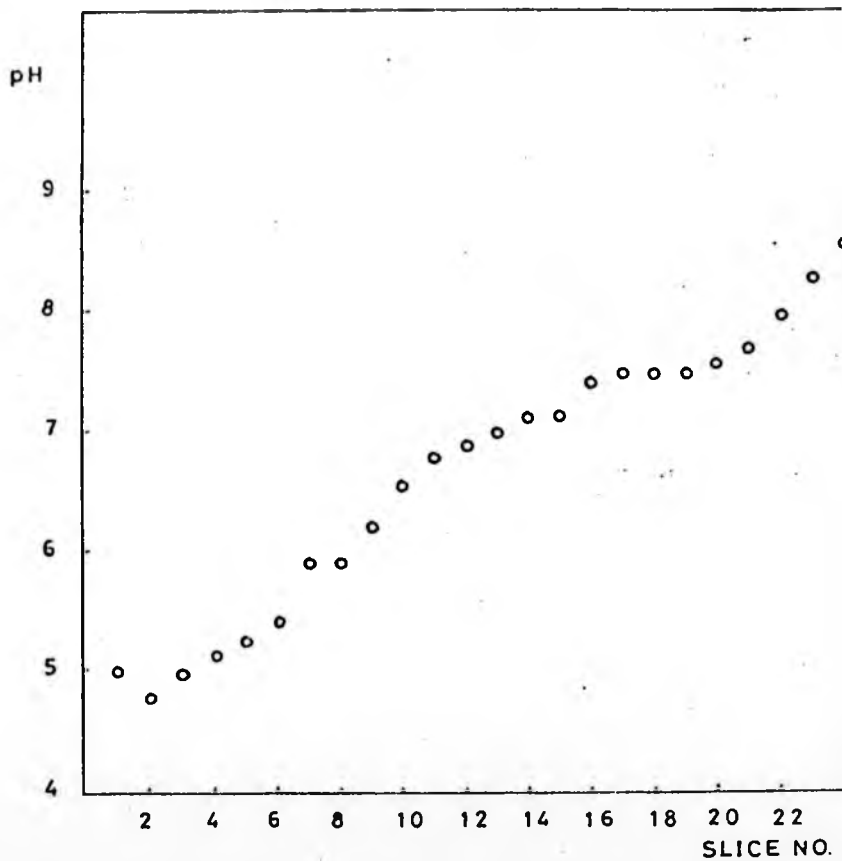


FIGURE 4:7

pH Gradient Established in Isoelectric focussing Gels

Gels were run as described in the legend to figure 4:6. 5 mm slices were cut manually and agitated in degassed water for 20 minutes. The pH was measured with a Beckman pH meter. The top of the gel is to the right.

0.03 M hydrochloric acid, the peptides dansylated and analysed on polyamide plates (208). The OL_I mutant shows essentially the same cleavage pattern as the wild type, but the OL_{III} map reveals a new peptide derived from the hydrolysis. This is consistent with the electrofocussing data, in which the pI of A21 is not markedly changed implying that a major charge substitution has not occurred. The presence of more Aspartate residues in D22/61, as suggested by the peptide map, is mirrored in the larger shift of pI towards the acid pH. The extent of the shift in isoelectric focussing gels is influenced by the size, number of ionizable groups and presence of strong dissociating groups in a protein (205). The low molecular weight, hydrophobic protein subunit 9 will be strongly influenced by an amino acid substitution resulting in a new strongly ionized group such as the Carboxyl of Aspartate. A definitive answer to this however can only be made after amino acid composition and sequencing studies.

In order to detect pI changes in other ATPase subunits, the purified enzyme from D22, A15 and A21 were focussed on gels as above. At least 30 bands were detectable on staining with Coomassie blue, but the pattern was too complex to detect any small charge differences. A scan of a D22 ATPase gel is shown in figure 4:8. Further resolution of these components according to molecular weight on SDS gradient slab gels is a possibility. The low solubility of mitochondrial proteins in the non ionic detergents used in isoelectric focussing has hitherto been a major obstacle in the analysis of these components. The solubilization method developed here is capable of dissolving about 20% of the applied sample. The use of highly radioactive samples containing little protein is an obvious example of a way in which this could be improved. To date, the only other work describing isoelectric focussing of yeast mitochondrial proteins has been described by Fiechter *et al* (213). These authors have obtained

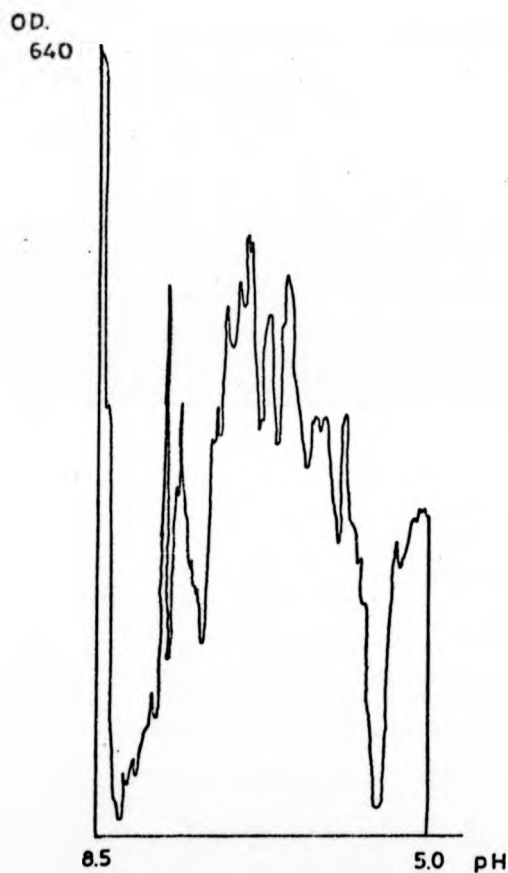


FIGURE 4:8

Isoelectric Focussing of OS-ATPase

OS-ATPase was prepared by the method of Tzagoloff (206). Preparations dissolved in 0.5% Triton X-100 were made 9.5 M in Urea and an equal volume of sample buffer A added (205). 50 μ g samples were focussed as described before. After staining with Coomassie blue, gels were scanned at 640 nm in a Gilford gel scanner.

similar gel profiles to that obtained here with ATPase, but have not characterized the proteins further.

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