A model for mitochondrial disease associated with rearrangements of the mitochondrial genome

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by

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Dedicated to Kieran

The research reported in this thesis is my own original work, except where otherwise stated, and has not been submitted for any other degree.

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Abbreviations

12S rRNA	small subunit mitochondrial ribosomal RNA
16S rRNA	large subunit mitochondrial ribosomal RNA
ADP	adenosine 5'-diphosphate
adPEO	autosomal dominant PEO
ATP	adenosine 5'-triphosphate
ATPase 6 and 8	ATP synthase subunits 6 and 8
BSA	bovine serum albumen
CMS	cytoplasmic male sterility
COXI, II, III	cytochrome *c* oxidase subunits I, II, III, in all organisms discussed, and the genes encoding these subunits, in mammals and fungi
coxI, II, III	genes encoding cytochrome *c* oxidase subunits I, II, III in plants
CO_2	carbon dioxide
CPEO	chronic progressive external ophthalmoplegia
CSB	conserved sequence block
cyt *b*	cytochrome *b*
dH_2O	distilled water
D-loop	displacement loop
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
EtOH	ethanol
FCS	foetal calf serum
Fig.	figure
HSP	heavy strand promoter
hsp	heat shock protein
H-strand	heavy strand
IPTG	Isopropyl-ß-D-thiogalactopyranoside
KSS	Kearns-Sayre syndrome
log	logarithm/logarithmic
LSP	light strand promoter
L-strand	light strand
MCS	multiple cloning site

MELAS	Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes
mhsp	mitochondrial heat shock protein
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
mtTFA	mitochondrial transcription factor A
NCS	non-chromosomal stripe phenotype (maize)
ND1-ND6, ND4L	NADH dehydrogenase subunits 1-6, 4L
O_H	origin of replication of the heavy strand
O_L	origin of replication of the light strand
ORF	open reading frame
OXPHOS	oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEO	progressive external ophthalmoplegia
pH	negative $\log_{10}$ [H+]
ρ^0	cells lacking mtDNA
RNA	ribonucleic acid
RNAse	ribonuclease
rRNA	ribosomal ribonucleic acid
RRF	ragged red fibres
SDH	succinate dehydrogenase
SDS	sodium dodecylsulphate
SSC	standard saline citrate
TEMED	N, N, N', N'-tetramethylenediamine
Tris	tris (hydroxymethyl) amino ethane
tRNA	transfer ribonucleic acid
urf	unidentified reading frame
UV	ultra-violet
X-gal	5-bromo-4-chloro-3-indolyl-B-galactopyranoside

Units

bp	base pairs
^{0}C	degrees Celsius
Da	Dalton
g	grammes
g	centrifugal force equivalent to gravitational acceleration
kb	kilobases/kilobase pairs
kDa	kiloDaltons
l	litres
M	molar (moles per litre)
mg	milligrammes
min	minutes
ml	millilitres
mM	millimolar
µCi	microCuries
µg	microgrammes
µl	microlitres
µM	micromolar
ng	nanogrammes
nt	nucleotides
rpm	revolutions per minute
rpm	revolutions per minute
sec	seconds
u	units
V	Volts
W	Watts

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Summary

Individuals affected by mitochondrial diseases such as Kearns-Sayre syndrome (KSS) frequently possess a heteroplasmic population of mitochondrial DNA molecules consisting of multiple copies of both a wild-type mitochondrial genome and a mitochondrial genome which has undergone a major rearrangement. One hypothesis to account for the pathogenic effects of rearranged mtDNA molecules is that in affected individuals abnormal proteins, encoded by chimeric mitochondrial genes created by the rearrangement of mitochondrial DNA, contribute to mitochondrial dysfunction. In order to investigate the relationship between the expression of a chimeric peptide in mammalian mitochondria and mitochondrial dysfunction, three synthetic genes were constructed. The first, designated PI ("Patient I construct"), was a chimeric version of mouse cytochrome *c* oxidase I (COXI) based on a rearranged COXI gene found in the mutant mtDNA of a KSS patient (Poulton *et al.*, 1989). The second, PIZ, was an epitope-tagged version of PI, and the third was a full length, universal-code version of mouse COXI.

As no transfection system for mammalian mitochondria has been developed, it was necessary to construct genes which could be transcribed in the nucleus and translated in the cytosol, with subsequent import to mitochondria of the encoded protein. The alteration of the coding sequence of COXI from the mammalian mitochondrial genetic code to the universal code required 43 programmed point mutations using site-directed mutagenesis. In order that the proteins encoded by the synthetic genes could be targeted to mitochondria, a sequence encoding the cytochrome *c* oxidase IV mitochondrial transit peptide was subcloned 5' to the code-corrected genes. The epitope-tagged gene PIZ incorporated an epitope recognised by a mouse monoclonal antibody against a herpes simplex viral protein, to facilitate expression analysis

Expression of PI, PIZ and the universal-code version of mouse COXI all appeared to be highly toxic to *E. coli*, based both on difficulties experienced in subcloning, and on expression analysis of the
constructs. Several hypotheses to account for this are discussed, including the possible interference with assembly/activity of the bacterial cytochrome *c* oxidase, and potential effects of the amphipathic transit peptide on membrane integrity.

Mouse cell-lines which had incorporated both the PI and PIZ constructs were generated. However, the lack of an effective antibody with which to detect PI prohibited characterisation of the PI-transfected cell-lines at the protein level. Northern and Western analysis suggested that PIZ was expressed at both the RNA and protein levels in several PIZ-transfected 3T3 clones. Western analysis also suggested that the PIZ protein may have been correctly processed proteolytically, which would indicate that the protein may be imported into the mitochondria.

In order to test whether the chimeric peptide conferred a respiratory phenotype in the 3T3 cell line, assays of growth in media containing one of two alternative carbon sources (glucose or galactose) as the catabolic substrate were performed. Statistical analysis of the cell growth assays suggests that PIZ expression was inhibiting respiration. Staining with mitochondrial-specific dyes also suggested that respiration was inhibited in the PIZ-transfected cell-line so tested (clone PIZ8.3).

Clone PIZ8.3 appeared to have incorporated multiple copies of the PIZ transgene. Under growth conditions which increase the requirement for respiration (galactose as the carbon source), clone PIZ8.3 was apparently deleting some copies of the transgene. This would support the conclusion that expression of the transgene is detrimental to respiration and cell survival or growth.

The synthetic genes and cell-lines which have been created in this study can contribute to further investigation of the relationship between a chimeric mitochondrial peptide and mitochondrial dysfunction by enabling biochemical analysis of the observed respiration deficiency. They may also facilitate analysis of the pathogenicity in an organism through the creation of transgenic mice. The results obtained in this study support the view that the expression of chimeric genes encoded across rearrangement break-points in mitochondrial DNA has a deleterious effect on mitochondrial function, and hence could play a significant role in mitochondrial disease pathogenesis.

Chapter 1

Introduction

1.1 Mitochondria - structure and function

A common analogy is that mitochondria are the powerhouse of the eukaryotic cell. By the process of oxidative phosphorylation they produce the bulk of the cell's ATP, the form in which energy is stored. Mitochondria also perform other biochemical functions vital to the cell. The citric acid cycle is the pathway by which all fuel molecules, such as fatty acids, amino acids, and carbohydrates, are oxidised to CO_2. Deficiencies in mitochondrial function have been linked to a growing number of human diseases. In order to investigate a possible mechanism of pathogenesis for one of these diseases, it is necessary to review what is presently known of both normal and abnormal mitochondrial function.

Structurally, the mitochondrion is a double membrane-bound compartment in which the membranes define two internal spaces, the intermembrane space and the matrix (reviewed in Alberts, 1983). The mitochondrial outer membrane is permeable to most small molecules and ions, whereas the inner membrane is virtually impermeable to all molecules, except those for which specific transport systems exist. The inner membrane is convoluted, forming structures known as cristae, and contains proteinaceous carriers to transport specific molecules, e.g., ADP and long chain fatty acids, into the mitochondrial matrix. Mitochondria are the only organelles in the mammalian cell apart from the nucleus to contain DNA, and it is in the matrix compartment that the mitochondrial DNA (mtDNA) molecules are located. In yeast, the mtDNA is attached to the inner membrane (Nunnari *et al.*, 1997). It is within the inner membrane that the enzyme complexes of the respiratory chain are situated and oxidative phosphorylation occurs (fig. 1.1). Mitochondria can differ both structurally and physiologically depending on the cell type and tissue they are situated in.

Aerobic respiration is the primary function of mitochondria (fig. 1.1). Pyruvate (formed during the metabolisation of sugars) and fatty acids are imported into the

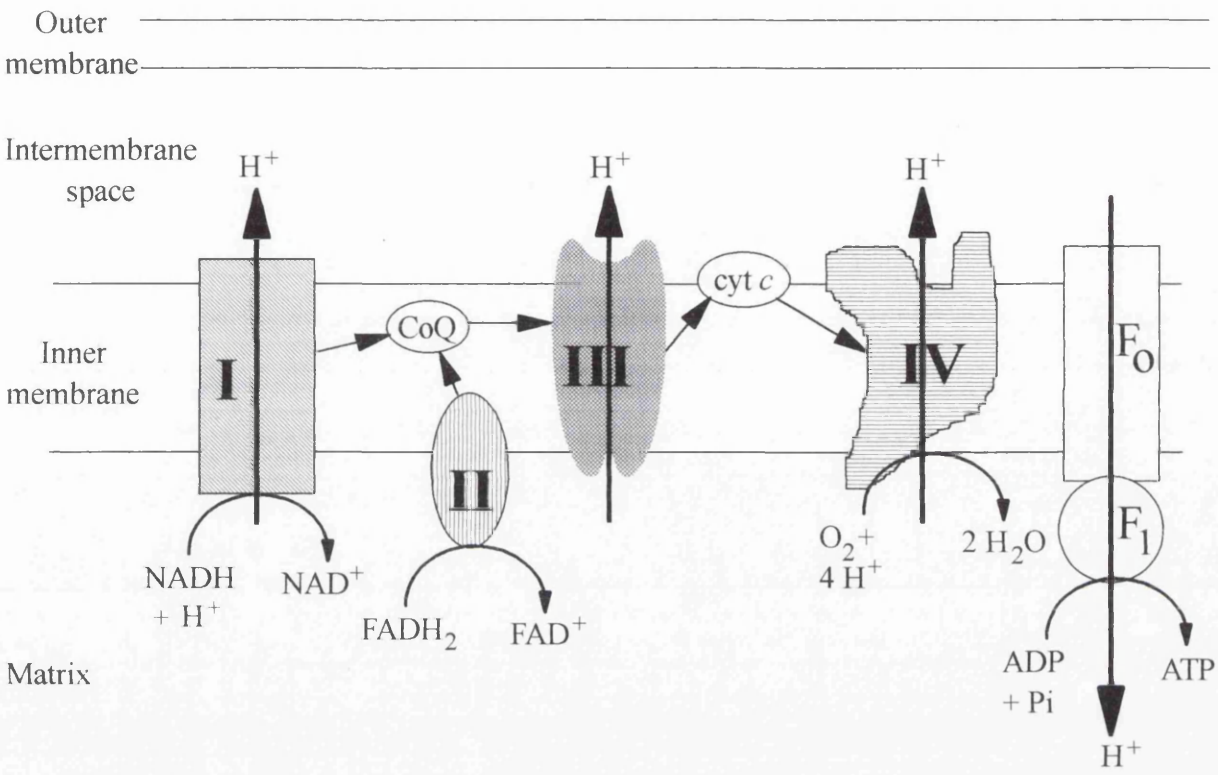


Figure 1.1 Summary of the reactions involved in aerobic metabolism

A schematic diagram of the mitochondrial double membrane boundary is shown. The components of the electron transport chain are situated in the inner membrane. The enzyme complexes of the electron transport chain are shown as :

I NADH dehydrogenase (Complex I)
II succinate Q reductase (Complex II)
III cytochrome c reductase (Complex III)
IV cytochrome c oxidase (Complex IV)

F_o and F_1 are components of the ATP synthase complex (Complex V)

CoQ coenzyme Q
cyt *c* cytochrome *c*

Electron transfer between the components of the respiratory chain

Proton transfer across the inner mitochondrial membrane

Metabolic reactions

mitochondrial matrix where they are converted to acetyl coenzyme A, the substrate for the citric acid (TCA) cycle. NADH and $FADH_2$ (reduced cofactors, products of the TCA cycle) are re-oxidised by the transfer of electrons via a series of electron carriers in the inner membrane enzyme complexes to the final acceptor, molecular oxygen. The movement of electrons along this electron transport chain is accompanied by the translocation of H^+ ions from the matrix to the inter-membrane space, which generates an electrochemical proton gradient. The flow of these ions through the ATP synthase complex back to the matrix causes the release of ATP generated by the phosphorylation of ADP. Thus the processes of oxidation and phosphorylation are coupled by the proton gradient formed across the inner membrane (reviewed in Stryer, 1988).

Historically there have been 3 models proposed for the configuration of mitochondria within the mammalian cell:- (1) the conventional model is that mitochondria are present in multiple discrete granular units, classically described as rod-shaped structures, about 0.5 μm in diameter. (2) A second model is that one giant mitochondrion, existing in the form of a network, is distributed throughout the cell. (3) An intermediate model consists of a parental intracellular network which is fluid in that smaller units can become separated and, conversely, assimilated back into the parental network. A growing body of evidence supports the third model. In a study using fluorescence labeling to analyse the shape and distribution of organelles in relation to the cytoskeleton in fibroblasts, mitochondria appeared to form an interconnected network of elongated tubules, similar to and perhaps associated with the endoplasmic reticulum. Mitochondria were additionally found to be present in vesicular form (Soltys and Gupta, 1992). It was unclear from this study whether the two forms have the potential for interconversion, whether the two forms are in any way functionally different or, indeed, if the observation of the vesicular form is an artifact. However, experiments utilising mitochondria with different mitochondrial DNA (mtDNA) genotypes have found evidence for genetic complementation within

the mitochondrial compartment (Takai *et al.*, 1997). Cybrids were constructed by introducing mitochondria from enucleated HeLa cells into recipient cells; both mtDNA molecular forms carried identifiable genetic markers. For the observed genetic complementation to have occurred, the 'foreign' mitochondria must have interacted with the endogenous mitochondria, enabling the matrix compartments of the respective mitochondria to have been in contact. Thus, assuming mitochondrial fusion was not caused by the technique utilised for cybrid formation, a mechanism may be inferred to exist for fusion of mitochondria with each other. This supports a model of a fluid network of mitochondria which can 'bud off' into smaller units, or reaggregate with the parental network. It is also possible that different cell types may have different mitochondrial configurations.

1.2 Mitochondrial genome - coding information and organisation

One distinguishing feature of mitochondria is that they contain DNA. Plastids, such as chloroplasts found in higher plants, are the only other organelles to contain genetic information, apart from the nucleus. The mitochondrial genomes of most organisms studied to date are circular, double-stranded DNA molecules. In humans, the two strands are conventionally referred to as the heavy- and light-strand (H- and L-strand) because of the disparity in guanine content, enabling separation of the two mtDNA strands on caesium chloride gradients. The genetic information contained encodes components of the mitochondrial translational machinery and subunits of the electron transport complexes and ATP synthase, although the exact complement varies between organisms. The mtDNAs of all organisms studied to date encode a minimum of 2 rRNAs (large subunit and small subunit which are the equivalents of the *E. coli* 16S and 23S rRNAs), cytochrome *b* and cytochrome *c* oxidase subunit I (COXI). The complement of transfer RNA (tRNA) genes varies considerably, from kinetoplastids, in which the mitochondria appear to import all tRNAs from the cytosol, to those of plants, which import a few nuclear-encoded tRNAs, to vertebrate mtDNA, which

encodes 22 tRNAs - sufficient to decode the mitochondrially encoded protein genes. (Review Gray, 1992). Plant mitochondrial DNA also encodes a 5S rRNA. Both plant and many protistan mtDNAs contain genes for ribosomal proteins. Some fungal and plant mitochondrial genomes contain coding sequences for maturases, which are required for post-transcriptional processing of precursor RNAs. RNA editing of mitochondrial transcripts is required for appropriate gene expression in kinetoplastids and in plants

The role of the mitochondrial genome is essentially consistent in all these organisms, that is to specify a limited number of constituents of the oxidative phosphorylation system and of an apparatus which enables the synthesis of polypeptide subunits from the mitochondrially encoded mRNAs. Given that all known respiration-competent mitochondria contain DNA, it is notable that at least in most organisms, mtDNA does not encode either the replicative or transcriptional machinery. Therefore, either mtDNA has always been dependent on nuclear-encoded machinery, or at some point in time the mitochondrial genome was independent of the nuclear genome, encoding all the necessary components for biogenesis, but has since lost this information to the nucleus.

1.2.1 The endosymbiont hypothesis

Mitochondria are believed to be relics of an endosymbiotic relationship between a primitive eukaryote and an engulfed prokaryote (Gray, 1992). This theory was based initially on the similarity between organellar and eubacterial translation systems, but has been more recently supported by comparative sequence analysis of prokaryotic, nuclear and organellar genomes. These phylogenetic analyses have primarily used ribosomal RNA sequences and respiratory chain coding sequences. The modern genome bearing coding sequences most similar to mtDNA belongs to the α-Proteobacteria, e.g. *Agrobacterium* (Yang *et al.*, 1985; Raitio *et al.*, 1987). Another

study has utilised the protein sequences of the mitochondrial heat shock proteins (Hsp 60s), which suggests that the chaperonins of the Ehrlichia/Rickettsia cluster of the α-Proteobacteria show the closest relationship to the mitochondrial chaperones of eukaryotes from all kingdoms (Viale and Arakaki, 1994). However, it has been proposed by some authors that multiple primary endosymbiotic events may have occurred, leading to the progenitors of the fungal, plant and metazoan kingdoms (Kochel and Kuntzel, 1982; Gray *et al.*, 1984). One analysis based on the relative abundance of di-, tri-, and tetranucleotides in mitochondrial genomes suggests that the animal mitochondrial genome may be descended from a *Sulfolobus*- or *Mycoplasma*-like endosymbiont, rather than an α-Proteobacteria (Karlin and Campbell, 1994).

If the endosymbiont hypothesis is correct, then the original genome of the engulfed prokaryote should have encoded all components necessary for biogenesis. Therefore, it has been proposed that many genes (e.g., those encoding the majority of the oxidative phosphorylation system) have been transferred to the nuclear genome from the organellar genome. However, all eukaryotes studied to date possessing respiration-competent mitochondria contain a mitochondrial genome, however minimal. Why has a mitochondrial genome persisted in the eukaryotic cell? One theory is that some proteins encoded by mtDNA must be produced within the mitochondrion because they would be toxic when cytosolically expressed, or their importation into the organelle is for some reason not possible. As a general theory this can only apply to two proteins, cytochrome *b* and cytochrome *c* oxidase subunit I, because the genes encoding them have been found in the mtDNAs of all eukaryotes to date. Alternatively it has been suggested that at a given point in cellular evolution the transfer of genetic information from organelles to the nucleus ceased, perhaps due to increasing divergence in the gene expression systems used in the two compartments. However, within relatively recent evolutionary time, i.e., during angiosperm evolution, the gene encoding the cytochrome *c* oxidase II subunit has been transferred to the nuclear genome in legumes, probably via an RNA-mediated mechanism

(Nugent and Palmer, 1991). In both cowpea and soybean the functional copy of the gene for *coxII* is located in the nuclear genome (Covello and Gray, 1992). Another possible explanation for the retention of a mitochondrial genome is that by some mechanism it confers greater sensitivity to respond to the energy requirements of the cell.

1.2.2 Organisation of the mammalian mitochondrial genome

The mammalian mitochondrial genome encodes 2 ribosomal RNAs, 22 transfer RNAs (sufficient for mitochondrial translation), and 13 protein subunits of the electron transport complexes and ATP synthase (fig. 1.2). The proteins encoded are subunits 1-6 (including 4L) of NADH dehydrogenase (complex I), cytochrome *b* (complex III), subunits I-III of cytochrome *c* oxidase (complex IV) and subunits 6 and 8 of ATP synthase. The coding information is tightly packed in the ~16 kb genome, with very little intergenic sequence. Many protein-coding genes end with a 'T' or 'TA' after the final sense codon, and the actual stop codon ('TAA') is generated by polyadenylation of the messenger RNA.

Only two non-coding regions are contained within the mammalian mitochondrial genome, and only one is of significant length. The major non-coding region is called the control region or D-loop region, for displacement loop. It spans 879 bp in the mouse mitochondrial genome, and 1122 bp in the human mitochondrial genome (Bibb *et al.*, 1981; Anderson *et al.*, 1981). This region contains the transcriptional promoters for both the H- and L-strands (mitochondrial transcription is polycistronic, producing primary transcripts the length of the entire genome) and the replication origin for leading-strand synthesis (replication initiates in a unidirectional fashion). The D-loop is so called due to the unusual 3-stranded structure characteristic of the region; the H-strand is displaced by a nascent segment of H-strand (also known as 7S DNA, extending 520-700 nt in mouse, 570-655 nt in human) which is repeatedly

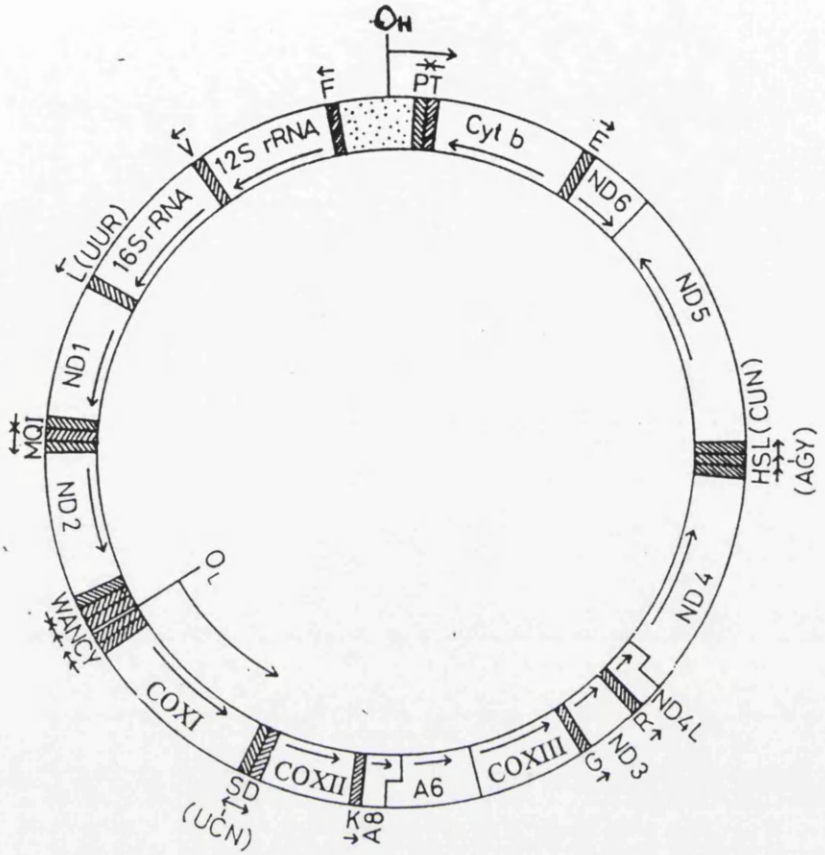


Figure 1.2 Structure of the mammalian mitochondrial genome

The origins of heavy strand and light strand replication are noted as O_H and O_L, respectively. The D-loop is indicated by the shaded box. The large and small ribosomal subunits are designated 16S rRNA and 12S rRNA, respectively. Genes for the tRNAs are designated by the standard one-letter symbol. Protein coding genes are designated as follows: ND1, ND2, ND3, ND4, ND4L, ND5, ND6 are subunits of NADH dehydrogenase; cyt *b* is cytochrome *b*; COXI, COXII, COXIII are subunits I, II and III of cytochrome *c* oxidase; A6 and A8 are subunits 6 and 8 of ATP synthase. The direction of transcription for both tRNA and protein coding genes is indicated by the accompanying arrows.

synthesised and degraded (Clayton, 1982). The shorter non-coding region (32 nt in mouse, 31 nt in human) contains the origin of lagging-strand (L-strand) replication and is surrounded by a cluster of tRNA genes (Bibb *et al.*, 1981; Anderson *et al.*, 1981).

1.2.3 Organisation of the plant mitochondrial genome

Plants are reported to contain a large range of linear and circular mtDNA molecules that may be hundreds of kilobases in size. Some plant species also contain mitochondrial plasmids - small linear or circular autonomously replicating molecules. A distinctive feature of plant mtDNA is the presence of repetitive sequences, frequently ranging from 1-10 kb in size, that facilitate recombination events. These recombination events generate rearranged mitochondrial genomes as well as subgenomic sized molecules. The "master circle" is the proposed mitochondrial genome which is the smallest size of molecule that can accommodate all the mtDNA mapped by restriction analysis for a given species. It is used as a conceptual reference point, although it may not in fact exist *in vivo*. Data from electron microscopy and pulse-field gels are not conclusive on this point. Not all repetitive sequence elements appear able to function as recombination sites, but no consensus sequence has been identified which could act as a substrate for a site-specific recombination system. Homologous recombination is the probable mechanism, although no *in vitro* assay has yet demonstrated this process in action (Hanson and Folkerts, 1992).

Although the plant mitochondrial genome is large (10 - 100-fold larger than the animal mitochondrial genome), it encodes essentially the same complement of respiratory chain genes as the mammalian mitochondrial genome (discussed in section 1.2.2) which is only ~16 kb. The plant mitochondrial genome encodes a few additional components of the translational apparatus, including multiple ribosomal

proteins. However, it is estimated that plant mitochondrial genomes contain ~100 ORFs, and may therefore encode a variety of other gene products (Oda *et al.*, 1992). The entire 367 kb mitochondrial genome of *Arabidopsis thaliana* has been sequenced (Unseld *et al.*, 1997). 57 genes have been identified, and another 85 potential ORFs of 100 codons or more are present. Whereas the variety in gene content between organisms studied to date is low, the amount of non-coding sequence in mtDNA varies substantially. In plants it is estimated that less than 10% of mtDNA is coding sequence, whereas in vertebrates coding sequence accounts for more than 90% of the genome; the distribution of coding sequence also differs, in that in plant mitochondrial genomes the genes are scattered, interspersed with non-coding sequences, whereas in animals the genes are contiguous. In contrast to animals, plants show great variability in the gene order between closely related species and within a species. This is the main source of diversity between plant mitochondrial genomes, as the mutation rate of plant mtDNA is estimated to be 0.2 substitution/site/billion years, roughly 100-fold less than animal mtDNA, and 10-fold less than nuclear DNA (Hanson and Folkerts, 1992; Gray, 1992; Albert *et al.*, 1996).

1.3 Mitochondrial biogenesis

As discussed in section 1.1, a growing body of evidence supports the view of the mitochondrial compartment within the mammalian cell as a dynamic and continuous network. This is compatible with what is known of the biogenesis of mitochondria, i.e., that organelles arise through growth and division rather than by *de novo* formation within daughter cells. The amount and composition of mitochondria is dependent on the specific needs of the given cell-type, e.g., an increased energy requirement as a result of repeated muscular contraction results in mitochondrial proliferation (Hood *et al.*, 1994). In order for the organelle to be responsive to cellular demands, it is likely that some mechanism exists for the nuclear genome to

influence mitochondrial activity. Conversely, it is possible that some mechanism exists for the mitochondrial genome to communicate with the nuclear genome.

1.3.1 Replication of mammalian mtDNA

Within the dynamic network of mitochondria reside multiple mtDNA molecules, of the order of 10^3 per cell (Clayton, 1982; Robin and Wong, 1988)). During the course of the cell cycle, mitochondrial DNA undergoes replication such that mtDNA copy number remains constant through generations of cell growth and division. However, any one molecule may be replicated multiple times or not at all within a given cycle (Flory and Vinograd, 1973). It has been reported that the replication event is not regulated temporally with respect to the phase within the cell cycle (Bogenhagen and Clayton, 1977), but the approach adopted may lack sensitivity given that the cultures were asynchronous. Other data suggests that the replication of mitochondrial and nuclear DNA may be coordinated (Van den Bogert *et al.*, 1993), but it is possible that the coordination observed in this case is an artifact caused by synchronisation of the cell culture. There may be a spatial determinant which affects whether a given molecule replicates within a cell cycle; in cultured rat neural cells it was shown that replication of mtDNA is first observed in molecules near to the nucleus (Davis and Clayton, 1996). It has been proposed that newly replicated mtDNA radiates outwards throughout the organelle network, and that this is achieved by movement of replicated molecules rather than transport/diffusion of a signal/factor required for the more peripheral molecules to replicate. The authors acknowledge that in other cell systems no such spatial determinism has been observed, perhaps due to the smaller size of the cell. It is therefore possible that the observation represents a special property of neural cells resulting from their shape/topology, rather than a general feature of mitochondrial replication.

Replication of mtDNA occurs within the mitochondrial matrix. It is dependent on the importation of protein, and possibly RNA from the cytosol, since all characterised components of the replication machinery are nuclear-encoded (reviewed by Clayton 1982, 1991). The control region for mitochondrial DNA synthesis is located within the non-coding region of the genome termed the D-loop (displacement loop), a 3-stranded structure in which a short segment of daughter H-strand DNA is hybridised to the L-strand displacing the parental H-strand. Replication by the mitochondrial DNA polymerase (DNA polymerase γ) is initiated at the heavy-strand origin (O_H) utilising a pre-existing RNA primer, which is generated by the transcription machinery. The existence of several discrete nascent L-strand transcripts terminating within the D-loop region has been demonstrated (Chang and Clayton, 1985; Chang *et al.*, 1985). Initiation of synthesis of the RNA primer for heavy strand synthesis and transcription of the L-strand of mtDNA both occur at the LSP sequence (L-strand promoter) and there are no known distinguishing features between the two events (Chang and Clayton, 1985; Chang *et al.*, 1985). Transcription proceeds through a region of three short conserved sequence elements called conserved sequence blocks (CSB) III, II, and I. During *in vitro* transcription by human mtRNA polymerase, persistent RNA-DNA hybrid structures are generated in the CSB region, referred to as R-loops (Xu and Clayton, 1996). It is within or downstream of CSB I that the transition from RNA synthesis to DNA synthesis occurs. It has been proposed that an endonuclease recognises the RNA-DNA hybrid molecule in this region, and cleaves the nascent transcript, freeing the 3' OH group for priming DNA synthesis (Chang and Clayton, 1987).

The processing of the putative RNA primer is the subject of controversy. It has been suggested that RNase MRP (characterised in several species by Clayton and co-workers) is the endonuclease responsible for primer formation; however, the majority of RNase MRP is isolated from the nuclear fraction of the cell (Topper and Clayton, 1990) and has been localised to the nucleolus (Yuan *et al.*, 1989). Using *in situ*

hybridisation a mitochondrial localisation for a minor fraction of RNase MRP has been shown in mouse (Li *et al.*, 1994). Utilising model R-loops, mouse RNase MRP has been shown to cleave the RNA-DNA hybrid molecule appropriately for the generation of replication primers including the site appropriate to the major species of H-strand DNA that has been isolated, which has a 5' end located within or downstream of CSB I (Lee and Clayton, 1997). The low abundance of MRP RNase in the mitochondrial compartment of cells such as HeLa (Kiss and Filipowicz, 1992) can be accounted for by imputing to the endonuclease a regulatory role in the rate of mtDNA replication (Topper *et al.*, 1992).

It is possible that there is no universal mechanism for the initiation of mitochondrial DNA replication. Another endonucleolytic enzyme has been localised to both mitochondrial and non-mitochondrial compartments within the cell, endonuclease G. This enzyme, isolated from calf thymus, is capable of generating RNA primers suitable for the initiation of mtDNA replication. An RNA-DNA heteroduplex which contained the mouse mtDNA control region was recognised as a substrate (Cote and Ruiz-Carrillo, 1993). However, none of the cleavage sites generated by this enzyme corresponds to the exact terminus of the major, nascent H-strand species (Chang and Clayton, 1985; Chang *et al.*, 1985). The detection of a possible transcription pause site in CSB I (using an *in organello* system with rat liver mitochondria) suggests that endonuclease activity may not be required for primer formation at this site (Cantatore *et al.*, 1995). *In vitro* transcription experiments demonstrated the formation of persistent RNA-DNA hybrids. The RNA species had 3' ends which mapped within or downstream of CSB I, supporting the hypothesis for a transcription pause site here (Xu and Clayton, 1996). Thus, the actual role of MRP RNase could conceivably be for primer removal.

DNA synthesis proceeds unidirectionally from OH by strand displacement. It is unclear whether leading-strand replication occurs via *de novo* DNA synthesis from

the RNA primer, or by extension of the pre-existing D-strand in the D-loop. Once DNA synthesis has reached approximately two-thirds of the way around the genome, the O_L (light-strand origin) is exposed on the displaced strand. Although the sequences for the light-strand origins are highly diverged between species, a potential stem-loop secondary structure is conserved (Martens and Clayton, 1979, Tapper and Clayton, 1981). Here a mtDNA-specific primase initiates priming and DNA synthesis of the lagging strand (Wong and Clayton, 1985a, b). In human and mouse no alternative priming site for lagging strand synthesis has been detected. It is believed that the O_L plays no regulatory role, i.e., if replication is initiated at O_H, second strand synthesis will begin automatically once the O_L is exposed. Segregation of the two daughter molecules precedes the completion of L-strand replication.

Replication of mtDNA is controlled by the nuclear genome. There are several potential points of regulation in this model of replication. Both the hypothetical 5' and 3' processing events of the primer for H-strand synthesis are obvious candidates. Another is the point at which DNA synthesis extends beyond the D-loop; some mechanism must regulate whether DNA synthesis terminates to form the D-strand, proceeds beyond that point to replicate a full strand of the genome, or indeed uses the D-strand as a substrate for elongation. A bovine protein has been isolated which binds to the replication pause site on both bovine and human template DNA in a sequence-specific manner (Suzuki *et al.*, 1996). The provision of one or more components of the system (e.g., the DNA polymerase) by the nuclear genome may be regulated. As an example, functional binding sites for nuclear respiratory factor-1 (NRF-1), a transcription activator, have been demonstrated in the regulatory region upstream of the genes encoding both human and mouse RNase MRP RNA. NRF-1 is a candidate for playing a coordinating role in mitochondrial and nuclear respiratory gene expression (Evans and Scarpulla, 1990, Virbasius *et al.*, 1993). Finally, mitochondrial transcription factor A (mtTFA) may play a role in RNA primer

formation due to its involvement in promoter selection by the transcriptional apparatus (see section 1.3.2).

1.3.2 Transcription and processing of mitochondrial RNA

As discussed in section 1.3.1, the D-loop region is the control region for mitochondrial replication and transcription. The promoters for both L-strand and H-strand transcription (LSP and HSP, respectively) are located within this region. Both promoters contain binding sites for the transcription factor mtTFA, which has been shown to activate transcription *in vitro* (Fisher *et al.*, 1987) and *in vivo* (Montoya *et al.*, 1997). mtTFA has the capacity to unwind and bend DNA, which may be necessary for precise transcriptional initiation to take place (Fisher *et al.*, 1992). The binding affinity of mtTFA for the LSP is greater than for the HSP, which corresponds to the LSP being more active *in vivo* and *in vitro.* However, the exact role of mtTFA has yet to be determined. Human mtTFA (h-mtTFA) binds to the D-loop region of mtDNA at regularly phased intervals of ~40-50 bp apart in a non-sequence specific manner, both *in vitro* and *in organello.* These intervals coincide with the spacing of transcriptional and replicative start sites (Ghivizzani *et al.*, 1994). h-mtTFA contains two high-mobility-group-like domains (Parisi and Clayton, 1991). Thus, one of the main functions of h-mtTFA appears to be the packaging of the mtDNA in the control region.

Transcription in mitochondria is polycistronic, thus, the L-strand is transcribed as a single transcriptional unit, beginning upstream of the replication origin for the H-strand. This transcript has a low informational content, in that it contains only 8 tRNAs and the mRNA for ND6. If the model of replication priming by a processed L-strand transcript is correct, then a full-length, polycistronic, mitochondrial transcript could also provide the H-strand replication primer. Alternatively, an independent initiation event could provide RNA primers for replication. The

existence of several species of nascent L-strand RNAs terminating within the D-loop region has been shown (Chang and Clayton, 1985; Chang *et al.*, 1985).

There are two major species of H-strand transcripts; one spans the rDNA region only, and the other the entire genome. The rRNA genes and adjacent tRNA genes are transcribed more frequently than the other H-strand genes; estimates range from ~10 times more frequently (in primary rat hepatocytes, Cantatore *et al.*, 1987) up to ~50 times more frequently (in HeLa cells, Gelfand and Attardi, 1981). The 3' ends of the 16S rRNA exhibits heterogeneity, suggesting it is the product of a transcriptional termination mechanism rather than a precise processing event (Dubin *et al.*, 1982). Using an *in vitro* system derived from human mitochondria, a bidirectional termination sequence was identified at the 16S rRNA-tRNA$^{Leu(UUR)}$ gene boundary (Christianson and Clayton, 1986; Christianson and Clayton, 1988). A DNA-binding protein, mTERF, which binds to this region with high affinity and promotes transcriptional termination has been identified (Kruse *et al.*, 1989; Micol *et al.*, 1997).

The literature is contradictory about the site of transcriptional initiation for the polycistronic H-strand transcript. Research originating from Attardi's group indicates that there are two initiation points for H-strand transcripts; one is proximal to the 5' terminus of the 12S rRNA gene, responsible for the transcript which contains the mRNAs for the protein-coding genes, the other is located 20-40 bp upstream of the tRNA^{Phe} gene, and is responsible for the synthesis of the rRNAs only (Montoya *et al.*, 1982; Montoya *et al.*, 1983; Micol *et al.*, 1997). Interpretation of the data by Clayton's group suggests that the template-directed transcriptional termination (mediated by mTERF) at the 16S rRNA-tRNA$^{Leu(UUR)}$ gene boundary is necessary and sufficient to explain the increased levels of rRNA transcription in comparison to the transcription of the protein-coding genes (Christianson and Clayton, 1986; Clayton, 1991). However, it was reported that mtTFA/H-strand promoter complexes did not give clear footprints *in vitro*, and involvement of another factor/s in transcriptional

initiation was postulated (Fisher *et al.*, 1987). Thus, the sensitivity of rRNA synthesis (but not mRNA synthesis) to ATP levels at the point of initiation could indicate the involvement of additional factors in transcriptional initiation , and supports the theory of an independent H-strand promoter for rRNA synthesis (Micol *et al.*, 1997).

The processing of the polycistronic mitochondrial transcripts requires the activity of at least one endonuclease to cleave precisely at the junctions between mRNAs, tRNAs and rRNAs. The tRNA punctuation model of mitochondrial RNA processing proposes that the secondary structure of the tRNA sequences, which are interspersed amongst the mRNA and rRNA sequences, may represent the primary recognition signal for processing (Ojala *et al.*, 1981). It is noteworthy that a point mutation, identified in a case of human disease, which localises to the aminoacyl stem of a mitochondrial tRNA gene, has been associated with RNA processing defects (Bindoff *et al.*, 1993). Northern blot analysis of RNA prepared from skeletal muscle and cultured skin fibroblasts indicate that RNA processing occurs in a tissue-specific manner. At junctions which lack a tRNA, it has been proposed that a stem-loop structure resembling a portion of a tRNA could function as the recognition signal (Ojala *et al.*, 1981). A mitochondrial ribonuclease P has been identified and characterised as the mitochondrial processing endonuclease responsible for the cleavage on the 5' side of the tRNA sequences (Rossmanith *et al.*, 1995). Cleavage of the 5' end of the tRNA precedes cleavage of the 3' end by a separate endonuclease. All mitochondrial tRNAs require the post-transcriptional addition of the 3' terminal CCA, as it is not encoded by the mitochondrial genome. As mammalian mitochondrial DNA does not contain any introns, no splicing machinery is required. mRNAs and the rRNAs are polyadenylated, and it has been suggested that polyadenylation may be associated with the processing step which releases the 3' end of these RNA products from the polycistronic transcript (Ojala *et al.*, 1981).

It has been reported that transcription of mtDNA in both HeLa cells (Gelfand and Attardi, 1981) and in rat liver cells (Cantatore *et al.*, 1987) is in excess of cellular requirements, and is not rate-limiting for mitochondrial protein synthesis.

1.3.3 Mitochondrial translation

Processing of the polycistronic RNA transcripts produces 11 mature transcripts, 9 of which are monocistronic mRNAs, and 2 of which are bicistronic mRNAs encoding overlapping reading frames. The mRNAs are uncapped and contain essentially no 5' untranslated sequence that could, for example, promote ribosome binding. This distinguishes mitochondrial mRNA from both cytosolic and bacterial ribosomal recognition systems. Analysis of secondary structure and RNAse protection experiments suggest that secondary structure in the 5' terminal region of mitochondrial mRNAs is integral to recognition by mitochondrial ribosomes, and mitochondrial initiation factors may be required for proper recognition and melting to allow translational initiation (O'Brien *et al.*, 1990).

Translation of the mitochondrial mRNAs by mitochondrial ribosomes yields 13 subunits of the mitochondrial respiratory chain. All protein components of the protein synthetic apparatus are nuclear-encoded; this includes ~85 different ribosomal proteins (nearly twice as many as in bacterial ribosomes) (O'Brien *et al.*, 1990). In yeast, many mRNA-specific translation factors have been identified (Dunstan *et al.*, 1997; Steele *et al.*, 1996) and such factors may also be involved in mammalian mitochondrial translation. In yeast, the putative RNA stem-loop structure found in the 5'-untranslated leader of the COXIII mRNA, which interacts with a COXII-specific translational activator (Dunstan *et al.*, 1997), may be functionally homologous to the putative stem structure identified at the 5' terminus of the bovine COXII mRNA (O'Brien *et al.*, 1990). Also in yeast are nuclear-encoded protein factors required for the post-translational assembly of specific mitochondrial subunits,

e.g., the SCO1 protein which is required for the correct assembly of COXI and COXII (Krummeck and Rodel, 1990).

Both of the ribosomal RNAs and all 22 transfer RNAs are mitochondrially encoded. In mammals only 22 tRNAs are required to decode the mitochondrial mRNAs because the genetic code differs slightly from the universal genetic code (Table 1.1), and because of expanded wobble that permits a single tRNA to decode all four codons, specifying some amino acids. Although most mitochondrial tRNAs can form the conventional cloverleaf structure, many deviations are observed as regards secondary structure (e.g., stem length) and sequence (nucleotides which are invariant in non-mitochondrial tRNAs and are involved in the tertiary structure) (Sprinzl *et al.*, 1985). It has been proposed that N-formyl-methionine is the amino terminal amino acid in mtDNA-encoded proteins, (this has been confirmed for COXI, Hensel and Buse, 1990) suggesting initiation of mitochondrial protein synthesis may require N-formyl-methionyl-tRNA (Chomyn *et al.*, 1981).

	TGA	ATA	AGA	AGG
Universal	stop	Ile	Arg	Arg
Mammalian mt	Trp	Met	stop/NF	stop/NF

Table 1.1 Mammalian mitochondrial genetic code

The table lists the differences between the universal genetic code and the mammalian mitochondrial genetic code. The four codons which specify different amino acids, or translational stops, within the two systems head the columns. NF indicates the codon is not found in the mitochondrial genomes of some mammalian species.

Given that some components of the respiratory chain are encoded by the nuclear and some by the mitochondrial genome, and that the assembled complexes require the protein subunits in equimolar amounts, it is puzzling as to why the mRNAs for the

mitochondrial subunits are transcribed at a much higher level than those for the nuclear-encoded subunits. It has been suggested that only a small proportion of mitochondrial mRNA is destined to be translated. Alternatively, it has been suggested that mitochondrial translation may be inefficient (in comparison to that in the cytosol) and that the relatively high levels of mitochondrial transcripts are necessary to ensure equimolar amounts of the subunits for the respiratory chain complexes (Ostronoff *et al.*, 1996; Cantatore *et al.*, 1987; Van den Bogert *et al.*, 1993).

One hypothesis to account for developmental and/or physiological regulation of mitochondrial gene expression is that it is simply a gene dosage effect (Williams, 1986). However, this does not appear to apply to all physiological conditions (Van den Bogert *et al.*, 1993). As an example, altering the thyroid state in rats did not change the copy number of mtDNA in various tissues despite substantial changes in the content of mitochondrial mRNAs (Wiesner *et al.*, 1992). These authors found that mitochondrial gene expression in this situation is controlled directly at the transcriptional level. During cellular differentiation, mitochondrial gene expression may also be regulated at the level of translation, in contrast to the regulation of mitochondrial transcription which appears to occur during organelle proliferation (Ostronoff *et al.*, 1996).

1.3.4 Protein import

As only 13 mitochondrial proteins are actually synthesised within the mitochondria, hundreds of nuclear-encoded mitochondrial proteins need to be synthesised within the cytosol, then imported into mitochondria. The protein import machinery within yeast has been extensively characterised, and, as far as is known, the general mechanism is broadly applicable to mammalian mitochondria as well (reviewed by Neupert, 1997; Kubrich *et al.*, 1995). Although some proteins are imported by mechanisms requiring

specific carrier proteins, there is a general import mechanism for the majority of imported proteins. Once the nuclear encoded mitochondrial proteins have been imported, intra-mitochondrial sorting and processing occurs.

Nuclear-encoded mitochondrial precursor proteins generally have amino-terminal extensions which serve as mitochondrial targetting sequences, although there are exceptions to the rule (Mitoma and Ito, 1992). These presequences are positively charged and are capable of forming amphipathic α-helices (the positively charged and hydrophobic residues facing opposite sides of the helix) (von Heijne, 1986). These presequences have been shown to be necessary and sufficient for mitochondrial import using the cytosolic protein dihydrofolate reductase as a reporter (Horwich *et al.*, 1985; Hurt *et al.*, 1985). Although there is no consensus sequence, a three-amino acid motif within the transit peptides has been identified as common to a set of 50 surveyed leader sequences (Hendrick *et al.*, 1989).

In order to be targeted correctly, the cytosolically synthesised mitochondrial precursor must adopt a conformation such that the targeting peptide is presented on the surface of the molecule. Cytoplasmic chaperone proteins associate with the precursor proteins in order to maintain a loosely folded, import-competent conformation (Murakami *et al.*, 1988; Murakami and Mori, 1990; Caplan *et al.*, 1992; Hachiya *et al.*, 1993). Receptor proteins in the mitochondrial outer membrane interact with the precursor protein which is then delivered to a protein import channel across the outer membrane (Lill and Neupert, 1996). Translocation across the inner membrane initially requires membrane potential (which drives the presequence across) (Martin *et al.*, 1991). One subcomplex of the inner membrane complex forms a protein-conducting channel; the other subcomplex involves mitochondrial chaperones on the matrix side, which promote translocation across the membranes (Berthold *et al.*, 1995; Blom *et al.*, 1993; Gambill *et al.*, 1993). mhsp70 can mediate both transport across the inner membrane and protein folding in the matrix, dependent on which

complex it is within (Horst *et al.*, 1997). Cleavage of the transit peptide occurs during or after translocation (Hawlitschek *et al.*, 1988; Yang *et al.*, 1988). Refolding of proteins in the matrix is mediated by a variety of molecular chaperones (Rospert *et al.*, 1996; Matouschek *et al.*, 1995; Rassow *et al.*, 1995). Precursors of subunits of the electron transfer system, which are assembled into the respiratory complexes in the inner membrane, may either be targeted to the matrix for processing and sorting, or targeted directly to the inner membrane, by a hydrophobic stop-transfer signal (Hurt *et al.*, 1984; Miller and Cumsky, 1991).

1.3.5 Transmission of mammalian mitochondrial DNA

1.3.5.1 Germ-line transmission

It is conventional to describe the inheritance of mtDNA as strictly maternal in mammals. However, the failure to detect transmission of paternal mtDNA could be due to the limitations of technique. It has been estimated that there are 10^5 mitochondrial DNA molecules in the egg, both of mouse and of human, whereas an individual sperm contains only about 10^2 mtDNA molecules (Piko and Matsumoto, 1986; Chen *et al.*, 1995). Utilising PCR, a very low level of paternal transmission has been demonstrated in interspecific, but not intraspecific, crosses in mice (Gyllensten *et al.*, 1991; Kaneda *et al.*, 1995). Gyllensten *et al.*, (1991) propose dispersion of the paternally-inherited mtDNA throughout the tissues of hybrid mice and persistent transmission to successive generations. However, data presented by Shitara *et al.*, (1998) shows that paternally-derived mtDNA is rarely present in ovarian tissue (6/91 F1 hybrid females), and the paternally-derived mtDNA was not present in 78 unfertilised eggs obtained from F1 hybrid females which were positive in the ovarian tissue (Shitara *et al.*, 1998). In intraspecific crosses Kaneda *et al.*, (1995) have shown that paternal mtDNA is detected at the early pronucleus stage, but is then eliminated. They then crossed a congenic strain (B6.mt^{spr}), which has a *Mus musculus* (B6) nuclear background but *Mus spretus* mtDNA, with B6 females, and demonstrated

elimination of the paternal mtDNA as in normal intraspecific crosses. They therefore propose a species-specific mechanism for the elimination of paternal mtDNA by the oocyte which recognises one or more nuclear-encoded factor(s) (Kaneda *et al.*, 1995). If this is the case, one wonders how the mechanism evolved, for intuitively a sperm which had demonstrated its respiratory fitness by successfully reaching an egg should contain functional mitochondria which would contribute to the fitness of the offspring. It has been suggested that maintaining homoplasmy of mtDNA (which would necessitate uniparental transmission) may better enable it to interact effectively with the nuclear genome (Poulton, 1995).

Whereas a great deal of speculation has focussed on a maternal inactivation mechanism of the paternally-derived mtDNA, recent data suggests that the male germline may inactivate its own mtDNA. Mitochondrial transcription factor A (mtTFA) is a transcriptional activator of mammalian mtDNA, and thus plays a contributory role in mtDNA replication (chap.1.3.1). In both mouse and human it has been shown that there are testis-specific mtTFA transcripts. In mouse the testis-specific protein isoform is imported to the nucleus rather than to the mitochondria of spermatocytes and spermatids (Larsson *et al.*, 1996). In humans, however, no such nuclear isoform is predicted from the sequence of the testis-specific transcripts. In differentiated human male germ cells a high level of testis-specific mtTFA transcripts correlates with a down-regulation of mtTFA protein levels and a parallel decrease in the amount of mtDNA (Larsson *et al.*, 1997).

In the female germline it has been proposed that mtDNA transmission to the subsequent generation proceeds through a genetic bottleneck, also referred to as the ploidy paradox. The mechanism of this bottleneck seems to be mitotic segregation during germ-cell development (see following section). The number of independently segregating units in oocyte mitochondria has been inferred to be approximately 200, based on experiments with heteroplasmic mice (Jennuth *et al.*, 1996). This genetic

founder effect would result in rapid fixation of a mutant genotype if the mutant were amongst the molecules replicated in a 'successful' oocyte. Rearranged mtDNA molecules have been detected in oocytes (Chen *et al.*, 1995). However, the authors suggest that the low levels detected (maximally 0.1%) support the idea of a genetic bottleneck which filters out deleterious molecules. In cows heteroplasmic for a neutral point mutation the mtDNA genotypes of the progeny could segregate for either of the maternal mtDNA variants, and could become homoplasmic within three generations (Ashley *et al.*, 1989). An interesting implication of maternal inheritance is that, if a mutation in mtDNA had a neutral effect on the phenotype in females, but a deleterious effect in males, selection pressure could not act directly to stop the mutation spreading in a population (Avise, 1991)

1.3.5.2 Mitotic segregation

Mitochondria, and the DNA molecules they contain, appear to be randomly partitioned between daughter cells (Papa *et al.*, 1996). In mammals, normally all the mitochondrial genomes found within a given organism will be identical, a situation known as homoplasmic. If a cell harbours a mixed population of genetically different mitochondrial genomes, it is called heteroplasmic. In humans, this situation has been reported only in cases of disease-associated mutations. Over multiple generations of random partitioning during cellular division of heteroplasmic cells, the complement of mitochondrial DNA can shift, as a result of random partitioning, accompanied in some cases by selection, towards a higher proportion of mutant or wild-type mtDNA. This process is referred to as mitotic segregation.

1.4 Rearrangements in mitochondrial DNA

1.4.1 Rearrangements in fungal mtDNA

The mitochondrial genomes of fungi, as of plants, exhibit great diversity. Closely related species can have mitochondrial genomes which vary in size by a factor of three, and even different strains of the same species can exhibit size variation due to the presence or absence of introns, and the number of repeat units found in the intergenic regions (Clark-Walker, 1992; Tzagoloff and Myers, 1986). Also in contrast to animal mitochondrial genomes is the variation in gene order which can be observed between similar species. As with plants, both linear and circular mitochondrial plasmids are commonly observed in natural isolates of fungi (Meinhardt *et al.*, 1990; Yang and Griffiths, 1993). The majority of these plasmids do not appear to be associated with an identified phenotype of the host organism. Likewise, although there is evidence for the mobility of intronic elements, most do not appear to confer a particular phenotype on the fungal "host".

However, there are several examples whereby mitochondrial introns or plasmids do confer a phenotype on their particular fungal host. One example is found in *Podospora anserina*. This ascomycete has been used as a research model for senescence. During aging, an intron located in the COXI gene becomes liberated, either through precise excision or via reverse transcription of an RNA intermediate, to form a circular plasmid (Osiewacz and Esser; 1984; Kuck *et al.*, 1985; Cummings *et al.*, 1985; Belcour and Vierny, 1986). The amplification of this plasmid is associated with senescence (Stahl *et al.*, 1978; Cummings *et al.*, 1979). In longevity mutants this plasmid DNA (plDNA or αsenDNA) is either absent, or amplification is delayed, suggesting that amplification of plDNA is integral to senescence (Vierny *et al.*, 1982; Koll *et al.*, 1985; Schulte *et al.*, 1988; Hermanns *et al.*, 1994). It is possible to

transform juvenile protoplasts of *P. anserina* with plDNA and thereby induce premature senescence (Tudzynski *et al.*, 1980).

Loss of mitochondrial function during aging of *P. anserina* cultures has been demonstrated (Tudzynski and Esser, 1979). The cessation of vegetative growth has also been correlated with multiple recombination and deletion events of the mitochondrial DNA (Belcour *et al.,* 1981; Kuck *et al.,* 1981). It should be noted that two longevity mutants have been isolated which are associated with multiple recombination events in the mutant mitochondrial genome (Schulte *et al.,* 1988). However, the rearranged mitochondrial genomes are stably inherited. In these mutants the senescence-associated intron has been deleted as well as a portion of the COXI gene. Although the cells have no functional COXI, *Podospora* possesses an alternative enzyme pathway which allows these mutants to survive. It has been hypothesised that senescence is due to the loss of mitochondrial function associated with inactivation of mitochondrial genes by integration of plDNA (Osiewacz and Esser, 1984; Kuck *et al.,* 1985). Alternatively, a plDNA-encoded polypeptide may act as, or may induce, a recombinase, causing multiple rearrangements in the mitochondrial DNA of senescent mycelia (Schulte *et al.,* 1988).

In *Neurospora* as well there are examples of a senescent phenotype associated with the integration of circular (e.g., Mauriceville and Varkud) or linear (*kalilo* and *maranhar*) plasmids (Akins *et al.,* 1986; Bertrand *et al.,* 1985; Court *et al.,* 1991). As with the *Podospora* plDNA, these integration events are associated with rearrangements of the mitochondrial genome, and may produce the senescent phenotype via resultant mitochondrial dysfunction. The integration of *kalilo* or *maranhar* DNA causes the proliferation of defective mitochondrial DNA molecules resulting in the displacement of wild-type DNA. This leads to a decline in mitochondrial functions, senescence and eventual death (Bertrand *et al.,* 1985).

However, subgenomic molecules have also been found in small amounts in a wild-type strain of *Neurospora* (Gross *et al.,* 1984).

In *S. cerevisiae*, cells harbouring deleted mitochondrial DNA (referred to as ρ^-*petites*) arise spontaneously at a frequency of about 1% (Piskur, 1994). They can also be induced with ethidium bromide treatment and other chemicals, or can result from defective mitochondrial protein synthesis (Stribinski *et al.,* 1996). The molecules consist of a deleted portion of the mitochondrial genome, frequently maintained in the form of a large repetitive DNA molecule. The repeating unit may originate from any region of the mtDNA molecule, and may be organised in tandem array or in palindromic repetitions. Cells of ρ^- *petite* mutants contain the same total amount of mtDNA as wild-type strains (Sanders *et al.,* 1973; Faye *et al.,* 1973). The most common class of spontaneous ρ^- *petite* are termed suppressive *petites* because when crossed with wild-type strains they primarily produce *petite* progeny.

The ploidy paradox (discussed in section 1.3.5) also aplies to yeast; in yeast 50 mtDNA molecules is the average number per cell, 100 molecules in a diploid zygote. Although ~40 molecules enter the first bud, the genetically segregating units are estimated to be only 3-4 in number (Dujon *et al.,* 1974; Birky *et al.,* 1978). It has been postulated that one possible mechanism to achieve this would be sequestration of the replication machinery, i.e., if a molecule has already replicated it has an enhanced probability of replicating again due to access to the replication machinery (Gingold, 1981; Birky, 1983). Thus, a form of competition amongst mtDNA molecules may exist in which *cis* elements are important in determining transmission to progeny; in yeast ρ^- *petites* the intergenic regions seem to be important (Piskur, 1988; Blanc andDujon, 1980). In yeast, another pertinent factor is the site of budding; although mitochondrial proteins disperse and mix soon after parental mitochondria fuse, the mtDNA appears to be attached to the mitochondrial membrane (Azpiros and Butow, 1993; Nunnari *et al.*, 1997).

Recombination of yeast mtDNA in respiration-competent cells can involve both homologous and site-specific recombination (Rayko *et al.,* 1993). The homologous recombination machinery is thought to be involved in *petite* formation (Boulet *et al.,* 1990). Recombination hotspots in *Neurospora*, *Podospora*, and *S. cerevisiae* occur in regions where single-strand breaks are deemed likely to occur or be stabilised, such as in hairpin loops and regions with extended repeats (Ripley, 1982). Repetitive sequences, both G/C clusters (Skelly and Clark-Walker, 1990) and A/T-rich repeats (De Zamaroczy *et al.,* 1983), are implicated in the recombination events that lead to ρ^- *petite* formation. As few as 6 bp of homology have been shown to be sufficient for the formation of ρ^- *petites* (Sor and Fukuhara, 1983). The involvement of a mismatch-repair mechanism in the intramolecular recombination process has been implicated in *Neurospora* (Gross *et al.,* 1989). A recombination/repair endo-exonuclease NUC2 is involved in the production of *petites*, both spontaneously and in the presence of ethidium bromide (Chow and Kunz, 1991). A cruciform-cutting endonuclease, CCE1, thought specifically to resolve Holliday junctions in mitochondria, has been localised to the yeast mitochondrial inner membrane (Ezekiel and Zassenhaus, 1993). The gene encoding the protein (also known as MGT1) had previously been shown to be required for biased transmission of ρ^- *petite* mtDNA in crosses between respiration competent and hypersuppressive *petite* cells (Zweifel and Fangman, 1991). However, the *mgt1/cce1* mutation elevates the frequency of *petite* molecules in respiration-competent strains, and homologous recombination is still observed (Piskur, 1997).

In summary, rearrangements of mtDNA correlate with mitochondrial dysfunction in *Podospora, Neurospora,* and *S. cerevisiae.* The mechanism by which mtDNA rearrangements arise in these organisms may involve mitochondrial DNA repair machinery, and repetitive DNA sequences.

1.4.2 Rearrangements in plant mtDNA

As discussed in section 1.2.3, rearrangements of the mitochondrial genome are not necessarily associated with a mutant phenotype in plants. However, rare recombination events do occur at sites other than in the large, recombinational repeat sequences, which lead to the creation of chimeric genes. These events often involve short homologous repeats. Two classes of maternally inherited disorder are linked to rearrangements of mtDNA in plants; the first is cytoplasmic male sterility (CMS), the second is abnormal growth associated with striped leaves (non-chromosomal stripe, NCS).

1.4.2.1 Cytoplasmic male sterility

CMS mutants are frequently healthy and vigorous apart from a deficiency in pollen development, although the morphology of the inflorescence may be more widely affected in some species (for reviews see Pring and Lonsdale, 1989; Hanson and Folkerts, 1992). In many systems, the CMS phenotype involves aberrations in parental anther tissues just prior to male meiosis, particularly tapetal tissue. The tapetal layer both synthesises and stores large amounts of nutrients (e.g., nucleic acids, proteins and lipids) which are used by the pollen mother cells and the developing pollen grains (Conley and Hanson, 1995). Male sterility can be caused by nuclear mutations, through nuclear/mitochondrial incompatibility, or by rearrangements of the mitochondrial genome. In the case of mitochondrial rearrangements, the genotype is usually homoplasmic and tends to be stable over many generations. The rearrangements create novel ORFs which segregate with the CMS phenotype in a number of systems, including *Petunia*, maize, *Brassica*, radish, sunflower and rice (reviewed by Conley and Hanson 1995). The first two mitochondrial loci shown to correlate with the CMS trait were found in the CMS-T strain of maize (Dewey *et al.*, 1986) and the S-pcf strain of *Petunia* (Young and

Hanson, 1987). In both cases, multiple recombination events have led to a stably inherited, novel, chimeric gene which is expressed, producing a chimeric polypeptide and resulting in a tissue-specific phenotype.

In *Petunia*, CMS is associated with the presence of the novel, chimeric gene *pcf* (Boeshore *et al.*, 1985; Young and Hanson, 1987). The gene is composed of a copy of the first portion of the ATP synthase subunit 9 gene (5' flanking region and 35 codons), noncontiguous portions of exons 1 and 2 of *coxII* (158 codons), and an unidentified reading frame, *urf-s* (157 codons) (Young and Hanson, 1987). Although no sequences homologous to *urf-s* were found in the database, it has been reported that an *urf-s* probe hybridised to total DNA prepared from fertile petunia lines, and the progenitor sequence may therefore be present in 1 of the 3 plant genomes (Nivison *et al.*, 1994). Northern analysis has demonstrated that in CMS *Petunia* the *pcf* gene is transcribed. Three 5' termini have been mapped for the *pcf* transcripts, and normal *atp9* and *coxII* transcripts are also present. The major species of *pcf* transcript (which is the shortest transcript) is present at a 5-fold higher level in anthers compared to leaves, whereas the *atp9* transcripts are roughly equivalent between tissues. The absolute level of the *pcf* transcript is also high in ovaries, but the authors discount the significance of this due to the increase in absolute levels of all mitochondrial RNA species in ovarian tissue. The *pcf* transcript is not present in fertile lines (Young and Hanson, 1987). In fertile revertant lines restored by the nuclear gene *Rf*, RNA levels of the shortest of the 3 mapped *pcf* RNA species are reduced (Pruitt and Hanson, 1991). The *pcf* gene is cotranscribed with the downstream genes *nad3* and *rps12* (Rasmussen and Hanson, 1989). The *atp9-coxII* portion of the *pcf* transcript, as well as the downstream *nad3* and *rps12*, are edited normally (Lu and Hanson, 1992; Nivison *et al.*, 1994).

The predicted molecular mass of the *pcf*-encoded polypeptide is 43 kDa. The apparent molecular weight of this protein on denaturing gels is 55 kDa. However, the

full-length protein was only detected by immunoprecipitation of protein from purified mitochondria, as it is a precursor protein which is rapidly processed (Nivison *et al.*, 1994). The mature protein, referred to as PCF, which had previously been shown to be specific to CMS lines, has an apparent molecular weight of 25 kDa (Nivison and Hanson, 1989) and an actual molecular mass of 19.5 kDa. Amino-terminal sequencing of PCF demonstrated it was encoded entirely by the *urfS* portion of the chimeric gene (Nivison *et al.*, 1994). The other putative processing product(s) of the precursor protein are not detectable by anti-ATP9 or -COXII antiserum, although the anti-COXII antiserum could detect the full-length precursor protein (Nivison *et al.*, 1994). PCF has been detected in both soluble and membrane fractions, and is therefore thought to be peripherally associated with the inner membrane rather than an integral membrane protein (Nivison and Hanson, 1989; Nivison *et al.*, 1994). The abundance of PCF is reduced in fertility restored *Petunia* lines (Nivison and Hanson, 1989). The protein is highly expressed in sporogenous tissue and the tapetal layer of the premeiotic and meiotic anthers in sterile plants, as well as in vascular tissue, an expression pattern similar to ATP synthase (Conley and Hanson, 1994).

Mitochondrially targeted PCF has been expressed in transgenic plants using both a constitutive promoter and a tapetal-specific promoter, but the plants were male fertile (Wintz *et al.*, 1995). As both promoters exhibit low activity in sporogenous tissue in early meiosis, the constructs may have failed to mimic an essential factor in the production of the CMS phenotype. Additionally, the expression pattern differed from that observed in CMS plants as all transgenic PCF protein was present in the soluble fraction. The constructs contained only the *urf-S* region which encodes the 25 kDa protein; thus, the amino-terminal portion of the chimeric protein may include membrane-targetting information integral to the phenotype (Wintz *et al.*, 1995).

In the CMS petunia strain a reduction in electron transport through the cyanide-resistant alternative oxidase pathway has been documented by oxygen electrode

analysis of respiration in suspension cells and suspension cell mitochondria (Connett and Hanson, 1990). The alternative oxidase pathway allows biosynthetic activity to continue in the presence of high levels of ATP, which may be important to tissues such as the tapetal layer and sporogenous tissue. The presence of the nuclear gene *Rf* restores fertility, reduces the abundance of the *pcf*-encoded peptide, and restores the activity of this pathway. Connett and Hanson (1990) propose that the activity of this pathway may be critical to pollen development and that PCF protein may directly disrupt its regulation.

Three strains of maize have been identified which carry the CMS trait. In the CMS-T strain, the associated chimeric mitochondrial gene is *urf13*, which is derived from the 5' flanking region of *atp6*, the 3' flanking region of the 26S rRNA gene (88 amino acids), 9 amino acids derived from an unknown sequence, and part of the 26S rRNA coding region (18 amino acids). *Urf13* encodes a 13 kDa integral membrane protein which has been detected in all tissues of CMS-T maize plants tested (etiolated seedlings, immature cobs, green leaves, roots). The presence of the nuclear restorer gene *Rf1* results in a decrease in abundance of the 13 kDa protein, and an alteration in the pattern of observed transcripts to that of fertile revertants(Dewey *et al.,* 1987). However, the presence of a second nuclear gene *Rf2* is required to restore fertility. Alternatively, fertility can be restored by further rearrangement of the mitochondrial genome as in the maize CMS-T revertant line analysed by Fauron *et al.,* (1990) in which a 165 kb duplication event was associated with a 423 bp deletion comprising the *urf13* locus (Fauron *et al.,* 1990). In the CMS-T strain of maize, sensitivity to a fungal toxin (called both BmT-toxin and HmT toxin in the literature, dependent on the nomenclature used for the fungal pathogen) is associated with the male sterile phenotype. In the presence of the insecticide methomyl or the fungal toxin, CMS-T mitochondria exhibit uncoupling of oxidative phosphorylation and leakage of molecules such as NAD^+. Expression of the 13 kDa protein in *E. coli* in the presence of the fungal toxin or methomyl inhibits respiration (Dewey *et al.*, 1988). The

universal code equivalent of *urf13* has also been expressed in *S. cerevisiae*. When targetted to the mitochondria by a transit peptide and expressed in the presence of toxin/insecticide it mimics the effects seen in maize (Glab *et al.,* 1990). It has been suggested that the 13 kDa protein encoded by *urf13* forms a channel in the mitochondrial membrane in the presence of the insecticide methomyl or the fungal toxin, thus permeabilising the membrane. Thus, the phenotype, protein expression, and an environmental factor have been shown to be associated in three systems.

Several theories have been proposed as to the molecular mechanism which causes the CMS phenotype. At the RNA level it has been suggested that interference with the transcription or processing of cotranscribed genes could play a role, as in Polima CMS *Brassica* (Singh and Brown, 1991). However, this does not appear to be the case in *Petunia* or maize CMS-T. The extreme demands placed on tapetal and sporogenous tissue may make them more vulnerable to disturbance of mitochondrial function, assuming a 'general' detrimental effect of the chimeric peptide. The reliance on the alternative oxidase pathway may be tissue-specific, and integral to pathogenesis (Connett and Hanson, 1990). An increased abundance of the detrimental protein in reproductive tissue as compared to other tissues may be significant (e.g., *Petunia*). It is possible that a tissue-specific gene product is involved (anther-specific and pollen-specific isoforms of proteins have been identified, Houlne and Boutry, 1994; DePaepe *et al.,* 1993). In maize anthers some natural compound may act in a manner analagous to the fungal toxin or methomyl, thereby causing respiratory deficiency in a tissue-specific manner. It is conceivable that the second requisite restorer allele *Rf2* may alter the expression of this hypothetical anther-specific factor.

1.4.2.2 Nonchromosomal stripe mutants

The other class of maternally inherited disorder which has been identified in plants is the non-chromosomal stripe (NCS) phenotype in maize. NCS plants exhibit stunted growth, aborted kernels, and a characteristic striping of the leaf. The striping can vary in colour from yellow to pale green/white, or the stripes can be necrotic. The mitochondrial genotype of these plants is heteroplasmic and the severity of the defect appears to be related to the relative amount of mutant mtDNA present. Maternal inheritance of the defect is demonstrated by reciprocal crosses with normal plants. An individual cross between an NCS plant and a plant lacking the NCS mtDNA will generate a range of phenotypes in the progeny, including normal derivatives which have no striped leaves. Normal derivative plants do not transmit the phenotype and have been shown to be homoplasmic for wild-type mtDNA. The mutant mtDNA is generated by the deletion of a segment of DNA bordered by short repeat sequences of less than 100 bp. The deletion events in the NCS lines studied have generated chimeric genes. All the NCS lines referred to here are derived from line WF9, which also produced CMS-T; this suggests there may be a factor in the nuclear background which predisposes this line to mtDNA rearrangement (Newton and Coe, 1986). The NCS3 deletion involves the 5' region of the ribosomal protein gene *rps3*, and exhibits necrotic sectors in leaves. The NCS5 and 6 deletions involve the 5' end of *coxII* and exhibit yellow leaf sectors. The NCS2 deletion involves the 3' end of *nad4* and exhibits broad, pale green stripes. (Hunt and Newton, 1991; Lauer *et al.*, 1990; Newton *et al.*, 1990; Marienfeld and Newton, 1994).

The sectored phenotype is due to somatic segregation of the mutant and wild-type mtDNA molecules; in NCS6 mutants only the mutant mtDNA is detectable by Southern blotting of DNA isolated from within the yellow stripes characteristic of the line, while green sectors of leaf harbour equivalent amounts of the two molecules. However, aborting kernels from this line are not homoplasmic mutant, which suggests

that a threshold exists - either for the number of copies of wild-type mtDNA molecules required to encode the *coxII* message, or for the number of mutant molecules encoding the abnormal transcript that can be tolerated. In culture, heteroplasmic callus tissue can be maintained, but if respiratory stress is induced (e.g., by infrequently changing the media) there is a strong selection for the wild-type genome (Gu *et al.*, 1993; Newton, 1994)

The aspect of the phenotype seen as striping of the leaves indicates a pleiotropic effect of the mtDNA lesion. In NCS6 both the structure and function of chloroplasts are altered in the yellow leaf sectors. These sectors contain about 30% of the normal levels of chlorophyll, and fluorescence data suggest a limitation, though not a block, in photosynthetic electron transport after PSII. Electron microscopy shows the chloroplasts to be small with a poorly developed thylakoid membrane system and no accumulation of starch granules. CO_2 fixation data shows no detectable labelling of yellow leaf sectors with ^{14}C. Since the fluorescence and protein data would indicate a reduced level of photosynthesis, the implication is that mitochondria have a role in the development of chloroplasts and carbon metabolism. Chloroplast biogenesis and function may be affected by intracellular ATP levels based on previous studies using oligomycin as an inhibitor of mitochondrial ATPase (Kromer and Heldt, 1991; Gu *et al.*, 1993).

The cases of CMS presented here demonstrate how a mitochondrial mutant can have a tissue-specific phenotype, either through interaction with a tissue-specific factor or possibly where available levels of cellular energy are critical to the development or performance of a given tissue. The NCS mutants show us that where heteroplasmy exists, different tissues may have varied threshholds for exhibiting a mutant phenotype. They also demonstrate how gross the pleiotropic effects of a mitochondrial defect can be. The question of why variation in the specific site of the mitochondrial lesion produces subtle variation of the phenotype needs to be

addressed. The mechanism which generates the illegitimate rearrangements in plants frequently involves short repeat sequences (6-100 bp) at the deletion junctions. Hunt and Newton (1991) suggest that intramolecular recombination between the short reiterated sequences is a more likely mechanism than replication slippage, due to examples such as NCS3 where the progenitor regions are separated by 100 kb on the mastercircle map. These examples of mitochondrial mutants found in plants may provide a useful conceptual model for the analysis of pathogenic mtDNA mutations in humans (section 1.5).

1.4.3 Mitochondrial DNA rearrangements in animals

Deletions in mitochondrial DNA occur in animals as diverse as mice, *Drosophila* and *C. elegans* (Boursot *et al.,* 1987; Volz-Lingenhohl *et al.,* 1992; Melov *et al.,* 1994). A mutant strain of *Drosophila subobscura* has been isolated from the wild which possesses a heteroplasmic mtDNA population in which approximately 80% of the molecules contain a 4.9 kb deletion (Beziat *et al.,* 1993). The deletion was equivalent to more than 30% of the genome, and encompassed subunits of complex I, complex III, and several tRNAs. The mutant displays no overt whole-organism phenotype; it is comparable to the wild-type strain for fertility (both number of eggs laid and number of offspring produced), its lifespan is normal, and its flight capacity is unaffected (Volz-Lingenhohl *et al.,* 1992). The mutation is genetically stable. There is some variation in the proportions of mutant to wild-type mitochondrial DNA between tissues, with a small decrease in the proportion of mutant mtDNA in the male abdomen as compared to thorax and head, and a substantial decrease in the female abdomen (Beziat *et al.,* 1997). When comparing levels of mtDNA to nuclear DNA, the mutant was found to have an elevated proportion of mtDNA (1.5x) as compared to the wild-type (Beziat *et al.,* 1993). Given that the number of mitochondria per cell is reportedly unchanged, the authors suggest that the upregulation of mtDNA copy

number may be a mechanism to compensate for deficiency of intracellular energy or a low redox state.

An anlysis of steady-state levels of mitochondrial transcripts showed no change as compared to wild-type in the levels of transcripts from genes not involved in the deletion. The genes which are deleted in the mutant genome have transcripts present at ~35% (ND5) - ~65% (cyt *b*) of the wild-type level. The fusion transcript was present at an intensity approximately equivalent to that of ND1 (involved in the deletion), which was ~45% of wild-type (Beziat *et al.,* 1993). Thus, the transcript levels are not directly proportional to the copy number of the mtDNA. The authors suggest that the relatively low level of the fusion transcript could be due either to a reduced rate of transcription of mutant mtDNA molecules, or instability of the transcript. The authors also suggest that "overtranslation" of wild-type transcripts may protect them from degradation leading to an elevated proportion of transcripts relative to the proportion of the wild-type and deleted genomes. However, this would suggest that the translation mechanism can preferentially utilise transcripts which are in short supply, since the undeleted region of the genome produces transcripts present at the same steady-state levels as in wild-type flies.

The authors have also assessed the activity of the respiratory complexes in different tissues (head, thorax and male/female abdomen). Complex I activity is decreased in the mutant in all tissues. Complex III activity is reduced in thorax (muscle tissue-enriched fraction) and the female abdomen (germ cell-enriched). Neither complex IV activity, nor ATP synthase activity were compromised with respect to the wild-type strain (Beziat *et al.,* 1997). Thus, the biochemical defects were apparently localised to the molecular defect, and in a tissue- and sex-dependent manner. The authors suggest that loss of tRNAs has not affected translation, as complex IV activity is normal. However, it is also conceivable that there is overcapacity of protein synthesis

within the mitochondrial system, or that a mechanism to extend the lifespan of functional subunits exists.

C. elegans has been presented as a suitable animal model in which to study the mechanism by which mitochondrial DNA deletions accumulate with age. Melov *et al.,* (1995) utilised a PCR screen to assay 900 animals from 3 different strains of *C. elegans* for age-associated deletions of the mitochondrial genome. Two of the strains were wild-type, whilst the third was an *age-1* mutant displaying an increased lifespan. The *age-1* mutation is a single gene defect producing a phenotype of stress-resistance; biochemically the organisms have increased levels of Cu/Zn superoxide dismutase and catalase in older animals, compared to wild-type, the maximum lifespan is doubled, whilst the mean lifespan is increased by 65%. The primers used for PCR spanned a 6.3 kb region, roughly half the genome, which encompassed subunits from all 4 complexes to which the mitochondrial genome contributes. Many amplified products were generated which were non-mitochondrial in origin; however, the authors did not amplify any of these molecules from suitable controls and maintain that they are not artifacts. Of the products which were shown to be mitochondrial by southern blotting, at least 10 discrete deletions were detected in this region of the mitochondrial genome. None of the rearranged molecules were present at sufficient levels to be detected by southern blotting, raising questions about their significance.

In the wild-type strains there was a statistically significant difference in the frequency of deletions detected at the mean lifespan (day 9) when compared to young animals (day 3). It should be noted that the number of deleted mtDNA molecules observed varies greatly from day to day in all 4 experiments reported. Furthermore, in 1 of 3 experiments with the wild-type strains a statistically significant increased in deleted mitochondrial DNA molecules compared with day 3 animals is already present at day 5. The authors postulate that the observed variability could be due to death of cells with elevated levels of deleted mitochondrial DNA, or elimination of deleted

mitochondrial DNA via some mechanism. Despite the variation in data, making the authors' conclusions questionable, there is a trend which suggests that the *age-1* mutant, although it does exhibit deletions, develops them later and at a lower frequency than the wild-type (Melov *et al.,* 1995). This would indicate that an elevation in the level of antioxidant enzyme synthesis protects against damage to mtDNA.

Melov *et al.,* have also analysed the breakpoints of some of the deletion junctions. Of 6 breakpoints reported, 5 involved direct repeats of 4-8 bp, whereas the 6th occurred between 2 sites which exhibited 18/25 bp homology (Melov *et al.,* 1994, 1995). Of the 4 breakpoints reported in the earlier paper, 6/8 sites involved tRNAs. The authors suggest that stem-loop structures may therefore be involved in the deletion process (Melov *et al.,* 1994).

The third animal model I shall discuss is a mouse model for cardiomyopathy, which has been associated with mitochondrial DNA deletions (Adachi *et al.,* 1993, and references therein). Doxorubicin (DOX) is a glycoside antibiotic which can cause cardiotoxicity and heart failure in humans as well as several animal models, including mouse. One suggested mechanism is that DOX causes production of free radicals which attack mtDNA, leading to mitochondrial DNA deletions. Adachi *et al.,* assayed for a 4 kb mtDNA deletion induced in cardiomyocytes by doxorubicin administration in a dosage- and time-dependent manner. They also tested for protection from mtDNA deletion by the free radical scavenger coenzyme Q10 at 2 dosage levels of DOX. The incidence of myocardial mtDNA deletion did correlate with DOX administration in both a dosage- and time-dependent manner (3 time points of 4, 8, and 12 weeks treatment). Coadministration of Q10 with DOX did significantly decrease the incidence of mtDNA deletions detected. The authors propose that the mtDNA deletion is involved in cardiomyopathy (Adachi *et al.,* 1993). However, the PCR protocol used to amplify the mtDNA was not quantitative, so the

level to which the deletions accumulated is not known. The physiological significance of the data has not been shown.

1.5 Rearrangements of mitochondrial DNA in humans

Mitochondrial disorders are predominantly encephalomyopathies due to the high energy requirements of brain and muscle tissues (reviews by Schon *et al.*, 1994; Shoubridge, 1994; Larsson and Clayton, 1995; Papa *et al.*, 1996). Because these tissues are largely non-dividing in the adult the founder population of mitochondrial DNA with which they are endowed is critical. If the founder population is heteroplasmic and includes mutated mitochondrial DNA molecules, these will persist in the organ; they cannot be lost through mitotic segregation unlike in rapidly dividing tissues such as blood or stem cells. Biochemically, most mitochondrial diseases present with respiratory chain deficiency and lactic acidosis. Biopsies of skeletal muscle typically display a pathology of ragged red fibres (RRF), which are characterised by the proliferation of morphologically and biochemically abnormal mitochondria. Common clinical manifestations include the ocular myopathies (ophthalmoplegia and ptosis), skeletal muscle weakness, seizures, dementia, and stroke-like episodes.

1.5.1 Diseases

Deletions of mitochondrial DNA were first reported in association with mitochondrial myopathies by Holt *et al.*, 1988. The relationship between clinical presentation and molecular defect was clarified by Zeviani *et al.*, 1988, in a report which linked mtDNA deletions with Kearns-Sayre Syndrome (KSS) specifically, as opposed to the other mitochondrial myopathies and encephalomyopathies which they investigated. Since then, deletions have also been reported in cases of sporadic Progressive External Ophthalmoplegia (PEO) (Holt *et al.*, 1989; Moraes *et al.*, 1989) and

autosomal dominant PEO (Zeviani *et al.*, 1989). Although heteroplasmy normally refers to one mutant mtDNA species and one unmutated species, in autosomal dominant PEO (adPEO) multiple forms of deleted mtDNA coexist with wild-type mtDNA. In this disorder, a nuclear mutation predisposes the affected individual to deletions in mtDNA. Partial duplications of mtDNA in mitochondrial encephalomyopathies were first reported by Poulton *et al.*, (1989a, b) in 2 KSS patients with diabetes mellitus. The symptoms exhibited in the myopathies which are associated with mtDNA rearrangements thus range from the relatively mild ocular myopathy PEO to the relatively severe KSS. KSS by definition has 3 invariant symptoms, ophthalmoplegia, pigmentary retinopathy, and onset before age 20, with at least one of the following symptoms: cardiac conduction block, cerebellar syndrome, cerebrospinal fluid >100 mg/dl. Other frequently associated clinical features include deafness, proximal limb weakness, endocrinopathies and renal tubular dysfunction.

Other disorders which have since been found to be associated with mtDNA rearrangements include Pearson's bone marrow/pancreas syndrome (Rotig *et al.*, 1989), (onset is in infancy/early childhood and presents with sideroblastic anemia and exocrine pancreatic dysfunction); adult onset diabetes mellitus and deafness (AODMD) (Ballinger *et al.*, 1992); inclusion body myolitis (IBM), a sporadic inflammatory myopathy (Oldfors *et al.*, 1993); diminished male fertility (Kao *et al.*, 1995).

Two other mutations which are associated with a similar range of phenotypes *in vivo* include the nuclear defect which leads to mitochondrial DNA depletion, and one of several point mutations in the $tRNA^{Leu(UUR)}$ gene. The former is characterised by respiratory chain defects, mitochondrial proliferation and severe depletion of mtDNA in the affected tissue. Thus, *in vivo*, mitochondrial proliferation can occur independently of mtDNA replication. In cases of later onset, mtDNA depletion exhibits a segmental pattern in muscle tissue, and can display 70-90% reduction in

mtDNA (Larsson and Clayton, 1995, Schon *et al.*, 1994). Thus an absolute reduction of wild-type mtDNA can result in mitochondrial dysfunction. The mutation at nucleotide (nt) 3243 in the $tRNA^{Leu(UUR)}$ gene typically presents as MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes). Although roughly 1/2 of PEO patients possess mtDNA deletions, of the remaining half approximately 1 in 3 has the nt 3243 MELAS mutation. These cases are clinically indistinguishable from each other apart from their molecular genetic etiology; most mtDNA deletions are sporadic in origin, whereas most point mutations are inherited through the maternal lineage. In addition, diabetes and deafness have been associated with this same point mutation (van den Ouweland *et al.*, 1992).

1.5.2 Correlation of phenotype with genotype in human diseases

Over 100 mtDNA rearrangements, including many different deletions and potential duplications, have been linked to mitochondrial degenerative diseases; the two replication origins are not involved in the majority of these rearrangement events in that all rearranged molecules contain at least one copy of each origin. The molecular mechanism responsible for these rearrangements has not been determined, and several possibilities have been proposed, e.g., slipped mispairing between distant repeats during mtDNA replication, homologous recombination, or breakage and ligation as a result of the activity of an enzyme such as a topoisomerase or a catenane resolvase. Over 95% of mtDNA deletions occur within the major arc - the section defined by the Heavy- and Light-strand origins, which encompasses ~ 2/3 of the mitochondrial genome. All large-scale rearrangements have encompassed tRNA genes as well as protein-coding genes. A 4,977 bp deletion which spans nt 8,482-13,460 and is bordered by 13 bp direct repeats has been observed so frequently it has been dubbed the common deletion (Schon *et al.*, 1989). Although the majority of rearrangements detected have been sporadic in origin, and were therefore thought to be somatic mutations, germ-line transmission was first documented by Poulton *et al.*, (1991), and

subsequently demonstrated by others (Rotig *et al.,* 1992; Ballinger *et al.,* 1992). It may be significant that partial duplications have been detected in germ-line transmission more frequently than would be predicted purely on the basis of the number of rearrangements reported (Rotig *et al.,* 1992; Ballinger *et al.,* 1992; Dunbar *et al.,* 1993).

Genetic rearrangements of mtDNA are linked to specific diseases, albeit with a broad clinical spectrum. The rearranged molecules of mtDNA have only been detected when coexisting at the cellular level with unmutated mtDNA, a condition known as heteroplasmy. It is generally held that the specific tissue-distribution of the mutant molecule in the affected individual contributes to the phenotype. It is controversial as to whether the relative proportions of mutant to wild-type molecules contribute significantly to clinical presentation (discussed in detail in section 1.5.3), but the concept of a threshold at which mitochondrial energy output becomes insufficient to meet cellular demand is often used. Attempts to quantitate this threshold will be discussed with regard to rearrangements in section 1.5.3; it is useful to consider that in cellular model systems a threshold of approximately 90% mutant mtDNA has been observed for several tRNA point mutations, above which protein synthesis is impaired and respiratory activity compromised (Moraes *et al.*, 1993; Attardi *et al.*, 1995). This implies that 10% wild-type mtDNA is sufficient to complement the remaining mutant molecules, or that 10% of the wild-type mtDNA normally found in the cell is sufficient for cellular requirements in these cell systems.

A threshold is a useful conceptual tool for interpreting the phenomena observed in mitochondrial diseases. Identical deletions have been reported in individual cases of KSS, CPEO, and Pearson's Syndrome. Survivors of Pearson's syndrome may develop KSS later in childhood (Larsson *et al.*, 1990). Thus the same genetic defect may manifest as a different disease, and in cases of maternal transmission, the same genetic defect may be present in apparently unaffected relatives. Within the affected

tissue it would appear that a certain amount of the rearranged molecule must be present in a focal manner (either intramitochondrial or intracellular) to produce a phenotype. This threshold amount may depend on multiple factors, including the nuclear background. Each individual patient will possess a slightly different nuclear background which may contain alleles that alter their susceptibility to the mitochondrial genetic lesion. Nuclear background has been shown to influence the segregation of mutant and wild-type mtDNA in cybrids harbouring molecules with the MELAS 3243 mutation (Dunbar *et al.*, 1995). As a result of mitotic segregation (see section 1.3.5.2), the proportion of rearranged mtDNA can exceed the critical threshold for a given tissue in a focal lesion despite the overall proportions of mtDNA for the tissue being within the limits capable of sustaining cellular function.

Some tissues will present with a clinical symptom at a lower proportion of mutant mtDNA than others. An example is the typical progression of AODMD; progressive sensory neural hearing loss commonly presents in the second decade of life, usually followed by the development of type II diabetes mellitus in the third or fourth decade. This may imply that auditory neural tissue has a greater reliance on the oxidative energy supply than the pancreas, or alternatively that it is more sensitive to oxidative damage caused by dysfunctional mitochondria. Northern analysis of cells derived from a patient with a tRNA point mutation provided evidence that fibroblasts and muscle tissue process the mitochondrial polycistronic transcript differently (Bindoff *et al.,* 1993). The intermediate species 'RNA 19', (identified by King *et al.*, 1992), accumulated to very high levels in skeletal muscle, but was only slightly elevated in fibroblasts compared with controls. Therefore, an abnormal transcript resulting from a mtDNA mutation could disrupt RNA processing differently, depending on cell type. In mice heterozygous for deletion of the nuclear gene which encodes mitochondrial transcription factor A (mtTFA, see section 1.3.2), which led to a partial depletion of mtDNA, compensation for decreased transcription by regulation of mRNA stability and translation occurred to varying extents in different tissues (Larsson *et al.,* 1998).

Larsson *et al.*, observed that in these mice skeletal muscle was normal with respect to respiratory activity and transcript levels. However, heart tissue displayed decreases in the level of mRNA, the protein level of at least one mitochondrially-encoded subunit, and in the respiratory activity of complexes I, III, IV, and V. Thus, cardiac tissue may be more sensitive to the disruption of mitochondrial gene expression than skeletal muscle tissue.

To draw analogies from other mitochondrial diseases discussed above, gross rearrangements of mtDNA could lead to mitochondrial dysfunction in several ways. One possibility is that the production of abnormal transcripts might interfere with RNA processing. Alternatively, a relative depletion of some of the normal transcripts could be disruptive in itself. Another possibility is that an imbalance in encoded tRNAs could disrupt mitochondrial translation. A possibility which is specific to these diseases is that the creation of a novel chimeric gene at the site of the mtDNA rearrangement encodes a novel peptide which is disruptive to normal mitochondrial function. It is conceivable that the mode of disruption may be specific, in that the chimeric peptide disrupts assembly/activity of the respiratory complex in which the subunit it most closely resembles would have inserted. It is also possible that the mechanism could be more general, and the presence of any abnormal hydrophobic peptide is disruptive to mitochondrial function, perhaps by insertion into the inner membrane, or by sequestering mitochondrial chaperones.

As discussed in the section on CMS (section 1.4.2.1), a novel chimeric protein in plant mitochondria leads to mitochondrial dysfunction in a tissue-specific manner. Mammals express tissue-specific isoforms of some nuclear-encoded components of the respiratory chain (reviewed by Lomax and Grossman, 1989), and it is possible that these play a role in the phenotype of mitochondrial diseases. The presence of isoforms indicates a requirement for specialisation of the protein function or, in the case of 'silent' isozymes with identical coding sequences, a specialisation of the

regulatory function. One example is that of cytochrome *c*, the substrate for cytochrome *c* oxidase. It has been shown in mouse that a testis-specific isoform, differing at 13 of 104 amino acid residues from the somatic isoform, is expressed early in mouse development, and is present only in germ cells (Virbasius and Scarpulla, 1988). A testis-specific cytochrome *c* has also been found in rat, rabbit, and bull (Kim and Nolla, 1986). The development of male gametes is affected in organisms as diverse as mammals and plants in diseases which involve rearrangements of the mitochondrial genome. A second example involves subunits of the COX complex. At least three nuclear subunits (VIa, VIIa, and VIII) exist as tissue-specific isoforms, which are restricted to contractile muscle tissue (Schlerf *et al.*, 1988). Tissue-specific isoforms of protein subunits could reflect a higher energy requirement of these tissues, or serve as a target for the disease mechanism.

1.5.3 Distribution of rearranged mtDNA within muscle fibres

Many studies have now been done to analyse the distribution and expression of rearranged mtDNA in skeletal muscle. No strict correlation has been found between the proportion of COX deficient fibres and the total fraction of rearranged mtDNA in muscle tissue as detected by Southern blotting (Oldfors *et al.*, 1992). *In situ* hybridisation has demonstrated a large focal accumulation of rearranged mtDNA, predominantly in the subsarcolemmal region of RRFs, where mitochondrial proliferation occurs (Mita *et al.*, 1989; Shoubridge *et al.*, 1990; Collins *et al.*, 1991; Hammans *et al.*, 1992b; Prelle *et al.*, 1994). The rearranged mtDNA is transcribed in RRFs, as evidenced by the detection of mRNAs from the undeleted region of the mitochondrial genome at levels corresponding to the proportion of rearranged molecules detected (Mita *et al.*, 1989; Shoubridge *et al.*, 1990; Oldfors *et al.*, 1992; Hammans *et al.*, 1992b; Moslemi *et al.*, 1996). A 6-fold overall increase in mitochondrial mRNA levels has been estimated in RRFs (Shoubridge *et al.*, 1990; Hammans *et al.*, 1992b). This figure tallies with the 4-6 -fold increase in the amount

of total mtDNA in RRFs estimated by quantitative PCR (Sciacco *et al.,* 1994). *In situ* hybridisation of longitudinal sections indicates the segmental nature of the elevated expression of the rearranged mtDNA molecules (Shoubridge *et al.,* 1990). In adPEO, although multiple species of deleted molecule are present in affected patient tissues, only one form of deleted molecule can be detected in each affected segment using single fiber PCR (Moslemi *et al.,* 1996). This suggests that affected fiber segments contain clonal populations of mtDNA produced by single somatic deletion events. Linkage analysis has implicated at least three autosomal loci in the generation of mtDNA deletions in adPEO (Suomalainen *et al.,* 1995; Kaukonen *et al.,* 1996). It should be noted that 3 patients in which the deletion encompasses the heavy-strand promoter region were found to have elevated light-strand transcription in RRFs, as expected, given the accumulation of rearranged mtDNA, but heavy-strand transcripts were only detected at low levels (Moraes *et al.,* 1991; Hammans *et al.,* 1992b).

The proportion of wild-type mtDNA in RRFs is agreed to be low, but the absolute amount is in question. Some studies have indicated that wild-type mtDNA and the corresponding transcripts are present at normal levels (Shoubridge *et al.,* 1990; Collins *et al.,* 1991), whereas others have indicated that the unrearranged molecules and the corresponding transcripts are present, but at decreased levels in both cytochrome *c* oxidase-deficient fibres and RRFs (Hammans *et al.,* 1992b; Mita *et al.,* 1989; Oldfors *et al.,* 1992; Prelle *et al.,* 1994). Two of the above studies have found variaton between and within patients such that wild-type transcripts were sometimes elevated in affected fibres (Collins *et al.,* 1991; Oldfors *et al.,* 1992). Differences of opinion can partly be attributed to differences in methodology (such as what range is considered 'normal' in Type I fibres). Utilising PCR on single fibres one study detected no wild-type mtDNA in some COX-deficient fibres (Moslemi *et al.,* 1996).

In fibres normal for COX activity most studies have shown normal levels of wild-type mtDNA, with little or no rearranged mtDNA (Mita *et al.,* 1989; Shoubridge *et al.,*

1990; Hammans *et al.,* 1992b; Oldfors *et al.,* 1992; Prelle *et al.,* 1994). Using semi-quantitative PCR 2/7 non-RRFs tested contained 18% and 27%, respectively, rearranged mtDNA molecules (Shoubridge *et al.,* 1990). The authors presume these to be fibres intermediate in character, i.e., cytochrome *c* oxidase-deficient, but not progressed to RRF. However, a subsequent study utilising quantitative PCR has detected ~15-75% rearranged molecules in fibres normal for cytochrome *c* oxidase activity (Sciacco *et al.,* 1994). One of four patients in this study (all of whom had the common deletion) consistently showed a higher proportion of rearranged mtDNA in cytochrome *c* oxidase-normal fibres. This suggests 2 possibilities; 1) nuclear background is integral to clinical presentation, or, 2) loss of COX activity is not an absolute indicator of mitochondrial dysfunction. If partially duplicated molecules were present in these patients, for example, the actual proportion of rearranged molecules would have been underestimated by the authors by the protocol used.

Sciacco *et al.,* 1994, conclude that the accumulation of the rearranged molecules precedes the biochemical phenotype, and have postulated a threshold of 85% rearranged mtDNA in muscle fiber segments before COX activity is impaired, and mitochondrial proliferation is observed. However, Shoubridge *et al.,* 1994, argue that although there is a correlation between the accumulation of deleted mtDNA and the abnormal COX phenotype, COX deficiency does not necessarily correlate to the accumulation of deleted mtDNA. Thus it is argued that a deficiency in OXPHOS may be a signal to induce mitochondrial proliferation (Shoubridge *et al.,* 1990; Hammans *et al.*, 1992b). This does not exclude the possibility that, as other authors have inferred, a (local) reduction in the absolute levels of wild-type mtDNA may be critical to pathogenesis.

A decrease in COX activity is frequently used as a biochemical marker for mitochondrial dysfunction, and if detected in parallel with increased succinate dehydrogenase (SDH) activity is considered an indicator of RRFs (mitochondrial

proliferation in tandem with loss of mtDNA-dependent mitochondrial function). However, Holt *et al.*, 1989, showed that some RRFs do exhibit COX activity. Reichmann (1992) demonstrated that enzyme activity (SDH and COX) can be highly variable along individual fibres in patients (which would reflect the variable distribution of mutant mtDNA molecules), although some fibres lack COX activity altogether (Taanman *et al.*, 1996). It was also shown that one CPEO patient (whose deletion spanned at least one COX subunit) had high levels of SDH and COX activity in RRFs compared to her own normal fibres (Reichmann, 1992). Thus, some of the disagreement on mutant *versus* wild-type mtDNA distribution could also be attributed to erroneous categorisation of fibres based on this enzyme assay.

Polarographic analysis has suggested that in patients whose deletions encompass no subunit of the cytochrome *c* oxidase complex, the biochemical defect can be limited to complex I (NADH dehydrogenase; ND) (Morgan-Hughes *et al.*, 1990; Hammans *et al.*, 1992a). Additionally, within RRFs, a more profound reduction in COX activity has been observed in patients with deletions involving cytochrome *c* oxidase subunits than in those where cytochrome *c* oxidase subunits are not involved (Hammans *et al.*, 1992b). This provides indirect evidence that translation of a proportion of transcripts from deleted mtDNA occurs in some patients. As all of the deletions in question (5/21 and 4/20, respectively) also involve the loss of genes encoding tRNAs, and one also included the 2 rRNAs, this would necessitate intraorganellar complementation for protein synthesis to occur. If this is the case, it raises the question of why the tRNAs (and rRNAs) would be present at sufficient levels to complement at a genomic ratio of wild-type to mutant that would be inadequate to observe complementation of the mRNAs (the ND subunits within the deleted region of the mtDNA). It is conceivable that an abnormal ND chimeric peptide could interfere with normal subunit assembly/function of the complex.

Immunohistochemistry has been used to confirm that some mitochondrial translation occurs within RRFs (Mita *et al.*, 1989; Prell *et al.*, 1994; Taanman *et al.*, 1996). All 3 studies assayed subunits of cytochrome *c* oxidase, and subunits encoded by both deleted and non-deleted genes were detected. The first study detected low levels of COXII protein (the gene is not within the deletion), the second study detected COXII and III (COXIII was deleted in 8/8 patients, COXII in 2/8), the third study detected low levels of COXI and II (COXIII was deleted). Whereas Mita *et al.*, (1989) consider the low level of detected protein as evidence of a deficiency in mitochondrial translation, Taanman *et al.*, (1996) suggest additionally that limiting amounts of the subunit encoded by the deleted region of the mutant genome may prevent assembly of the remaining mitochondrially encoded subunits (making them less stable). However, the data presented by Prell and co-workers suggests that loss of oxidative function cannot be attributed directly to the loss of mitochondrially-encoded subunits in all cases. They suggest further that expression of the deleted genomes may be functionally dominant. The nuclear-encoded subunit COXIV is overexpressed in RRFs (Prell *et al.*, 1994; Taanman *et al.*, 1996) and it has also been suggested that the imbalance of nuclear to mitochondrially-encoded subunits could alter the assembly of complex IV (Prell *et al.*, 1994). However, Taanman *et al.*, (1996) have shown that RRFs lack the nuclear-encoded subunits COXVa, Vb, and VIc; thus, the common interpretation of COXIV immunoreactivity as indicative of normal levels of the nuclear-encoded subunits of complex IV is not reliable.

As discussed previously, the rearranged mtDNA molecules are transcriptionally active. This has been shown conclusively by the detection of the abnormal transcript encoded by the chimeric gene in 2 KSS patients, at levels proportional to the level of rearranged mtDNAs (in muscle tissue and in derived fibroblast clones) (Nakase *et al.*, 1990). However, a study which looked at transcripts in muscle tissue from one KSS patient found a 10-fold increase of the intermediate processing product 'RNA 19' (Heddi *et al.*, 1993). It also appears that levels of individual transcripts, whether

derived from genes internal or external with regard to the deleted region, are not strictly proportional to the amount of the respective mtDNA (e.g., the mRNA for COXI, a gene external to the deletion, is at lower levels than the chimeric transcript across the deletion, whereas the mRNA for ND5, which is internal to the deletion, is decreased by 30% from the control level, whereas deleted mtDNA constitutes 74% of the total). Heddi *et al.*, (1993) observed an accumulation of unprocessed transcripts in skeletal muscle of a young, unaffected son of a KSS patient and speculate that an RNA processing defect could be involved in KSS pathogenesis.

1.5.4 Effects on translation of pathological mtDNA rearrangements

The perturbation of steady-state RNA levels reported by Heddi *et al.*, (1993) might be expected to manifest as an imbalance of the encoded protein subunits. One study has looked at translation in mitochondria isolated from muscle biopsies of 3 CPEO patients, each of whom had a different site of deletion in the mutant population of mtDNA (Sudoyo *et al.*, 1993). The rate of protein synthesis was within the range of 10 normal controls. However, the relative amounts of the mitochondrial translation products were radically different from the controls. This observed perturbation of the proportions of the subunits did not correlate directly with the site of the deletion, nor with the overall amount of deleted mtDNA. As an example, COXIII protein levels were disproportionately high in one patient in whom the deleted region of mtDNA included the COXIII gene, and COXIII protein levels were relatively decreased in another patient although the gene encoding COXIII was not deleted. As a trend, it would appear that levels of the larger proteins are decreased and the smaller proteins increased compared with controls; however, Sudoyo *et al.*, (1993) discounted increased proteolytic activity in the mitochondria of patients as a cause of the abnormal patterns of mitochondrial protein synthesis. A novel mitochondrial translation product was observed in one patient, but the authors did not sequence the

abnormal junction and could not therefore judge whether this could be a chimeric protein.

Whilst Sudoyo *et al.*, (1993) analysed protein synthesis in mitochondria isolated from patient tissues, other groups have focused on cellular model systems. Nakase *et al.*, 1990, reported the results of an assay of mitochondrial translation in SV40-transformed fibroblast clones derived from a KSS patient. Clone f1 was essentially homoplasmic for undeleted mtDNA, whereas clone f4 had approximately 60% deleted mtDNA. The figure showing the PAGE of ^{35}S Met-labelled translation products from the two clones is open to some interpretation. The authors detected no novel band in the region of the gel where the fusion protein is predicted to run. Additionally, the authors observed no qualitative difference in the patterns of translation products between the two clones. They concluded that this is indicative of functional segregation of the abnormal and wild-type mitochondrial genomes, and that the transcripts from deleted genomes are not translated. As this paper is frequently cited with regard to the first point, it is worth considering the data presented in some detail. No indication of statistical error is given, nor any mention of repeated observations, therefore it is possible that the data presented are the result of a single experiment and should be regarded with due caution. As the authors acknowledged, the results could be specific to the one clone analysed. It is also possible that, since the control cell-line was patient-derived there were subtle defects of translation also present in the "control" mitochondria.

The first observation I would make is that, contrary to the authors' assertions, there does appear to be both a quantitative and a qualitative difference between the mitochondrial translation products of the two clones. The methods section of Nakase *et al.*, (1990) indicates that equal amounts of mitochondrial protein were loaded in the two lanes; however, there is clearly a lower level of signal from the heteroplasmic clone. If this is not a loading error, it indicates either that mitochondrially synthesised

proteins are less abundantly synthesised or less stable. Another qualitative difference is that, although the majority of protein bands appear less intense from the f4 clone than f1, the ATPase 6 band (the lowest molecular weight band identified on the gel) is more intense in the f4 lane.

Nakase *et al.*, (1990) have quantitated the ^{35}S signal from the protein bands for the three subunits of cytochrome *c* oxidase. The genes for subunits II and III are within the deleted region, whereas the gene for subunit I is completely external to the deletion. The authors normalised the raw data for subunits II and III respectively, and calculated the predicted ratios for the other two subunits in each case (COXI and III, or COXI and II, respectively) on the assumption that the proteins would be present in stoichiometric amounts to the DNA which encodes them. In both cases, COXI was not present at the high levels they predicted, and they concluded that this is indicative of no translation of transcripts from the deleted mitochondrial DNA molecules. However, if one approaches the data presented differently, by normalising for the level of the subunit which is not expected to change, i.e., COXI, one finds that there is evidence of translational cooperation between the two genomes. The proportion of radioactivity incorporated into COXI in the f4 clone/f1 clone is 37%; the same calculation for COXII and COXIII is 46% and 60% respectively. If only transcripts from wild-type mtDNA are translated, then the three subunits should have been present at the same proportions as in the control clone. If all transcripts are translated equally (deleted and non-deleted) there should have been a higher proportion of COXI protein than COXII or III. Yet, the observation was that proteins encoded by the deleted genes were present at a higher proportion than the COXI subunit relative to the control cell line. This would appear to support the data of Sudoyo *et al.*, (1993), that the presence of rearranged mtDNA leads to a disturbance of the mitochondrial translation pattern.

With respect to the presence or absence of a fusion protein encoded across the rearrangement break point, the fact that no prominent, discrete band was detected by Nakase *et al.*, (1990) is accepted. However, if the abnormal protein were present at low levels, it could be present in the smear between COXII and III, as this is within the author's predicted size range for the fusion protein's apparent molecular weight. If it were co-migrating with COXIII it would explain the anomalously high signal for the band relative to COXI. Alternatively, if the putative fusion protein were migrating with an even lower apparent molecular weight it could be co-migrating with ATPase 6, which would explain the extremely intense signal for that band compared to the control lane. These possibilities warrant a thorough reinvestigation, using other cell clones and other gel systems for resolution of the mitochondrial translation products.

Bourgeron *et al.*, (1993), also analysed clonal lines established from a Pearson's patient with deleted mtDNA. In fibroblast cells they found that the proportion of rearranged mtDNA decreased with time unless the medium was supplemented with uridine. In supplemented medium the proportion of rearranged mtDNA increased from 60% to 90% within 10 cell doublings, and the authors reported no change in total mtDNA content. Thus, the presence of the deleted mitochondrial DNA is inferred to be detrimental to cellular respiration, at least within the original nuclear background. In EBV-transformed lymphocytes the authors reported a stabilisation at ~60% rearranged mtDNA, with normal respiratory enzyme activities. Northern analysis shows a pattern of transcript abundances which does not strictly correlate with the proportions of wild-type and deleted mtDNA, but translation was reportedly normal for both total amount and pattern of products synthesised. In this case, although the putative fusion protein was reportedly undetected, it is predicted to be only ~4.7 kDa and may not have been visible by the gel system used.

Spelbrink *et al.*, (1994) also studied lymphoblast cell-lines derived from a Pearson's patient, which had 70% deleted mtDNA. By comparing the amounts of nuclear- and mitochondrially-encoded COX subunits synthesised to the amounts assembled, they concluded that normal respiratory activity was achieved in these cells due to overcapacity of mitochondrial gene expression in the wild-type cell. The transcription pattern of the patient-derived cell line (as in Bourgeron *et al.*, 1993) was not directly proportional to mtDNA, and is similar to that observed in the *Drosophila* model discussed in section 1.4.3. Likewise, an increase in the total mtDNA content is observed (Spelbrink *et al.*, 1994, 1997). They reported that the predicted fusion transcript was not detected (data not shown). A specific decrease in the synthesis of mitochondrial proteins encoded by the deleted region of mtDNA was observed. This supports the conclusion that, at intermediate levels of deleted mtDNA, reduced gene dosage and a reduction in steady-state levels of the corresponding mRNAs can lead to decreased synthesis of specific subunits before the tRNA concentration becomes rate-limiting. However, due to the overproduction of subunits, reduction in the synthesis of a subunit does not necessarily result in a reduction of the concentration of the corresponding enzyme complex.

Low levels of doxycycline, an inhibitor of mitochondrial translation, were administered in order to partially inhibit mitochondrial protein synthesis (Spelbrink *et al.*, 1997). They found that the proportion of deleted mtDNA declined steadily with time. Long-term treatment led to virtual depletion of deleted mtDNA, which was stable following the removal of the drug. Short-term administration (5-6 weeks) reduced the proportion of deleted mtDNA to ~ 5% of total mtDNA; withdrawal of doxycycline at this point was followed by reamplification of the deleted mtDNA molecules to ~ 50-60% of total mtDNA. These results support the hypothesis that deleterious mutations such as large mtDNA deletions can accumulate to a threshold level above which they would confer a phenotypic disadvantage on the cell. This

threshold can be altered by manipulating the respiratory capacity of the cell, and in this particular cell system the smaller mtDNA molecule has a replicative advantage.

In order to distinguish the phenotypic features which may be attributable to the presence of the rearranged mitochondrial DNA from the possible contribution of the nuclear genome, Hayashi *et al.,* 1991, introduced mitochondria derived from a CPEO patient's fibroblasts into ρ^o HeLa cells. The mtDNA was heteroplasmic for a 5.2 kb deletion, which created 2 chimeric genes (due to the overlapping nature of the *ATP6/8* genes); a) *ATPase 8/ND5* encoding a predicted fusion protein of 77 amino acids, and b) *ATPase 6/ND5* encoding a predicted fusion protein of 141 residues. Cybrid clones which were heteroplasmic for deleted mtDNA consistently displayed a propagational advantage over the smaller molecules in uridine-supplemented medium, up to an equilibrium of 75-85% deleted mtDNA, in all 3 clones and their derivatives over 10 weeks following cellular fusion. The observed threshold for deleted mtDNA content was reduced to ~50% if cells were cultured in medium containing galactose rather than glucose, suggesting that higher levels of rearranged mtDNA compromised the respiratory efficiency of the cells.

The authors reported that the increase in deleted molecules was accomplanied by a decrease in wild-type mtDNA such that, contrary to the observations *in situ* of diseased muscle fibres, the total mtDNA content was unchanged. However, the two clones compared were, respectively, homoplasmic for the wild-type mtDNA and a heteroplasmic clone containing 11% wild-type mtDNA. A Southern blot of *Pvu*II digested DNA probed with labeled total mtDNA gave a ratio of 1.12 for mtDNA content in the heteroplasmic clone relative to the homoplasmic clone. Although the authors concluded that this indicated the total amount of mtDNA per cell to be relatively unchanged, when the large deletion and high proportion of deleted mtDNA is taken into account, the data actually suggest a significant increase in copy number of mtDNA molecules in the heteroplasmic clone. Given that the signal for total

mtDNA was 10% higher in the heteroplasmic clone, the fact that the deletion covers nearly one-third of the genome, and that the deleted molecules constituted nearly 90% of the mtDNA population, this suggests an increase of over 50% in copy number in the heteroplasmic clone.

Hayashi *et al.*, (1991) reported that transcription, including that of the fusion transcript, was proportional to mtDNA content of the clones. Mitochondrial translation products were analysed by ^{35}S-Met labelling and bands corresponding to both of the putative fusion proteins were identified in clones harbouring 55% and 61% deleted mtDNA, although the amino acid sequences of the proteins were not determined to confirm their identity. This finding indicates translational cooperation between the two types of mtDNA molecule in this cell system. Clones containing proportions of deleted mtDNA exceeding 60% displayed decreased COX activity and a reduction in mitochondrial translation. The perturbation in the proportions of proteins translated observed by Sudoyo *et al.*, (1993) *in vivo* did not occur in this cell system. Electron micrographs of mitochondria from a clone harbouring 75% deleted mtDNA show a uniform loss of COX activity, accompanied by swelling and disorganisation of the inner membrane.

1.5.5 Large-scale duplications of mitochondrial DNA

The first report of a mtDNA mutation which was proposed to be disease-associated was the observation of dimeric molecules in tumour cells (Clayton and Vinograd, 1967; Clayton and Smith, 1975). In these molecules two complete copies of the mitochondrial genome were joined in tandem array. Partially duplicated molecules of mtDNA have since been shown to be associated with a range of diseases, such as KSS (Poulton *et al.*, 1989a, b), renal tubulopathy and cerebellar ataxia (Rotig *et al.*, 1992), and diabetes mellitus and deafness (Ballinger *et al.*, 1992, 1994). Maternal transmission of partially duplicated molecules has been documented (Ballinger *et al.*,

1992, 1994; Rotig *et al.,* 1992; Dunbar *et al.,* 1993). Reinvestigation of cases initially reported as deletions of mtDNA has shown that partial duplications of mtDNA are prevalent in KSS, but not in 'pure myopathies' (Poulton *et al.,* 1993, 1994). A partially duplicated mtDNA molecule is essentially a tandem dimer with a partial deletion in one copy of the genome, and it is possible for more than one form of a partially deleted molecule to coexist with the wild-type genome. Poulton and coworkers first documented families of rearranged molecules consisting of partially duplicated molecules, deletion monomers, and deletion dimers (two deletion monomers joined head-to-tail), and these molecular forms have since been demonstrated by other groups (Poulton *et al.,* 1993; Brockington *et al.,* 1995; Manfredi *et al.,* 1997). The abnormal junctions in the various species of rearranged molecules when sequenced have been shown to be identical, indicating that all 3 forms of the partially deleted molecules have arisen from one initial rearrangement event.

Several studies have now looked at the distribution of these various molecular species in patient tissues (Poulton *et al.,* 1995; Brockington *et al.,* 1995; Fromenty *et al.,* 1997). Blood can contain up to 85% partially duplicated mtDNA, whereas deleted molecules are not detected (Holt *et al.,* 1989; Brockington *et al.,* 1995). The correlation of partially duplicated molecules (Poulton *et al.,* 1989; Rotig *et al.,* 1992; Dunbar *et al.,* 1993; Ballinger *et al.,* 1992, 1994; Poulton *et al.,* 1994) rather than deleted mtDNA alone (Holt *et al.,* 1989) with diabetes mellitus, may be significant. One publication which has included post-mortem data for the pancreas reported that in that one KSS patient ~40% of mtDNA was rearranged, and about 1/4 of this was partially duplicated, the remainder of the rearranged species in the pancreas being deletion monomer/dimer (Poulton *et al.,* 1995). It is possible that in other cases of "mitochondrial" diabetes, the pancreas contains a mixed population of rearranged molecules.

Sequential biopsies and single fiber analysis from patients indicates that the partially duplicated species may be a transient form in skeletal muscle, and/or a progenitor of the deleted monomer/dimer species (Poulton *et al.*, 1993; Poulton *et al.*, 1995; Manfredi *et al.*, 1997). PCR of individual COX^- fibres from a patient with adult onset myopathy and a mixed population of mtDNA (~40% partially duplicated, ~1% deleted species) presented a mixed picture (Manfredi *et al.*, 1997). The authors reported that the majority of fibres tested possessed "nondeleted" mtDNA (i.e., wild-type and partially duplicated) at a level several fold lower than found in COX+ fibres, but 3/25 fibres contained "nondeleted" mtDNA at levels equivalent to COX+ fibres. This suggests that respiratory dysfunction might precede depletion of "nondeleted" mtDNA, and would imply a dominant negative effect on respiratory function of the presence of rearranged mtDNA. PCR amplification of deleted mtDNA was not reported in this study, so it is not possible to relate proliferation of deleted mtDNA to respiratory dysfunction in these fibres. The authors also report that all COX^- fibres showed reduced COXII antibody staining. However, the nuclear subunit tested was COXIV (see sect. 1.5.3) so it is not possible to distinguish between loss of mitochondrial translation, and general lack of COX subunits. Only a proportion of COX- fibres stained excessively with SDH; this also indicates that respiratory deficiency precedes mitochondrial proliferation (as SDH staining is used generally as an indicator of mitochondrial volume [Reichmann, 1992]).

In cardiac muscle, duplicated molecules appear to be more stable than in other long-lived post-mitotic tissues, such as skeletal muscle and brain (Fromenty *et al.*, 1997). These authors reviewed postmortem data which indicates that the overall mutant load of mtDNA (duplicated and deleted molecules) correlates with the severity of the cardiac conduction defect (Poulton *et al.*, 1995; Brockington *et al.*, 1995; Fromenty *et al.*, 1997). This indicates that duplications can be pathogenic.

A high proportion of deleted mtDNA has been shown to correlate with decreased cellular respiration and decreased/abnormal mitochondrial protein synthesis in cell culture. By contrast, few reports of cultured cells harbouring partially duplicated mtDNA are available. In one report, duplicated mtDNA was rapidly lost from both uncloned and cloned patient fibroblast lines (Poulton *et al.,* 1993); in contrast, partially duplicated mtDNA from different patients was reported to be stable in cultured myoblasts (Dunbar *et al.,* 1993; Brockington *et al.,* 1995). Unfortunately, media conditions were not reported in these 3 papers, so it is unknown whether uridine supplementation had any effect, or whether cell type was a determining factor.

In the first detailed cell-culture study to address the latter point (Holt *et al.,* 1997), enucleated cytoplasts from one such patient with partially duplicated mtDNA (Dunbar *et al.,* 1993) was fused with a ρ^o osteosarcoma cell-line (ρ^o 143B) and also with a ρ^o lung carcinoma cell-line (ρ^o A549). The two recipient cell-lines displayed quite different phenotypes; all clones analysed from the lung carcinoma cell line became homoplasmic for wild-type mtDNA, regardless of uridine supplementation. As the mitochondria introduced into the recipient A549 cell line were virtually homoplasmic for partially duplicated mtDNA, the authors suggest this is evidence that homologous recombination is active in this nuclear background. In the osteosarcoma background, further mtDNA rearrangements were observed on at least two occasions; in both of these cases a partially triplicated mtDNA molecule was produced. In the presence of uridine supplementation, random drift occurred such that mitochondria which were heteroplasmic could mitotically segregate to 100% wild-type, 100% partially duplicated, or ~100% partially triplicated. Thus, no systematic replicative advantage can be said to be gained by the presence of additional copies of the Heavy-strand replication origin. Serial transfer of homoplasmic partially duplicated mitochondria into ρ^o 143B cells could lead to mtDNA depletion, whereas serial transfer of wild-type mtDNA did not. A modest but statistically significant respiratory deficiency was observed in the osteosarcoma cybrids due to increased lactate:pyruvate ratios.

The observed similarities in the clinical features of patients with partial duplications and deletions of mtDNA suggest that duplications are in some way pathogenic (Holt *et al.,* 1997; Brockington *et al.*, 1995). It is frequently observed that deleted molecules rather than duplicated molecules correlate with mitochondrial proliferation in RRF, and, therefore, that deleted mtDNA is more pathogenic than duplicated mtDNA. A popular hypothesis is that up to a given proportion of deleted mtDNA, translational cooperation with coexisting wild-type mitochondrial DNA molecules masks any defect. However, above a critical threshold, competition for the rare tRNAs leads to a loss of mitochondrial protein synthesis, and respiratory dysfunction (Hayashi *et al.*, 1991). I would argue that this sequence of events probably occurs within RRFs, but it may not be the primary defect. The evidence presented in this section indicates that the mitochondrial proliferation which is observed in RRF occurs in response to preexisting mitochondrial dysfunction.

It has been suggested that partially duplicated mtDNA molecules are pathogenic only insofar as they are the progenitor molecules of deleted mtDNA (Poulton *et al.,* 1993, 1994); however, there are patients in whom the only species of rearranged mtDNA identified is duplicated molecules (e.g., Dunbar *et al.,* 1993). Partially duplicated mitochondrial DNA could be problematic to the mitochondrion for several reasons: 1) an imbalance of tRNAs leads to translational interference. This seems unlikely in that the mitochondria tolerate a relative excess of the 2 tRNAs which are encoded adjacent to the mitochondrial rRNAs, but this could relate to the "threshold" effect; 2) an imbalance of protein subunits could disrupt the kinetics of enzyme complex assembly. This seems possible, given the polarographic data discussed earlier; 3) the chimeric gene transcript interferes with normal mitochondrial function, possibly by disrupting processing of the polycistronic transcript; 4) translation of the novel chimeric protein encoded by the rearranged mtDNA. An abnormal polypeptide in the mitochondria could disrupt cellular respiration in several ways, e.g., by interfering with normal

translational mechanisms, disrupting complex assembly, or by inserting into the inner membrane, making it "leaky" to protons or other molecules, and disrupting the electrochemical gradient essential to OXPHOS. It is of course possible that any of these hypotheses may be applicable to certain cell-types only, and that all or at least more than one of the above mechanisms contributes to pathogenesis. As discussed in section 1.4.2, different cases of CMS in plants may be attributable to causes as divergent as an alteration of the transcriptional pattern (*Brassica*) and the presence of a fusion peptide (CMS-T maize, and *Petunia*).

1.5.6 The fusion peptide hypothesis

In the three model systems discussed in detail in section 1.4 (CMS-T in maize, CMS in *Petunia*, and NCS isolates of maize), the presence of a fusion peptide encoded by a chimeric gene generated by a rearrangement of the mitochondrial genome is inferred to be pathogenic. In both cases of CMS protein interaction with the membrane is implicated. In CMS-T maize, a tissue-specific factor may also be involved. Furthermore, the absence of a detectable fusion protein *in vivo* does not exclude a role for the putative polypeptide. In the CMS *Petunia* strain discussed in section 1.4.2, a novel protein of apparent molecular weight 25 kDa is easily detectable by PAGE; however, this protein is the processed carboxy terminal portion of the chimeric gene product. The unprocessed chimeric protein is detectable only by immunoprecipitation, and the processed amino terminal extension has not been detected by any immunological method. Analysis of transgenic plants which express the 25 kDa protein suggests, moreover, that the undetected amino terminal extension is involved in the generation of the CMS phenotype.

In yeast, it has been shown that the presence of an abnormal respiratory chain subunit (subunit 6 of complex III) can produce a *petite* phenotype when the absence of that same subunit yields no growth defect (Schmitt and Trumpower, 1991). This suggests

that the presence of an abnormal protein in mitochondria, and not simply the loss of function of the normal subunit, can cause dysfunction. Respiratory dysfunction in this case may be due to interference with complex assembly; western analysis found that some other subunits of the affected complex were absent or present in reduced amounts. However, overexpression of subunit 9 of the same complex can suppress the phenotype, perhaps due to some interaction between the two subunits. By analogy, the proliferation of mtDNA in diseased tissue in humans may be a nuclear response to mitochondrial dysfunction; not only is this a crude mechanism for increasing the levels of mRNA, but the overexpression of other subunits may act as a compensatory mechanism for the presence of abnormal subunits in some cases, or early in pathogenesis.

The evidence for the presence of a fusion protein in cases of rearranged mtDNA in humans may be summarised as follows: (1) transcription of rearranged mtDNA has been demonstrated *in vivo*; (2) translation occurs in ragged red fibres; (3) the chimeric gene transcript is translatable by the mitochondrial machinery in at least one cell-culture system; (4) cases of mitochondrial pathology associated with partially duplicated (but no deleted) mitochondrial DNA molecules have been reported; (5) polarographic data indicates that the respiratory deficiency is associated with the genetic lesion. It is likely that some biochemical deficiency is the signal to induce mitochondrial proliferation.

An abnormal protein would be encoded in most cases of rearranged mtDNA. In one of the studies which showed a polarographic defect specific to the complex affected by the molecular rearrangement, the sequence is reported for the rearrangement breakpoint found in the mtDNA of the 4 patients (Hammans *et al.*, 1992a). The chimeric gene would encode a putative fusion protein consisting of all but the final 8 residues of the ND4 subunit followed by 6 abnormal residues encoded by the ND6 gene (antisense). The presence of an abnormal protein in the mitochondria could

contribute to respiratory deficiency in multiple ways, as discussed in section 1.5.2. The overlap in the phenotypes associated with the MELAS mutation and that of mtDNA rearrangements could extend to the mechanism of pathogenesis. Misincorporation/ premature termination at Leu (UUR) codons (Flierl *et al.*, 1997) would generate abnormal peptides in the mitochondria, creating a situation analagous to that hypothesised for rearranged mtDNA.

Thus, there is reasonable cause to speculate that the presence of an abnormal protein could be a factor contributing to mitochondrial dysfunction in these cells. A direct test of this hypothesis is therefore warranted.

1.6 Aims of the project

The aim of the project was to test the hypothesis that abnormal proteins, encoded in affected individuals by chimeric mitochondrial genes, contribute to mitochondrial dysfunction and pathogenesis. The long-term aim of the project was to create transgenic mice expressing a chimeric mitochondrial gene product, to assess whether such a protein would be pathogenic in an organism. The suitability of mice as models for human disease is dependent on the nature of the disease under investigation (Erickson, 1989). With regard to this project, the three main points raised by Erickson are all relevant: variation in biochemical pathways between humans and mice (species-specific differences in oxidative phosphorylation capacity have been shown, (Van den Bogert *et al.*, 1993), variation in neurodevelopmental pathways between humans and mice, and the possibility that the rate of the pathological process in diseases associated with mitochondrial DNA rearrangements may be dependent on absolute time rather than physiological time. However, only by creating the mouse models could one address these questions.

The more limited, specific aim of this PhD project was to generate mouse cell-lines expressing chimeric mitochondrial peptides and assess the phenotype of the cell-lines produced. The strategy which I have adopted is to relocate the chimeric gene to the nucleus, and to target the encoded protein to the mitochondria. This makes it possible to analyse specifically, and in a controlled fashion, the effect of a chimeric protein on the cellular environment. The use of a controlled expression system should result in production of the abnormal protein at a level which can be detected by conventional methods. By selecting and characterising both low- and high-expressing cell-lines, a possible threshold effect of the chimeric protein level can be investigated. In addition, by using an inducible promoter, the phenotype can be ascribed precisely to the expression of the protein. If the expression of the protein is found to be highly detrimental to the cells, an inducible promoter should enable one to maintain the cell-line. Although positional effects of the transgene insertion site may affect expression of the transgene within a specific clone, by comparing the phenotype of multiple cell-lines all carrying the same transgene one can reduce the possibility of having created any observed phenotype by chance.

The molecular models used for the experimental work described in this thesis are two rearranged mitochondrial genes, characterised by Poulton *et al.*, (1989 a, b) each derived from a rearranged COXI gene. The chimeric genes were identified in two KSS patients, and are described in section 1.7.1. As the long-term aim of the project is to create a mouse model for Kearns-Sayre syndrome, mouse mtDNA and mouse-cell lines were used for this work. As no mitochondrial transformation system was available for mammalian cells, nuclear versions of the chimeric mitochondrial genes had to be created. The nuclear version of the full-length COXI gene required 43 point mutations to convert the nucleotide sequence from the mitochondrial to the universal genetic code. Additionally, it was necessary to insert a transit-peptide coding region 5' to the nuclear constructs, to enable mitochondrial targetting of the encoded protein.

A further consideration was to determine the apparent molecular weight of the chimeric peptides by SDS-PAGE analysis, as this was not known.

The work described in my thesis has been subdivided into three chapters. Chapter 3 describes the mutagenesis and subcloning required to create the nuclear versions of COXI and the patient-analagous constructs. Chapter 4 describes the use of an *in vitro* translation system and a prokaryotic expression vector to assay the available antisera for monitoring expression of the synthetic genes, and to ascertain the apparent size of the novel chimeric peptides by SDS-PAGE. Chapter 5 describes the generation and analysis of mouse cell-lines transfected with the patient-analagous constructs.

1.7 The molecular models

1.7.1 Pathology of patients 1 and 2

In this section are described the two Kearns-Sayre syndrome patients reported by Poulton *et al.,* (1989a, b), that are used as molecular models for the experimental work described in my thesis. Both patients exhibited clinical features representative of the syndrome - "ptosis, external ophthalmoplegia, retinopathy, ataxia, proximal muscle weakness and diabetes mellitus." In addition, patient 1 was "deaf and had a cardiac conduction defect," whilst patient 2 "was mentally retarded and had stroke-like episodes." Both patients had had muscle biopsies taken, and although patient 1 exhibited ragged red fibres, patient 2 did not (however, this patient was biopsied early on in the progression of the illness).

Partial duplication of mtDNA was demonstrated in both patients using Southern hybridisation (Poulton *et al.,* 1989a). Southern blotting was performed on *Bam*HI-digested mtDNA extracted from whole blood in both patients, and additionally EBV-transformed lymphocyte lines, muscle, urinary epithelium, and fibroblasts in Patient I.

The blot was probed with total mtDNA, and the relative proportions of normal to abnormal mtDNA measured densitometrically. Partially duplicated molecules were only detectable by Southern blotting in the blood and muscle tissue samples. Densitometry indicated that in Patient I muscle 20% of mtDNA was present in the duplicated form, whilst in blood 32% of mtDNA was present in the duplicated form. In Patient II 43% of mtDNA was present in the duplicated form in blood. Using PCR, rearranged molecules were detected in all tissues tested for Patient I. It must be noted that the PCR products generated did not distinguish between amplification from a template molecule which is a partially duplicated mtDNA and one that is partially deleted (i.e., the latter molecule lacking the "normal" copy). Given the detection limits of Southern blotting, it is possible that deleted molecules were also present in patient tissues.

In order to investigate whether there had been a single event or multiple events which generated the abnormal mtDNA molecules, DNA was extracted from single hairs and granulocyte clones. PCR was performed to amplify the mtDNA sequences across the abnormal junction, and the products were subcloned for sequencing. 3 of 5 recombinants obtained from cloned granulocytes, and 5 of 16 recombinants obtained from individual hairs contained an insert indicating that they arose from rearranged mitochondrial DNA molecules. All recombinant plasmids generated had similar insert sizes. Of those sequenced, a single rearrangement breakpoint was detected for each patient, although the possibility of additional variants, with minor sequence differences was not ruled out.

In both patients the COXI gene has been interrupted by fusion to another gene (figure 1.3). In patient 1, nt 6130 is joined to nt 15056 (from the cyt *b* gene). The abnormal junction in patient 2 is formed at a point where the two genes have 3 bp identity, so the actual breakpoint has occurred between nt 7354-7357, and nt 15914-15917 ($tRNA^{thr}$ gene). Analysis of the sequence in the immediate vicinity of the abnormal

a) patient 1

Reference Sequence - cytochrome *c* oxidase I - (bp 6120-6193)
ATCATAATCGGAGGCTTTGGCAACTGACTAGTTCCCCTAATAATCGGTGCCCCCGATATCGCGTTTCCCCGCATA
I M I G G F G N W L V P L M I G A P D M A F P R M

Patient Sequence at abnormal junction
ATCATAATCGGTACGGATCATTTCTCTACTCAGAAACCTGAAACATCGGCATTATCCTCCTGCTTGCAACTATAG
I M I G T D H F S T Q K P E T S A L S S C L Q L *

Reference Sequence - cytochrome *b* - (bp 15045-15119)
GAGGCCTATATTACGGATCATTTCTCTACTCAGAAACCTGAAACATCGGCATTATCCTCCTGCTTGCAACTATAG
G L Y Y G S F L Y S E T W N I G I I L L L A T M

b) patient 2

Reference Sequence - cytochrome *c* oxidase I - (bp 7347-7370)
GTCCTAATAGTAGAAGAACCCTCC
V L M V E E P S

Patient Sequence at abnormal junction
GTCCTAATAGTCTTGTAAACCGGA
V L M V L *

Reference Sequence - $tRNA^{thr}$ - (bp 15906-15929)
AATACACCAGTCTTGTAAACCGGA

Figure 1.3 Sequence data for patients 1 and 2

Sequence data for the cloned PCR products obtained from patients 1 and 2 (Poulton *et al.*, 1989b). The sequences of wild-type mtDNA from the breakpoint regions are indicated above and below the sequence which spans the abnormal junction in each clone. The inferred amino acid sequences (mitochondrial genetic code) of the wild-type and encoded chimeric peptide are also given. In patient 1 bp 6130 of COXI adjoins bp 15056 of cyt *b* and in patient 2 bp 7357 of COXI adjoins bp 15917 of $tRNA^{thr}$. COXI sequence is underlined.

junction reveals in the case of patient 1 that there is some sequence similarity (7/10 matches), although not in alignment with respect to the breakpoint. As noted by the authors the "GAGG" sequence implicated in nuclear DNA rearrangements (Picolli *et al.,* 1984) is present on both strands. In the case of patient 2, the sequence "CCTC" (the converse of "GAGG") is found on one of the parental strands near the breakpoint. Also, there is some sequence similarity (7/13 matches) between the two parental strands.

The DNA sequence of the recombinant plasmids obtained for each patient indicates that a truncated and terminally altered form of COXI is encoded by the partially duplicated mtDNA in each case. In patient 1 this chimeric peptide consists of the amino terminal 76 residues encoded by the COXI sequence, which are joined to 20 residues encoded by cyt *b*, out of frame. In patient 2, as already stated, there are 3 homologous base pairs between the two genes over which the rearrangement occurred. I shall refer to the breakpoint as occurring at base number 7357 in COXI, adjoining base number 15917 in $tRNA^{thr}$; this results in a chimeric peptide consisting of the first 485 residues from COXI followed by one abnormal amino acid before a stop codon is generated.

These two KSS patients were chosen as the molecular models for the study primarily because, in both cases, the mtDNA rearrangement clearly involved a duplication. Therefore, the hypothesis that rearranged mtDNA is associated with pathogenesis through a depletion of mitochondrially encoded constituents seemed an unlikely explanation in these two cases. There are several possible interpretations of the manner by which partially duplicated molecules could be pathogenic (discussed in section 1.5.5). However, it was (and remains) plausible that the novel protein encoded by the rearranged genes contributed to pathogenesis in these individuals. This hypothesis has become more likely since the reevaluation of 10 KSS patients

thought previously to harbour only deleted mtDNA, but now demonstrated to possess partially duplicated molecules as well (Poulton *et al.*, 1994).

1.7.2 Complex IV - cytochrome *c* oxidase

As the chimeric mitochondrial genes chosen as models for this project are subunits of cytochrome *c* oxidase, a few details about the function and composition of the enzyme are presented. Complex IV is the terminal enzyme of the electron transfer chain in aerobic bacteria as well as in eukaryotic mitochondria (Capaldi, 1990). In mitochondria, it catalyses the transfer of electrons from reduced cytochrome *c* to molecular oxygen, coupled to proton translocation across the mitochondrial inner membrane from the matrix to the inter-membrane space (the bacterial enzyme is a component of the plasma membrane).

The crystal structure of the oxidised form of bovine cytochrome *c* oxidase has been determined (Tsukihara *et al.*, 1996). It exists as a dimer, each monomer consisting of 13 different subunits. The transmembrane region of the complex has 28 α helices per monomer; COXI itself has 12 transmembrane domains, and no substantial extramembrane region. Subunits II and III both associate with the transmembrane region of COXI, with no direct contact to each other. The structure of the bacterial enzyme has been described, and the three subunits common to both complexes show structural similarities (Iwata *et al.*, 1995; Tsukihara *et al.*, 1996). These 3 mitochondrial subunits (COXI, COXII, and COXIII) are encoded by mtDNA in mammals, and together they perform the catalytic functions of the enzyme.

In eukaryotes, additional subunits of the complex, which appear to have no direct role in electron transfer or proton translocation, are encoded by the nuclear genome (Capaldi, 1990). They are assumed to have roles in the regulation of COX assembly and/or activity. Yeast and mammalian COX are reported to have 9 and 10 nuclear-

encoded subunits, respectively (Taanman and Capaldi, 1992; Capaldi, 1990). 3 of the nuclear-encoded subunits in mammals have tissue-specific isoforms which are designated 'heart' or 'liver' for historical reasons. The COXVIa heart isoform is expressed in heart and skeletal muscle, whereas the liver isoform is expressed in all tissues, although at a low level in contractile muscle (Schlerf *et al.*, 1988). 7 of the 10 bovine nuclear-encoded subunits have transmembrane domains (including all 3 which have tissue-specific isoforms), whilst 3 associate peripherally with the complex (Tsukihara *et al.*, 1996).

The assembly of cytochrome *c* oxidase in cultured human cells has been studied (Nijtmans *et al.*, 1998). Two stable assembly intermediates of the complex were identified by 2-dimensional PAGE and western blotting. It would appear that assembly of complex IV is initiated by the association of the mitochondrially-encoded subunit I with the nuclear-encoded subunit IV. Although intuitively one might suppose that subunits I, II and III would interact first (given that these subunits are homologous to the entire bacterial enzyme), this first assembly intermediate may be an evolutionary adaptation to ensure involvement of the nuclear genome. However, as discussed in section 1.5.3, the actual amount of subunit IV does not appear to be rate-limiting in assembly of the complex (Taanman *et al.*, 1996). It would appear that other nuclear-encoded subunits (such as VIa, VIIa or VIIb) may have a regulatory role in the assembly of cytochrome *c* oxidase (Nijtmans *et al.*, 1998).

1.7.3 Nomenclature of cytochrome *c* oxidase subunits

The nomenclature in usage for the subunits of cytochrome *c* oxidase varies within organisms, and the alternatives in use differ between organisms. To reduce the possibility of confusion, I have adopted the use of COX, with the subunit denoted by a roman numeral, to refer to the protein subunit for all organisms. The identical nomenclature is used for the respective gene, except in plants (because this format is

not used), for which I have adopted the use of *cox*, with the subunit denoted by a roman numeral. However, all oligonucleotides and plasmids generated during this work, which contain a reference to cytochrome *c* oxidase in the name, use CO rather than COX (e.g., the universal code equivalent of COXI is called *n-COI*).

Chapter 2

Materials and methods

2.1 *E. coli* strains

The *E. coli* strains used in this work are all derivatives of *E. coli* K12.

Strain	Genotype	Reference/Source
XL1-Blue	*RecA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac*[F' *proAB* $lacI^qZ$(M15 Tn10 (Tet^R)]	Stratagene
CJ236	F+ *LAM- dut*-1 *ung*-1 *relA*1 *spoT*1 *thi*-1 Cm^R	Joyce and Grindley (1984)
CB51	*dam*-3, *ara*-14, *thi*-1, Δ(*lac-pro*) Sm^R	Iain Hunter
M15	Nal^S Str^S rif^S, *lac- ara- gal- mtl-* F- *recA*+ *uvr*+	Qiagen

2.2 Mammalian cell lines

Mammalian cell-lines used in this work, other than those which were generated in the course of this study and are described in chapter 5.

LTK- A mouse cell-line with fibroblast-like morphology derived from cell-line L-M, which was derived from connective tissue from a 100 day old male C3H/An mouse. The European Collection of Animal Cell Cultures (ECACC) reference number is 85011432.

NIH 3T3 A mouse cell-line with fibroblast morphology derived from a Swiss NIH embryo. The ECACC reference number is 93061524.

2.3 Plasmids

Plasmids used in this study, other than those whose construction is described elsewhere in this thesis, are listed below.

Name	Use	Source
pBluescript II SK+, KS+	*E. coli* cloning vector	Stratagene
pQE32	*E. coli* expression vector	Qiagen
pMam*neo*	Mammalian expression vector	Clontech

2.4 Oligonucleotides

Oligonucleotides used in this study, grouped according to their application. All oligonucleotide sequences are given 5' to 3'. Reference coordinates for oligonucleotides corresponding to mouse mtDNA sequence are according to Bibb *et al.*, 1981. Oligonucleotides which are mutagenic will not be identical to the published sequence. Coordinates for oligonucleotides corresponding to the antisense strand are listed in reverse.

2.4.1 Oligonucleotides used for the mutagenesis of the mouse COXI gene

Oligonucleotides used for the mutagenesis of the mouse COXI gene as depicted in fig. 3.7.a. Nucleotides which determine the code-corrections are underlined.

A	GGTTGAGAATAAC<u>C</u>AACGATTAATG	5357-5333
B	GTACCCAC<u>C</u>ATTCCCGC<u>C</u>CAGGCTC	5419-5395
C	CAAAGCCTCCAAT<u>C</u>AT<u>C</u>ATTGG<u>C</u>ATTAC<u>C</u>ATGAAGAAAAT<u>C</u>ATAACAAAAG	5563-5512
D	GGCTCCGAT<u>C</u>ATTAGTGGGACAAG<u>C</u>CAGTTTC	5594-5564
E	GGTAGGAG<u>C</u>CAAAAACT<u>C</u>ATATTATT<u>C</u>ATTCGTGGGAATGC<u>C</u>ATATCTGG	5644-5595
F	CTCCTGCTTCTAC<u>C</u>ATTGATGATG	5691-5668
G	GTAGACTGT<u>C</u>CATCCTGTTCCTG	5714-5692
H	GTTTGATACTGTGT<u>C</u>ATGGCTGG	5869-5847
I	TAAGTACGGA<u>C</u>CAGACAAATAGTGGA	5895-5870
J	CTGTTAGTAG<u>C</u>ATAGTAATGCC	5961-5940
K	GCCCAAAGAA<u>C</u>CAGAACAG	6045-6027
L	CAATAGACAT<u>C</u>ATTGC<u>C</u>CATAC<u>C</u>ATTCC<u>C</u>ATATAGC	6168-6133
M	CTACTGTGAA<u>C</u>ATGTGGTGGGC<u>C</u>CATACAA	6213-6184

N	GCGATAATCATAGTGGCTG	6265-6247
O	GGTTGCAAGCCAGCTAAA	6305-6288
P	CCTAAGGCCCATAGCATAGCTGGAGACCATTTAA	6355-6322
Q	ATAATGGGAACCAGTGAACAAATCCTGCCATGATAG	6525-6490
R	GAAGTGGGCTTTTGCCCATGTGTC	6569-6546
S	GAAGAATGTCATGTTTACTCCTACGAACATGATGGC	6605-6570
T	GCGTCGTGGCATTCCTG	6644-6628
U	TAAATGATCCCATAGAAGAGACAGTGTTCCATGTGG	6705-6670
V	GCAAAGGCCTCCCAAATCATAAAGATC	6757-6731
W	CATACGATACTGACATTACTTCTCGTTTTGAA	6789-6758
X	CCATGAAGCCATTCTAAATTTGTTGAAG	6817-6790
L'	GCGAATAGACATCATTGCCCATACCATTCCC	6170-6140
Q'	CAAATCCTGCCATGATAGC	6507-6489

2.4.2 Oligonucleotides used to repair unprogrammed mutations

Oligonucleotides used to repair unprogrammed mutations as described in chapter 3, depicted in fig. 3.7.b and 3.16.

J'(repair)	GTAATGCCTGCCGCTAGTACTGGTAG	5947-5922
Q''(repair)	GAACCAGTGAACAAATCCTGCCATG	6518-6494
S'(repair)	CATGTTTACTCCTACGAACAT	6596-6576
Rpr/1178/A	GCGGTGCACAAATCCTGCCATGATAGC	6512-6489
Rpr/1178/B	GCGGTGCACTGGTTCCCATTATTT	6507-6527

2.4.3 Oligonucleotides used for sequencing COXI

S+1	GAGGATTTAAACCTCTG	5269-5285
S+2	CTTGTCCCACTAATGATC	5571-5588
S+3	CCACTATTTGTCTGGTC	5871-5887
S+4	CTTTCTAGGCTTTATTG	6170-6186
S+5	CTATCAATGGGAGCAGTG	6468-6485
Y	CATAGGTTGGTTCCTCG	6858-6842
Z	GATATGAGATTGGCTTG	6923-6907

2.4.4 Universal sequencing primers

Reverse primer	AACAGCTATGACCATG
M13 -20 primer	GTAAAACGACGGCCAGT

2.4.5 Oligonucleotides used to create the transit peptide coding region

Oligonucleotides used to create the COXIV transit peptide coding region as depicted in fig. 3.8.

TP1	GCGGTCGACCAGAAT
TP2	GTTGGCTTCCAGAGCGCTGAGCCTGATTGG
TP3	CAAGAGAGCCATTTCTACTTCGGTGTGCCTTCGAAGCG
TP4	CGCTTCGAAGGCACACCGAAGTA
TP5	GAAATGGCTCTCTTGCCAATCAGGCTCAGC
TP6	GCTCTGGAAGCCAACATTCTGGTCGACCGC

2.4.6 Oligonucleotides used to create the Patient I 3' coding region

Oligonucleotides used to create the Patient I 3' coding region as depicted in fig. 3.10.

PI1	GCGTGATCATTTCTCTACTCAGA
PI2	AACCTGAAACATCGGCATTATCCTCCTGC
PI3	TTGCAACTATAGGTCGACGCG
PI4	CGCGTCGACCTATAGTTGCAAGCAGGAGGATAA
PI5	TGCCGATGTTTCAGGTTTCTGAGTAGAGAAATGATCACGC

2.4.7 Oligonucleotides used for PCR-based subcloning

Oligonucleotides used for PCR-based subcloning as described in chapter 3

COI/Lsp	GCGTTCGAATGTTCATTAATCGTTGG	5328-5345
COI/PI3'	GGGATCCGTACCAATCATCATTGGCATT	5546-5537
5'TP/Xba	ATATCTAGACAGAATGTTGGCTTCCAG	
PI3'/ZIF-1	GGTCCTCGGGGTCGCCTAGTTGCAAGCAGGAGGA	
ZIF-2	TATCTCGAGTCAATCCAGGTCCTCGGGGTCGCC	
COI/WT3'/Xho	CGCCTCGAGTTATTTTACTTTTACATAGGT	6872-6852

2.4.8 Oligonucleotides corresponding to pMam*neo* sequences

pMam/5'MCS	GCTATCATCACAAGAGC	1501-1517
pMam/3'splice	TCAGTTCCATAGGTTGG	1728-1712

2.5 *E. coli* growth media and culture

2.5.1 Media

L-Broth: 10 g Tryptone, 5 g yeast extract, 5 g NaCl, made up to 1 litre with dH_2O, adjusted to pH 7.0 with NaOH.

L-Agar: As L-Broth with the addition of agar to 1.5%.

2xYT Broth: 10 g Tryptone, 10 g yeast extract, 5 g NaCl, made up to 1 litre with dH_2O, adjusted to pH 7.0 with NaOH.

2.5.2 Antibiotics

Ampicillin	Stock (1000x) 50 mg/ml in dH_2O
Carbenicillin	Stock (1000x) 50 mg/ml in dH_2O
Chloramphenicol	Stock (100x) 25 mg/ml in 100% ethanol
Kanamycin	Stock (1000x) 25 mg/ml in dH_2O
Streptomycin	Stock (1000x) 10 mg/ml in dH_2O
Tetracycline	Stock (1000x) 12.5 mg/ml in 50% ethanol/50% dH_2O

2.5.3 Growth conditions

L-broth was used for growth of most *E. coli* strains. 2 x YT was used for single-stranded DNA preparation. Incubation was at 37 ^{0}C (unless specified otherwise), with vigorous shaking. For long term storage,

0.75 ml of overnight culture was added to 0.75 ml of 30 % glycerol, mixed by inversion, quick frozen and stored at -70 ^{0}C.

2.5.4 Transformation of *E. coli*

Competent *E. coli* cells were prepared using the calcium chloride procedure (Sambrook *et al.*, 1989). For storage 100 µl aliquots had sterile glycerol added to 15 % and were then frozen in an ethanol/dry ice bath before storage at -70 ^{0}C in screw top 1.5 ml Eppendorf tubes. After heat shock (1 minute at 42 ^{o}C), 1 ml of L-Broth was added to each tube and the bacteria allowed to recover at 37 ^{o}C for 45 minutes before plating onto appropriate selective media.

2.5.5 Indicators

For blue/white selection of transformants 50 µl of 100 mM IPTG (Isopropyl-B-D-thiogalactopyranoside) and 50 µl of 50 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-B-galactoside) were spread onto 90 mm diameter L-agar plates and allowed to dry before spreading the transformed bacteria. The plates were incubated at 37 °C for 12-16 hours. To intensify blue/white colour selection, plates were sometimes placed at 4 °C for one hour prior to picking colonies.

2.6 Mouse cell growth media and culture

2.6.1 Cell culture media

The mouse cell lines NIH 3T3 and LTK- were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS).

Derivatives of these cell lines transfected with pMam*neo*, pM/PI, or pM/PIZ were maintained in supplemented medium referred to as "superglu".

DMEM-Superglu	DMEM + 10% FCS supplemented to a final concentration of 2 mM glutamine, 50 µg/ml uridine, and 1 mM sodium pyruvate.

2.6.2 Cell culture conditions

Cells in routine culture were maintained in 25 cm^2 plastic tissue culture flasks under 10 ml of the appropriate medium at 37 °C in an atmosphere containing 5% CO_2. The cells were passed every 3-4 days. In order to pass the cells the medium was aspirated off and the cells were washed with 10 ml phosphate buffered saline (PBS) which was

Ca^{+2} and Mg^{+2} free. The PBS was also aspirated off and the cells were detached by treatment with 1 ml trypsin-versene (0.025% trypsin, 500 μM EDTA, 0.0015% phenol red in PBS) for 1 minute. The cells were dislodged by tapping the flask and were dispersed in 9 ml of fresh medium. Fresh cultures were initiated with 1/10 to 1/30 dilutions made up to 10 ml with fresh medium.

2.6.3 Selective medium conditions

Geneticin (G-418 sulfate, Gibco BRL) was used in selections at a final concentration of 600 μg/ml for NIH 3T3 cells; 800 μg/ml for LTK- cells.

Dexamethasone was used at a final concentration of 0.2 μM where appropriate.

Ethidium bromide for the generation of ρ^0 derivative cell lines was used at a final concentration of 50 ng/ml (Desjardins *et al.*, 1985).

Derivatives of NIH 3T3 and LTK- cell lines transfected with pMam*neo*, pM/PI, or pM/PIZ were incubated in galactose medium when appropriate to experimental conditions as specified in chapter 5.

Galactose medium DMEM without glucose, 25 mM galactose, 10% FCS.

2.6.4 Transfection of mouse cell lines

All transfections were performed with DOTAP (Boehringer Mannheim), a cationic lipid which forms unilamellar vesicles (liposomes) in aqueous solution. The liposomes form complexes with DNA which, upon fusion with a cellular membrane, release the DNA into the cytoplasm of the cell. The manufacturer's protocol was followed precisely. The cells were transfected with circular plasmid DNA, prepared

using Qiagen midiprep columns (Qiagen). Cells were incubated overnight (15-16 hours) with the transfection reagent. The medium was aspirated off, cells were washed with PBS, then fresh medium was applied. After 24 hours recovery, the transfected cells were placed under selection with Geneticin G418-sulfate at a concentration of 600 μg/ml, the concentration previously determined as the lowest which caused cell death of untransfected NIH 3T3 cells, with no resistant colonies remaining after 2 weeks. After 2 weeks in selective media, colonies were cloned using sterile metal cloning rings coated with vaseline, by detaching the cells with trypsin and gentle pipetting up and down, and transfer to 6 well dishes where they were incubated in appropriate media.

2.6.5 Galactose assay for respiratory competence

Initially, the test media I used contained DMEM (without glucose), 10% dialysed Foetal Calf Serum (dFCS), 25 mM galactose. However, all cell-lines tested died rapidly in this medium, presumably due to unsupplemented factor(s) lost during the dialysis of the dFCS. For the test data presented in section 5.2.7, the galactose medium (gal medium) consisted of DMEM (without glucose), 10% FCS, and 25 mM galactose. All transfected cell-lines were incubated in media containing G418. Cell-lines were incubated with dexamethasone (dex) to upregulate transgene expression, where specified by test conditions.

The assays were conducted as follows; as stated previously, the cell-lines were maintained in "superglu" medium, which is supplemented with constituents identified as required for the growth of respiration-incompetent cells. Confluent cells were trypsinised, counted using an automated cell counter, and 1 x 10^5 cells were plated in "superglu" in 25 cm^2 flasks, for each of the four media conditions to be tested. Cells were allowed 3 hours to settle and adhere to the flask. Medium was then aspirated off, and after washing with PBS, the appropriate medium was added to each flask.

The clones were tested for any variation in adherence efficiency; this may have been important, given that PIZ is primarily derived from COXI, a membrane protein, and could conceivably be inserting into the plasma membrane when expressed at high levels. The media and PBS washes were centrifuged to pellet the cells which had not adhered within 3 hours. Very few cells had not adhered within this period, and no difference was observed between the clones. The four media conditions used were "superglu" without dexamethasone, "superglu" with dexamethasone, galactose without dexamethasone, and galactose with dexamethasone. These four conditions were intended to test the effect of transgene expression in low and high respiratory stress conditions. The media was changed on day 2, and the cells were harvested and counted using an automated cell counter after 4 days of growth.

2.7 Buffers and reagents

Church buffer	7 % SDS, 1 % BSA, 1 mM EDTA, 0.25 M Na_2HPO_4, pH 7.2
50 x Denhardts	10 g Ficoll, 10 g polyvinylpyrolidine, 10 g BSA, made up to 1 litre with dH_2O.
EDTA	A 0.5 M solution was made up in $dH_2O$and adjusted to pH 8.0 with 10 M NaOH.
20 x SET	3 M NaCl, 20 mM EDTA, 0.4 M Tris, pH 8.0
20 x SSC	175.3 g NaCl, 88.2 g Sodium citrate, made up to 1 litre with dH_2O.
50 x TAE	242 g Tris base, 57.1 ml glacial acid, 100 ml 0.5 M EDTA (pH 8.0), made up to 1 litre with dH_2O.
5 x TBE	54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8.0), made up to 1 litre with dH_2O.
TE	10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).

2.8 Nucleic acid isolation

2.8.1 Double-stranded plasmid DNA

Both large and small scale preparations of double-stranded plasmid DNA were performed by alkaline lysis. Single colonies of *E. coli* were picked with a sterile toothpick, and grown overnight in L-broth at 37 °C with constant shaking. Minipreps of 1.5 ml of culture were prepared either with stock solutions according to the protocol in Sambrook *et al.*, 1989 or using the Promega Magic miniprep kit and following the manufacturer's instructions. Maxipreps of 100 ml of culture were prepared with stock solutions and purified by CsCl density gradient centrifugation according to Sambrook *et al.*, 1989. For transfections into mammalian cells, DNA was extracted from 30 ml of overnight bacterial cell culture using Qiagen Midiprep columns (Qiagen).

2.8.2 Single-stranded phagemid DNA

Single-stranded DNA was prepared essentially according to the 1989 protocol recommended by Stratagene for use with pBluescript phagemids, and the method is described below. The bacterial strain used was XL1-blue, the phagemid from which single-stranded DNA was prepared was pBluescript II SK+, and the helper phage was VCSM13.

50 ml of 2 x YT media was inoculated with 5 ml of fresh overnight *E. coli* culture and incubated for 1 hour at 37 °C with vigorous shaking. prior to helper phage infection. The optimal multiplicity of infection (moi) was not constant throughout the work described; an moi of 10:1 (phage to cells) was used in early experiments,

subsequently an moi of 1:1 was found to be optimal. 30 minutes after infection kanamycin was added to a final concentration of 25 μg/ml, and incubation was continued for a further 6 hours. The culture was spun at 11,000 g for 5 minutes to remove bacterial cells, and the supernatant was transferred to a fresh tube. Phagemids were precipitated by the addition of 12.5 ml of 2.5 M NaCl, 20%PEG solution (PEG-8000), inverting to mix, allowing to stand at room temperature for 15 minutes, then spun at 11,000 g for 20 minutes. The pellet was resuspended in 1 ml of media, to which 250 μl of PEG solution was added, and reprecipitated as above. The pellet was resuspended in 500 μl digestion buffer as described below, and incubated at 65 ^{0}C for 45 minutes. An equal volume of buffer-saturated phenol/chloroform was added, the mixture was vortexed for 1 minute, then spun in a microfuge for 1 minute. The aqueous phase was removed and reextracted until there was no interface left. An equal volume of chloroform was added, vortexed for 1 minute, then spun in a microfuge for 2 minutes. The aqueous phase was removed to a fresh tube and DNA was precipitated by adding 1/10 volume 3 M sodium acetate and 2 volumes cold absolute ethanol. The mixture was left on ice for 15 minutes, then spun for 20 minutes 11,000 g, 4 ^{0}C. The pellet was washed with cold 80% ethanol. The dry pellet was resuspended in 50 μl TE.

Phagemid digestion buffer: 20 mM Tris, pH 8, 1 mM EDTA, 200 μM proteinase K, 0.1% SDS

2.8.3 Mouse cell DNA extraction

High molecular weight DNA was prepared from ~ 5 x 10^6 trypsinised cells which were washed with PBS and pelleted at 550 g for 5 minutes. Two protocols were used.

1 - Nucleon kit (Scotlab) as per manufacturer's protocol. Briefly, the cells were lysed in a TE buffer containing 150 mM NaCl and 1% SDS, the optional RNAse A treatment was used, and deproteination was performed with sodium perchlorate. The

DNA was extracted with a Silica suspension, and precipitated by centrifugation at 1700 g for 2 minutes, then washed with 70% EtOH.

2 - Cells were resuspend in 300 µl digestion buffer (as described below) and incubated overnight at 55 ^{0}C, with shaking. The solution was extracted with an equal volume of buffered phenol, and twice with 2FC (as described below) (mixed by inverting tubes, and spun at 10,000 g in a microfuge for 10 minutes). 30 µl 10 M NH_4OAc and 330 µl cold EtOH were added and the solution was mixed vigorously. This was spun immediately at 10,000 g for 2 min in a microfuge. The precipitate was washed with 70 % EtOH, respun, aspirated, and allowed to air dry. DNA was resuspended in 115 µl TE, and heated for 15 min at 65 ^{0}C.

Digestion buffer — 50 mM Tris, pH 8, 100 mM EDTA, 100 mM NaCl, 1 % SDS. Proteinase K was added to 10 µg/ml just before use.

2FC — 50 ml phenol, 50 ml chloroform, 0.1 g hydroxyquinoline, 1 ml isoamyl alcohol, sufficient 1 M Tris, pH 8 to layer overtop. The solution was mixed well, and left overnight before use. pH of solution should be 8.

2.8.4 Mouse cell RNA extraction

Cells were incubated in "superglu" medium supplemented with dexamethasone and G418 for approximately 40 hours prior to harvesting (the untransfected 3T3 cells did not have G418 in the medium). Total RNA was prepared from mouse cells by two alternative protocols, both of which used a guanidium thiocyanate denaturing solution, phenol extraction, and isopropanol precipitation. In both cases ~ 10^7 cells were lysed *in situ* using guanidium thiocyanate denaturing solution. The RNA pellet obtained was resuspended in 0.5% SDS and heated at 60 ^{0}C for 10-15 minutes.

1 - The method of Chomczynski and Sacchi, (1987), which was followed precisely.

2 - RNAzol B (Biogenesis Ltd.), following the manufacturers protocol precisely (which is based on the method above).

2.9 *In vitro* manipulation of DNA

2.9.1 Restriction digests and sub-cloning

Restriction enzyme digestion of plasmid DNA was carried out using the conditions recommended by manufacturers. Typically 0.5-2 μg of DNA were digested in 20 μl containing the recommended enzyme buffer and 5-10 units of enzyme. After 1 hour at the appropriate temperature the incubations were placed on ice. For sequential digests the first restriction enzyme was either heat inactivated or removed by Magic DNA Cleanup Columns (Promega) before the second digestion. Sub-cloning of DNA into plasmid vectors was performed essentially as described by Sambrook *et al.*, (1989).

2.9.2 Extraction of DNA fragments from agarose gels

Three methods were employed to extract DNA from agarose gels, as follows.

(1) Low melting point agarose (Sambrook *et al.*, 1989).

(2) Spin-X columns (Sigma) as per manufacturers instructions. The tube containing the agarose slice was frozen at -20 °C before being centrifuged at maximum speed in a microfuge for 15 minutes.

(3) Electroelution onto NA45 membrane (Schleicher & Schuell) as per manufacturers protocol.

For methods 1 and 2 - The bands of interest were cut from agarose gels under ultraviolet illumination with a sterile scalpel blade. For method 3, NA45 paper was

inserted into the gel immediately below the band so that the DNA would run onto the paper during electrophoresis.

2.9.3 Radio-labelling of nucleic acids

2.9.3.1 Labelling of DNA fragments by random priming

25 ng of gel-purified DNA was labelled using the Prime-it kit (Stratagene), with 50 μCi of α-^{32}P-dCTP. The unincorporated label was removed using Nuctrap columns (Stratagene) as recommended by the manufacturer. Before hybridisation the probe was denatured by boiling for 5 minutes, then spun down and kept on ice before adding to the hybridisation tube.

2.9.3.2 End-labelling of oligonucleotides

15 pmol of oligonucleotide was end-labeled with 50 μCi of γ-^{32}P-ATP using 10 units of polynucleotide kinase. The unincorporated label was removed by spinning the labeled oligonucleotide in TES buffer through a sephadex G-25 column at 1580 rpm for 4' at 15 ^{0}C.

TES 10 mM Tris-HCl (pH 8.0), 100μM EDTA, 20 mM NaCl

2.9.4 Sequencing of plasmid DNA

The Sequenase Version 2.0 (United States Biochemical Corporation) kit was used. No modifications to the protocol were made. Where compressions were detected, the dITP labeling and termination solutions were used, as recommended by the manufacturer. The radiolabel used was ^{35}S. In early experiments, single-stranded DNA template was prepared; subsequently,

double-stranded plasmid DNA was used employing a rapid alkaline denaturing protocol as recommended in the USB protocol and described below.

5 µl of template DNA (prepared using the Magic Miniprep columns, Promega) was added to 5 µl of dH_2O. To denature the DNA, 1 µl of 2 M NaOH was added and the mixture was incubated at 37 ^{0}C for 20 minutes. To neutralise the solution, 1.65 µl of 2 M sodium acetate, pH 4.5 was added, and the DNA was precipitated with 30 µl of cold EtOH at -70 ^{0}C for 15 minutes. After spinning at maximum speed in a microfuge at 4 ^{0}C for 30 minutes, the pellet was washed with 70% EtOH. The pellet was resuspended in 2 µl of Sequenase reaction buffer, 1 µl of primer, and 7 µl of dH_2O. Annealing was performed by heating the mixture to 65 ^{0}C for 2 minutes, then cooling slowly (15-30 minutes) to room temperature. It was then spun briefly, and kept on ice for sequencing.

2.9.5 PCR

2.9.5.1 Typical parameters of PCR

Typical PCR reaction components:

5 µl of each primer oligonucleotide (20 pM stock)
5 µl of 2 mM dNTP mix

5 µl of 10 x reaction buffer
0.5 µl of DNA polymerase (5 u/µl)
20 ng template DNA
dH_2O to 50 µl

Typically PCR conditions were:

Step 1	94 ^{o}C for 5 min	(initial denaturation)
Step 2	60 ^{o}C for 30 sec - 1 min	
Step 3	72 ^{o}C for 30 sec - 3 min	
Step 4	94 ^{o}C for 30 sec - 1 min	

(Steps 2-4 were repeated for 30 cycles)

Step 5 60 °C for 5 min
Step 6 72 °C for 10 - 15 min

Specific parameters depended on both the machine used and the size of the amplified fragment.

2.9.5.2 Cloning of PCR products

Pfu DNA polymerase (Stratagene) was used due to the reduced frequency of mutations introduced by this enzyme compared with *Taq* DNA polymerase (Cline *et al.*, 1996). After 5 µl of the reaction had been run on an agarose gel to check that a product of the desired size had been amplified, the rest of the amplified product was purified using Magic PCR cleanup columns (Promega).

2.9.5.3 PCR screening of bacterial transformants

A numbered bacterial colony was touched with a sterile toothpick. This was then dipped into 50 µl of dH_2O in an eppendorf tube and overlaid with mineral oil. The samples were denatured for 5 minutes at 94 ^{0}C and placed on ice. The rest of the PCR reaction components (as specified in section 2.8.5.1) were added to a final volume of 100 µl. *Taq* polymerase was used. 10 µl of each reaction was run on an applicable percentage agarose gel.

2.9.5.4 PCR of genomic DNA prepared from mouse cell lines

Typical PCR reaction components:

5 µl of each primer oligonucleotide (20 pM stock)
5 µl of 2 mM dNTP mix

5 µl of 10 x *Taq* buffer
0.5 µl of *Taq* DNA polymerase (5 u/µl)

1 μg template DNA
dH_2O to 50 μl

The PCR conditions were generally:

Step 1	94 °C for 5 min	(initial denaturation)
Step 2	60 °C for 45 sec	
Step 3	72 °C for 1 min 30 sec	
Step 4	94 °C for 45 sec	

(repeat steps 2-4 for 30 cycles)

Step 5	60 °C for 45 sec
Step 6	72 °C for 10 min

10 μl of the product was run on an appropriate percentage agarose gel.

2.9.6 Site-directed mutagenesis

Site-directed mutagenesis using single-stranded template was performed essentially according to the protocol recommended by Stratagene for use with pBluescript phagemids (1989), apart from the amendments detailed in section 3.2.2, (e.g., the DNA polymerase used initially was Klenow, subsequently Sequenase (USB) was used), the annealing procedure, and the temperature and duration of the primer extension reaction.

Protocol for site-directed mutagenesis:

100 ng of oligonucleotide was phosphorylated in 40 μl total volume, using T4 polynucleotide kinase, at 37 ^{0}C for 30 minutes. 50 ng of each mutagenic oligonucleotide was added to 1 μg of single-stranded DNA template in 1 x ligation buffer* (the total volume depended on the number of mutagenic oligonucleotides in the reaction). Annealing was performed by incubating the oligonucleotide/template mixture at 65 ^{0}C for 10 minutes, then allowing it to cool gradually from 65 ^{0}C to room temperature in the heating block. The primer extension reaction was carried out in 1

x ligation buffer*, 125 μM dNTP mix, 1 mM rATP, 100 μg/ml Gene 32 protein (Pharmacia), 1.5 units DNA polymerase, 4 units T4 DNA ligase. The primer extension reaction was initially executed at room temperature for 3-4 hours as per the protocol, but the procedure was later amended to an incubation of 15 ^{0}C overnight. 1/4 of the total synthesis reaction was used in each transformation.

*Initially, the ligation buffer used was as per the recipe provided with the protocol, which is given below. Subsequently, the ligation buffer provided by the manufacturer with the ligase was substituted.

10 x ligation buffer: 500 mM Tris-Cl, pH 7.5, 70 mM $MgCl_2$, 10 mM dithiothreitol

2.10 Gel electrophoresis

2.10.1 Agarose gels

0.7-4 % agarose gels were used for separating plasmid and PCR-derived DNA depending on the size of the expected fragments. The gels were normally run in 1 x TAE at 100-150 Volts and contained 0.1 μg/ml of ethidium bromide. Low melting point (LMP) agarose gels were run in 1 x TAE at 50 V.

5 x Loading Buffer (agarose) 0.25 % Bromophenol blue, 0.25 % Xylene cyanol FF, 10 mM EDTA (pH 8.0), 15 % Ficoll.

2.10.2 Agarose formaldehyde gels

Total RNA was analysed in 1% agarose formaldehyde gels as per Sambrook *et al.*, 1989. After running, the entire gel was washed three times for 20 minutes in 1 x TAE; during the second wash it was stained with ethidium bromide (1 μg/ml).

2.10.3 Polyacrylamide gels

Products of DNA sequencing reactions were separated on denaturing polyacrylamide gels; 6% acrylamide (acrylamide:bisacrylamide, 19:1), 7M urea, in TBE. Polymerisation was initiated by the addition of 1 ml of 10% ammonium persulphate and 35 µl of TEMED to 100 ml of 6% acrylamide/urea solution. Conventional spacers were used to give a flat gel. Sequencing gels were allowed to polymerise overnight before use. They were prerun for 1 hour at 60 W. Samples were denatured at 90 ^{0}C for 2 minutes in the buffer supplied by USB with the Sequenase kit, and quenched on ice, immediately prior to loading with the aid of a shark's tooth comb. Gels were run for the required amount of time (until the bromophenol blue had reached the bottom of the gel for short runs, approximately 4 hours for a long run) at 60 W. The gels were fixed in 10% acetic acid/10% methanol for 30 minutes, then dried onto Whatmann 3MM paper under vacuum. Autoradiography was carried out without intensifying screens at room temperature.

2.10.4 SDS-Polyacrylamide gels

Protein samples were separated by SDS-PAGE, using the discontinuous gel method (Laemmli, 1970).

Separating gel - 0.375 M Tris-HCl, pH 8.8, acrylamide as below.
Stacking gel - 0.125 M Tris-HCl, pH 6.8, 4.0 % acrylamide.

Depending on the size-range needing separation, 12 to 17.5 % acrylamide (acrylamide:bisacrylamide, 37.5:1) separating gels were used. Gels were run at 4 ^{0}C using minigel kits as per the manufacturer's protocol (Biorad). The samples were run in at 100 V, then voltage was increased to 200 V once samples had entered the

separating gel. Gels were run until the band of bromophenol blue had reached the bottom of the gel. The gels were either stained using Coomassie R-250 brilliant blue, or electroblotted for western blots.

For staining, the gels were placed in dH_2O containing 30 % methanol, 10 % glacial acetic acid and 0.1 % Coomassie R-250 brilliant blue for 30 minutes. Destaining took approximately 2 hours in frequent changes of dH_2O containing 30 % methanol and 10 % glacial acetic acid. The gels were then left in dH_2O overnight before being transferred to 3MM paper and dried down at 80 °C under vacuum for 45 minutes.

5 x SDS-PAGE Running Buffer	15 g Tris base, 72 g Glycine, 5 g SDS, made up to 1 litre with dH2O.
5 x SDS-PAGE Sample Buffer	1.25 ml 0.5 M Tris-HCl, pH 6.8, 1.0 ml glycerol, 2.0 ml 10 % (w/v) SDS, 0.5 ml 2-β mercaptoethanol, 0.25 ml 0.05 % (w/v) bromophenol blue, 5.0 ml dH_2O

2.11 Nucleic acid hybridisation

2.11.1 Screening of bacterial colonies by colony hybridisation

Colonies were patched out onto duplicate 90 mm agarose plates in a grid (120 colonies per plate). One plate was retained as a master plate. The other plate was used for colony lifts. 2 replica lifts were performed for each oligonucleotide being used to screen the colonies.

Pre-wetted Hybond-C filters (Amersham) were inverted onto the plate used for the lifts, oriented by punching a needle through the filter in several places, then replaced on LB/Amp plates colony side up, and incubated at 37 °C until the colonies were 1 mm in diameter.

Lysis of the bacteria and plasmid DNA denaturation were performed as per the 1989 protocol recommended by Stratagene for use with pBluescript phagemids; the filters were placed on 3MM (Whatmann) paper soaked in 0.5 M NaOH for 5 min, blotted dry 5 min, the filters were placed on 3MM paper soaked in 1 M Tris-HCL, pH 7.5 for 2 min, blotted dry 2 min, and the filters were placed on 3MM paper soaked in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.5) for 10-15 minutes, blotted dry 2 min. The DNA was UV crosslinked to the membranes.

Hybridisation was performed using oligonucleotide hybridisation buffer, as described below (prehybridisation for one hour, hybridisation overnight). Membranes were washed sequentially in oligonucleotide wash buffer (see below) twice at 5x, 1x and twice at 0.1x. The final wash was carried out at a few degrees below the calculated T_M for each mutagenic oligonucleotide used as a probe in final wash buffer using the formula below. %G+C refers to the percentage of G and C residues in the oligonucleotide. n refers to the total number of residues in the oligonucleotide. Obviously there would be no mismatch between a successfully mutated target sequence and the mutagenic oligonucleotide; however, use of this formula enabled the selection of oligonucleotides as probes with a wide differential between successfully mutated target DNA and unmutated DNA.

$$T_M = 81.5 + 1/2(\%G+C) + 16.6\log[Na+] - 600/n - \%mismatch$$

Oligonucleotide hybridisation buffer: 4 x SET, 5 x Denhardt's solution, 0.02 M sodium phosphate buffer, pH 6.8, 5 % dextran sulphate, 10 µg/ml each of poly A and poly C, 0.1 % SDS, 100 µg/ml denatured, sonicated salmon sperm DNA

Oligonucleotide wash buffer: 5 x / 1 x / 0.1 x SET, 0.02 M sodium phosphate buffer, pH 6.8, 0.1 % sodium pyrophosphate, 0.1 % SDS

Sodium phosphate buffer, pH 6.8: 100 ml each of 1 M Na_2HPO_4 and NaH_2PO_4 were prepared. The pH of the two solutions was ascertained. Whichever was closest to pH 6.8 was brought to pH 6.8 by addition of the other solution.

2.11.2 Northern blots

Blotting was performed both by electroblotting and by capillary action, as specified in the relevant figure legends in section 5.2.3. The membrane used was Boehringer nylon membrane. RNA was cross-linked to the membrane by baking at 80 ^{0}C for 2 hours. Church buffer was used for both prehybridisation, and hybridisation of the blots with the random-primed probes, at the temperature specified in the respective figure legends. Washes were performed at high stringency by sequentially washing at 1 x SSC at room temperature, 1 x SSC at 68 ^{0}C, then twice at 0.1 x SSC at 68 ^{0}C (all wash buffers contained 0.1% SDS).

2.11.3 Slot blots

To 120 µl aliquots of DNA was added 40 µl of 1 M NaOH. This was vortexed for 10 minutes at room temperature. Biodyne membrane (PALL) was presoaked in 1 x TAE for 30 minutes at room temperature. 160 µl of 10 x TAE was added to samples. The samples were vortexed, spun down for 2 minutes, then put on ice. Samples were loaded onto the blotting apparatus without vacuum. Vacuum was applied, then 1 drop of 5 x TAE was added from a pasteur pipette to each well. DNA was cross-linked to the membrane by UV irradiation using a Stratalinker (Stratagene).

2.12 Western blots

The western blots described in this work were predominantly performed using PVDF membrane (Bio-Rad) used as per the manufacturer's protocol, although initially Hybond-C membrane (Amersham) was used. Proteins from unstained gels were transferred to the membrane in SDS-PAGE Blot Buffer, at 100 mA overnight at room temperature, or 200 mA for 2 hours at 4 ^{o}C. The PVDF membrane was allowed to dry in order to fix the protein. The membrane was moistened with 100% ethanol and

Ponceau-S stained (Sigma) to allow detection of the protein markers. The blot was then rinsed with TBS-T. The ECL western blotting protocol (Amersham) was followed using TBS-T (0.1 %) with the following three amendments; no wash step was included between the blocking step and incubation with the primary antibody, 2.5% w/v non-fat milk was added to the primary antibody solution, although normally the blot was incubated with the primary antibody for one hour as per the Amersham protocol, initially a protocol was used which recommended an incubation period of 3 hours-overnight. All antisera used are described fully in section 4.1.2. Primary antibody dilutions varied; the monoclonal antibody Z1F11 was normally used at a 1/2000 dilution, the polyclonal rabbit antisera at 1/1000. The secondary antibodies (HRP-conjugates) were used at a dilution of 1/2000. Preabsorbtion of the patient 1 - analagous oligopeptide to the rabbit 3 antiserum (fig. 4.7) was performed by incubating the antiserum with 1 μg/ml of the oligopeptide in blocking solution at room temperature for 2 hours before it was applied to the membrane.

SDS-PAGE blot buffer	3.25 g Tris-HCl, 14.4 g glycine, 200 ml methanol, made up to 1 litre with dH_2O.
TBS-T	8 g NaCl, 2.42 g Tris base, adjust to pH 7.6 (~3.8 ml 1 M HCl), add dH_2O to 1 litre.
Tween-20 was added to	0.1 %.
Ponceau-S	0.5% Ponceau-S, 1% glacial acetic acid in dH_2O.

2.13 Cell labelling with mitochondrial dyes and Z1F11

Cells were plated onto coverslips in a petri dish and incubated in the appropriate medium until they had grown to ~70% confluent. Labelling with MitoTracker dyes (Molecular Probes, Inc.) was performed according to the manufacturer's protocol. Three concentrations of each dye were used; CMXRos at 100 nM, 200 nM, 500 nM and CXXRos-H_2 at 200 nM, 500 nM and 1000 nM. Cells were incubated *in situ* with

the dyes for 30 minutes, then in fresh medium for 30 minutes. Cells were washed in PBS, then fixed with freshly prepared 3.7% paraformaldehyde in PBS at room temperature for 10 minutes. Cells were washed with PBS, then permeabilised with 0.5% Triton-X-100 in PBS for 15 minutes at room temperature. Cells were washed with PBS then the coverslips were rinsed with 0.05% Tween in PBS (PBS-T). Cells were incubated with Z1F11 at 1/100 dilution in PBS-T for 1 hour in a humidified chamber. Cells were washed with PBS, rinsed with PBS-T, then incubated with FITC-labelled goat anti-mouse IgG (H+L) antibody at 1/100 dilution in PBS-T for 20 minutes. Cells were washed with PBS. Cells were treated with SlowFade-Light (Molecular Probes, Inc.) as per the manufacturer's protocol to extend the time of fluorescence emission of the fluorescein label. The mounting medium used was Mowiol (Harco).

2.14 Immunoprecipitation

Immunoprecipitation of PIZ was performed using 5 x 10^6 cells washed in PBS and pelleted. The cells were lysed in 1 ml RIPA buffer, with 2% aprotinin and 1 mM PMSF, then vortexed and left on ice for 5 minutes. This was then spun in a microfuge at maximum speed, 4 ^{0}C for 10 minutes. The supernatant was transferred to a fresh tube. 100 µl of 50% protein A-sepharose was added, and the tube was slowly rotated for 2 hours at 4 ^{0}C. This was spun down as before, and the supernatant transferred to a fresh tube. The antiserum was added (50 µl Z1F11), and the tube was slowly rotated overnight at 4 ^{0}C. 50 µl 10% protein A-sepharose, which had been preincubated with rabbit anti-mouse IgG*, was added to the mouse monoclonal fraction. This was rotated for 30 minutes at 4 ^{0}C. It was spun down (2 min), and the supernatant discarded. The pellets were washed twice with 1 ml NET:N, pH 8.0, and once with 1 ml TBS. The pellets were resuspended in 30 µl reducing sample buffer and vortexed, incubated for 5 minutes at 95 ^{0}C, and spun. The supernatant was then ready to be loaded on an SDS-polyacrylamide gel.

*For binding the mouse monoclonal, the protein A-sepharose had been preincubated with rabbit anti-mouse IgG overnight, and washed 3x with NET:N buffer, pH 8.8.

NET:N	150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 8.0 or 8.8), 0.05% NP40.
RIPA buffer	150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS.

2.15 Purification of poly histidine-tagged fusion proteins

For screening transformants, the rapid screening of small scale expression cultures protocol was used (Qiagen). Cells were resuspended in buffer B (described below). The Ni-NTA resin (Qiagen) slurry/lysate mixture was washed with buffer C, before eluting bound protein using buffer C with a final concentration of 100mM EDTA to chelate the Ni^{2+} ions.

buffer B	8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0.
buffer C	as buffer B, but pH 6.3.

Chapter 3

Creation of nuclear cytochrome *c* oxidase I constructs

3.1 Introduction

3.1.1 Overview of strategy

In order to investigate the relationship between the expression of a chimeric peptide in mammalian mitochondria and a disease-related phenotype (as discussed in section 1.6), the first stage of my project required the creation of a nuclear version of the mouse COXI gene (*n-COI*). A nuclear version was necessary because no reliable procedure for the transfection of animal mitochondria was (or is) available, whereas transfection of DNA targetting the nuclear genome is a standard procedure. The engineering of a nuclear version entailed alteration of the coding sequence from the mammalian mitochondrial genetic code to the universal code, using site-directed mutagenesis. The code corrections required for a mouse mtDNA sequence to be compatible with cytosolic translation are adenine to guanine transitions (for the mitochondrial codons Met (ATA) and Trp (TGA)). In order that the n-COI protein could be targeted to the mitochondrial compartment it was necessary to subclone a sequence encoding a transit peptide 5' to the code-corrected gene. The mouse cytochrome *c* oxidase subunit IV (COXIV) transit peptide was selected for the following reasons: (i) it targets a subunit of the same enzyme complex in the same organism (the mouse), (ii) it conforms to the stereotypic structural requirements for appropriate processing of a leader sequence (Hendrick *et al.*, 1989), (iii) the sequence of the translational start site contains the elements essential for efficient translation in eukaryotes (Kozak, 1986), (iv) the sequence of a cDNA for this subunit was available (Grossman & Akamatsu, 1990), (v) the yeast COXIV transit peptide was sufficient for importation of an attached cytosolic protein into the matrix of yeast mitochondria (Hurt *et al.*, 1985). Figure 3.1 shows the nucleotide and amino acid sequence of the portion of the COXIV gene encoding the transit peptide. The full length, code-corrected, mitochondrially targetted n-COI protein was intended to be used as a

a)

```
ATGTTGGCTTCCAGAGCGCTGAGCCTGATTGGCAAGAGAGCCATTTCTACTTCGGTGTGCCTTCGA
M  L  A  S  R  A  L  S  L  I  G  K  R  A  I  S  T  S  V  C  L  R
```

b)

	-3 +4
Kozak optimal sequence	ACC ATG G
Mouse COXIV	AGA ATG T

Figure 3.1 Sequence of COXIV transit peptide

a) Nucleotide and amino acid sequence of the mouse cytochrome *c* oxidase subunit IV mitochondrial targetting peptide, as determined from the sequencing of cDNA (Grossman and Akamatsu, 1990). Amino acid residues indicated by Hendrick *et al.*, (1989), as conserved for appropriate processing of the leader sequence are underlined.

b) The optimal sequence for translational initiation by eukaryotic ribosomes as defined by site-directed mutagenesis studies on the rat preproinsulin gene is shown (Kozak, 1986), and compared to the relevant section of the mouse COXIV gene (Grossman and Akamatsu, 1990). A purine at position -3 is essential for efficient translation, with an adenine optimal. If an adenine is present at -3, a T at position +4 is translated at ~1/2 the rate of a construct with a G at position +4. The Cs at positions -2 and -1 were not shown to enhance translation when the constructs tested had an A at position -3 and a T at +4. Although the T at position +4 is not optimal for translational initiation in the cell system used by Kozak (1986), it was decided to use the native sequence of COXIV rather than altering the leucine codon to a valine.

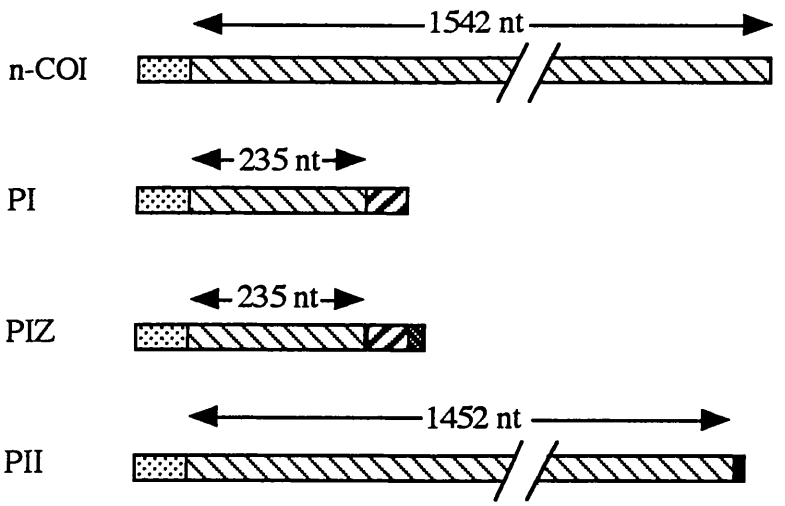


Figure 3.2 Nuclear cytochrome *c* oxidase I constructs

n-COI - the nuclear version of COXI
PI - Patient I construct
PIZ - epitope tagged version of the Patient I construct
PII - Patient II construct

mouse COXI-derived sequence

mouse COXIV-derived sequence (transit peptide - 22 aa)

human cyt *b*-derived sequence (out of frame - 20 aa)

DNA encoding the Z1F 11 epitope tag (7 aa)

human $tRNA^{thr}$-derived sequence (1 aa)

control for expression studies with the chimeric versions of n-COI depicted in fig. 3.2, and described below.

The chimeric mouse genes which I set out to construct were the equivalent of the chimeric COXI genes found in the partially duplicated mitochondrial genomes of KSS patients 1 and 2 (Poulton *et al.*, 1989) discussed in section 1.6. The first construct (designated PI for Patient I) consists of the 5' region of *n-COI*, plus a 3' region derived from the human cyt *b* gene, read out of frame (see fig. 3.2). In the mutant mitochondrial genome of KSS patient 1, COXI is joined to cyt *b* out of frame. If I had used the homologous mouse cyt *b* sequence to construct the chimeric mouse genes, a stop codon would have been generated after 6 abnormal residues rather than the 20 abnormal residues encoded by the chimeric gene found in the patient's mutant mitochondrial DNA (fig. 3.3). Also, as explained below, it was necessary for the mouse construct to encode the same carboxy-terminal protein sequence as the patient chimeric gene in order to utilise the antipeptide sera raised by J.Poulton and co-workers for analysing KSS patient 1 tissues. Therefore it was decided to use the human cyt *b* coding sequence for generating the PI construct. The second construct (designated PII for Patient II) would encode all but the final 33 amino acids of COXI, plus a single novel codon derived from the $tRNA^{Thr}$ coding sequence.

In order to analyse the expression of the three constructs based on *n-COI*, it was anticipated that a specific antibody would be necessary. No antibodies raised against mouse COXI were available. These would not have been useful for intra-mitochondrial analysis of the full-length n-COI anyway, due to the presence of endogenous COXI. In collaboration with Dr. J. Poulton rabbits were immunised with a peptide encoded by the *cyt b* region of the patient 1 chimeric gene. This should have generated PI-specific antibodies, because the *cyt b* region of the chimeric gene is read out of frame. However, it was decided also to "tag" the three n-COI constructs with a 3' terminal epitope to facilitate expression analysis. The epitope that was

(a) Human cyt *b* sequence (nt 15056-15121)

```
Y  G  S  F  L  Y  S  E  T  W  N  I  G  I  I  L  L  L  A  T  M  A
TACGGATCATTTCTCTACTCAGAAACCTGAAACATCGGCATTATCCTCCTGCTTGCAACTATAGCA
 T  D  H  F  S  T  Q  K  P  E  T  S  A  L  S  S  C  L  Q  L  *
```

(b) Mouse cyt *b* sequence (nt 14448-14513)

```
Y  G  S  Y  T  F  M  E  T  W  N  I  G  V  L  L  L  F  A  V  M  A
TATGGATCATATACATTTATAGAAACCTGAAACATTGGAGTACTTCTACTGTTCGCAGTCATAGCC
 M  D  H  I  H  L  *
```

Figure 3.3 Sequence comparisons of human and mouse frame-shifted cyt *b*

This figure depicts the region of the cytochrome *b* gene relevant to the discussion in section 3.1.1.

(a) gives a portion of the sequence of human cytochrome *b*. The top line shows the cyt *b* amino acid sequence, the middle line is the nucleotide sequence, and the bottom line is the amino acid sequence of the patient I 3' region, as specified by the frame-shifted cyt *b* nucleotide sequence.
The amino acid sequences are translated using the mitochondrial genetic code.

(b) gives the sequence of the corresponding portion of the mouse cytochrome *b*. The top line shows the cyt *b* amino acid sequence, the middle line is the nucleotide sequence, and the bottom line shows the predicted amino acid sequence if the nucleotide sequence were frameshifted as in the human patient 1. Translation of the top line uses the mitochondrial genetic code, translation of the bottom line uses the universal genetic code.

chosen is a seven amino acid epitope from the Herpes Simplex Virus - 1 65K DNA-binding protein ($65K_{DBP}$, encoded by the gene *UL42*), for which the sequence is GDPEDLD. This epitope was chosen because the monoclonal antibody designated Z1F11, which was reactive against $65K_{DBP}$, was available (courtesy of Dr. H. Marsden and co-workers), and had been characterised (Schenk *et al.*, 1988; Murphy *et al.*, 1989). The antibody was reported to recognise both the denatured protein on western blots and the native protein for the purpose of immunoprecipitation. The epitope had also been used previously as a tag for immunofluorescence-labelling of transgene-encoded proteins in a mammalian cell culture system (personal communication, Dr. R.Sutcliffe).

3.1.2 Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis is a technique which permits the programmed change of DNA sequence by *in vitro* procedures (Zoller and Smith, 1982). The technique requires the use of a cloning vector capable of generating single-stranded DNA. In general, bacteriophage M13 or M13-based phagemid vectors are used. pBluescript SK^+ (supplied by Stratagene), a phagemid, was chosen as the cloning vector, because it was reported to be less prone to deletion of insert DNA than M13, as well as more efficient at packaging the single-stranded DNA (due to its reduced size), and also because the polycloning site is situated between the T3 and T7 promoters, enabling *in vitro* transcription. In this technique, the mutagenic oligonucleotide is designed to be complementary to the single-stranded template, except at the site of the programmed mutation (which could be a point mutation, insertion or deletion of sequence). The mismatch region is usually centred between the two exactly matched anchor sequences. Figure 3.4 shows a schematic diagram of the basic technique, using a phagemid vector to create a single point mutation. The mutagenic oligonucleotide is hybridised to the single-stranded template and then extended using a DNA Polymerase (I initially used Klenow fragment) in the presence

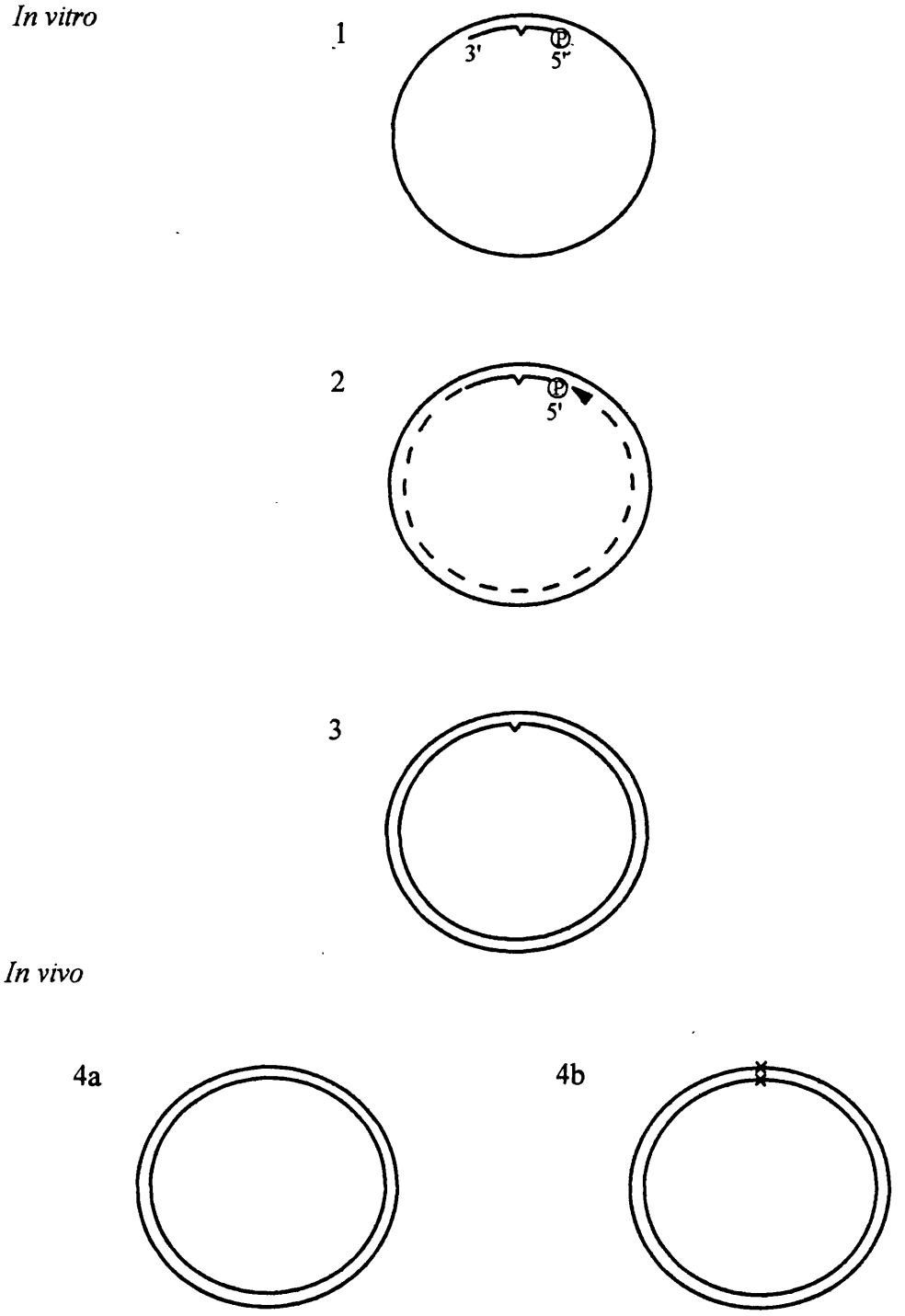


Figure 3.4 Oligonucleotide-directed mutagenesis with single-stranded DNA as template

1. A 5' phosphorylated oligonucleotide encoding a single mismatch is annealed to the single-stranded template DNA. 2. Primer extension using DNA polymerase. 3. Ligation of the nascent strand to form a closed heteroduplex molecule. 4. Replication/mismatch repair of the plasmid produces two possible daughter molecules. 4a is identical to the original plasmid. 4b contains the programmed mutation on both strands.

of T4 DNA ligase. This produces a double-stranded closed-circular DNA molecule which contains a mismatch at the site of mutation. Following transformation of a suitable host bacterial cell, the heteroduplex molecule will give rise to both mutant and wild-type progeny cells.

The diagram shown in fig. 3.4 implies that 50% of the daughter molecules will contain any given programmed mutation. However, *E.coli* contains a methyl-directed mismatch repair system which favours the repair of a non-methylated DNA strand over a methylated strand. In the host cell this system protects against mutation by preferentially repairing newly synthesised DNA which has not yet been methylated. Similarly, the non-methylated, *in vitro* synthesised strand is preferentially repaired by the cell, thus reducing the proportion of mutant progeny (Kramer *et al.*, 1984). The presence of any uncopied template or partially replicated molecules in the transformation reaction can also reduce the proportion of mutant progeny (Zoller and Smith, 1983). Klenow fragment is used as the DNA polymerase because it should lack the 5'-3' exonuclease activity. However, if any such residual activity is present in the enzyme preparation used, the mutagenic oligonucleotide is susceptible to digestion. Zoller and Smith (1984) developed a mutagenesis method using 2 primers to circumvent this problem. By having an accessory non-mutagenic primer upstream of the mutagenic oligonucleotide, which can be extended and covalently joined to the 5' end of the mutagenic oligonucleotide, the probability is increased that the mutagenic oligonucleotide will not be displaced by exonuclease activity. However, in practice, less than 10% of colonies obtained by oligonucleotide-directed mutagenesis contain a typical programmed mutation. Therefore, strand selection techniques have been designed to enrich the yield of mutants which were used in the project at specific stages (Kunkel, 1985; Vandeyar *et al.*, 1988).

3.1.3 Strand selection methods

One technique to increase the proportion of mutants obtained is the Kunkel procedure (Kunkel, 1985). This requires the sequential use of two *E. coli* strains. The template strand is prepared in a bacterial strain which is deficient in both dUTPase (*dut*) and uracil-n-glycosylase (*ung*). The enzyme dUTPase converts dUTP to dUMP, so a deficiency causes an increase in the cellular pool of dUTP leading to its incorporation in DNA at thymine sites. The second enzyme, uracil-n-glycosylase, would normally remove uracil residues incorporated into DNA. After the *in vitro* mutagenesis reaction using standard dNTPs, transformation of the heteroduplex molecules into an *ung*+ strain will remove the uracil from the template strand, thus blocking its replication and making it susceptible to cleavage by nucleases. The preferential destruction of the template strand increases the proportion of progeny containing the desired mutation. One drawback to this technique is that *dut*-, *ung*- strains are less viable than standard *E. coli* strains due to the increased potential for mutation of the host genome.

The converse of this approach is to include a modified nucleotide in the *in vitro* synthesis of the second strand, such as 5-methyl-dCTP or dCTPαS. The heteroduplex can then be nicked with an endonuclease (e.g., *Msp*I and *Nci*I respectively) which cleaves the non-modified strand preferentially, and exonuclease III digestion will then remove the parental strand (Vandeyar *et al.*, 1988, Eckstein/Amersham system). Other systems are now widely available.

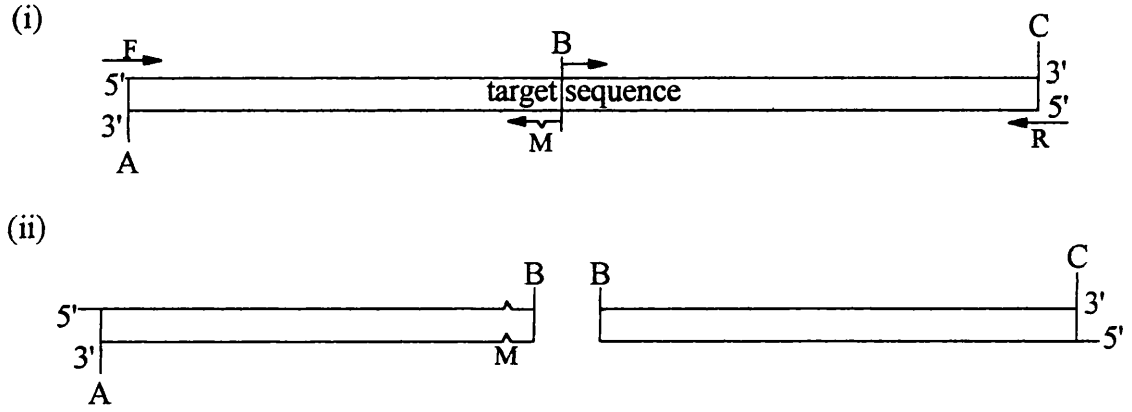
3.1.4 The use of PCR in oligonucleotide-directed mutagenesis

Another version of oligonucleotide-directed mutagenesis utilises the polymerase chain reaction. As with the technique already described, the programmed mutations are incorporated in the oligonucleotide sequence. Several variations of the technique

exist (see fig. 3.5). Mutagenic primers are used in PCR to create changes in the target sequence whilst amplifying the fragments which are then cloned by standard methods. Mutagenesis of an internal site will necessarily generate two fragments, because each oligonucleotide used for priming DNA synthesis forms an end of a product. The products can then be subcloned, either with a blunt end ligation at the junction formed by the two internal primers, or by "sticky-ended" ligation, if a suitable restriction site is present or can be generated. Although "sticky-ended" ligation is more efficient than blunt ended, the problem with the second strategy is that not all endonucleases are capable of efficient digestion near to the end of a fragment. Sequential PCR overcomes this by designing primers that overlap. This produces 2 PCR fragments which, when denatured and reannealed, can form the template for a second round of PCR. The product of this reaction can then be subcloned as a single fragment.

The major advantages of a PCR-based method for oligonucleotide-directed mutagenesis are the simplicity of the technique (there is no need to use special vectors and procedures for preparing single-stranded DNA), speed, and the high frequency of mutation incorporation. However, it is more costly than the procedure described in section 3.1.2 because 2 oligonucleotides are required for each mutagenic target site. Additionally, 2 primers external to the target sequence are required for amplification by PCR (although these could be standard primers such as M13 forward/reverse, which hybridise to vector sequence). The major drawback of a PCR-based approach is the fidelity of the DNA polymerase used. *Taq* DNA polymerase does not have a 3'-5' exonuclease (proofreading) activity. Thermostable DNA polymerases with proofreading activity are now commercially available, *e.g.*, *Pfu* polymerase supplied by Stratagene. However, a PCR-based approach was not deemed suitable to a project requiring the mutagenesis of multiple sites within a single gene such as this, although this approach was used for other steps in the creation of the nuclear-expressible transgenes.

(a) Blunt end



(b) Sticky end

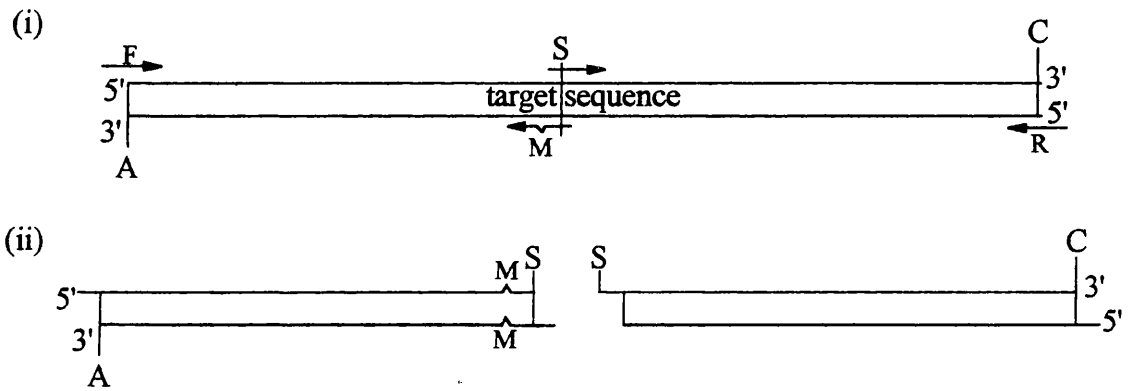


Figure 3.5 Oligonucleotide-directed mutagenesis by PCR

(a) Mutagenesis by PCR produces two fragments which can be subcloned by a blunt end ligation at the central junction.
(b) Mutagenesis by PCR produces two fragments which can be subcloned by a cohesive end ligation at the central junction.

(i) PCR of the template sequence is performed using two external primers, and two internal primers. The mutation is programmed by one of the internal primers for methods (a) and (b).
(ii) PCR yields two products. (a) digestion with restriction enzymes A and C enables directional subcloning of the two fragments into the vector, with a blunt end ligation at the junction of the fragments. (b) digestion with restriction enzymes A, C, and S enables directional subcloning of the two fragments in a vector.

A, C - flanking restriction sites. F, R - forward and reverse primers. B - blunt end junction of primers. M - point mutation introduced. S - sticky-end restriction site.

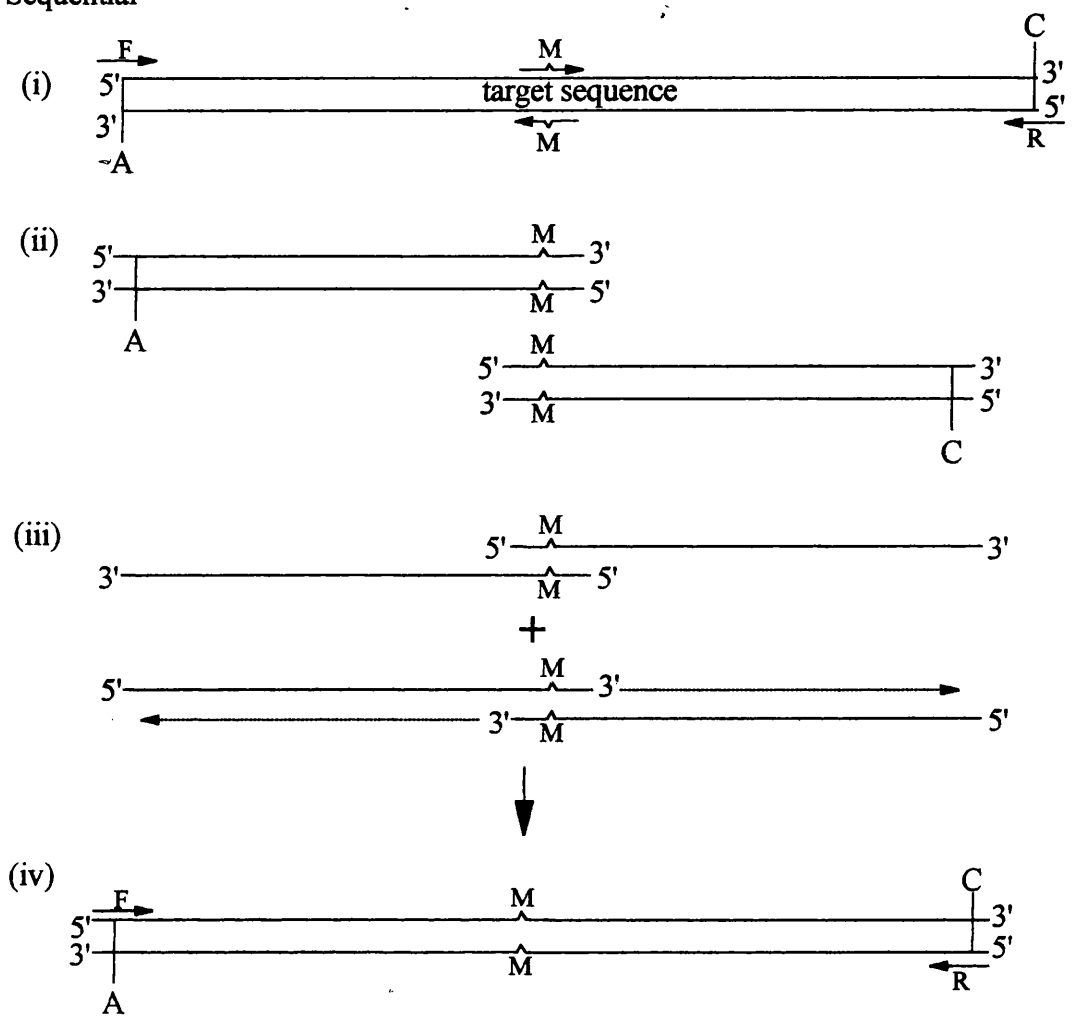


Fig. 3.5 continued

(c) Mutagenesis by sequential PCR is executed in two stages, producing a single fragment for subcloning.

(i) PCR of the template sequence is performed using two external primers, and two internal primers. The mutation is programmed by both internal primers for method (c).
(ii) PCR yields two products. (c) denaturing and annealing of the two products yields a mixture of molecules, including the two shown in (iii).
(iii) The reannealed product with the free 3' OH ends can serve as a substrate for DNA polymerase.
(iv) PCR of the new template sequence is performed using two external primers. Digestion with restriction enzymes A and C enables directional subcloning of a single fragment.

A, C - flanking restriction sites. F, R - forward and reverse primers. M - point mutation introduced.

3.2 Results

3.2.1 Subcloning of mouse COXI into pBluescript II SK+

Plasmid p5.0 (a gift from Dr. K. Fischer-Lindahl) contains the region 3565-8420 of the mouse mitochondrial genome, cloned as a *Bam*HI/*Pst*I fragment in pUC. The 2,448 bp *Lsp*I fragment from this plasmid contains the entire coding sequence of COXI, as well as 107 nt of 5' and 800 nt of 3' flanking DNA. Following digestion with *Lsp*I, the 5' overhanging ends of the fragment were endfilled using DNA polymerase I Klenow fragment, and subcloned into the *Sma*I site of pBluescript SK+. *Eco*RV digestion of plasmid DNA prepared from 12 recombinant colonies (as ascertained by blue/white colour selection of colonies grown in the presence of X-gal and IPTG) enabled assessment of the presence of an insert, and the orientation of the mouse mitochondrial DNA insert within the vector multiple cloning site (MCS). An *Eco*RV site is present in the MCS of pBluescript II SK+, and a single *Eco*RV site is present in the insert DNA, at nt 42 of COXI. In one orientation the 2 restriction fragments produced are 3.15 kb and 2.35 kb respectively, in the other orientation the fragments are 5.35 kb and 0.15 kb. 9 transformants contained the insert in the "sense" orientation for COXI, i.e., transcription from the β-galactosidase promoter would produce mRNA containing the coding sequence of COXI (the remaining 3 transformants contained no detectable insert). One transformant containing the mouse mitochondrial DNA insert was selected for use, and designated pLR (fig. 3.6).

3.2.2 Mutagenesis of mouse COXI

The pBluescript vectors are phagemids which can be used to prepare single-stranded DNA. The vector contains a 454 nt intergenic region from an f1 filamentous phage (f1IG), which is M13-related. This region encodes all of the *cis*-acting functions necessary for single-stranded DNA synthesis and packaging. The orientation of the

f1IG region in the vector determines whether the '+' or '-' strand is synthesised. The use of pBluescript vectors for single-stranded DNA preparation requires an F^+ *E. coli* host, because the sex pili encoded by the F plasmid are used by filamentous helper phage as the route of infection. The engineered helper phage encode *trans*-acting factors for the preferential packaging of phagemid DNA. pLR contains the mitochondrial DNA insert in the orientation which produces single-stranded DNA containing the sense strand of COXI. Therefore, all oligonucleotides designed for the site-directed mutagenesis are based on the antisense sequence (see fig. 3.7.a for a depiction of the plan; the sequences of the oligonucleotides are listed in full in section 2.4.1).

I devised a strategy using 24 oligonucleotides to create 43 point mutations in the COXI coding sequence which would enable correct cytosolic translation. Each oligonucleotide encoded between 1-5 programmed mutations. Where possible, they were designed to anneal precisely adjacent on the template, so that they could be ligated together prior to the addition of DNA polymerase. The "rules" used for oligonucleotide design were as stated in Sambrook *et al.*, e.g., 8-10 nt anchor sequence 5' of mutation (to prevent displacement by DNA polymerase), 7-9 nt 3' of mutation (to prevent exonucleolytic degradation), minimal length of the oligonucleotide while meeting the other criteria (in order to maximise the difference in thermal stability between a perfectly matched/mismatched hybrid for later screening). Also, C/G base pairing was preferred at termini to increase primer/template hybrid stability. Up to 14 oligonucleotides were used per primer extension reaction. Following transformation, 10^2-10^3 colonies were patched out in a grid format and colony lifts were screened with two ^{32}P end-labelled oligonucleotides of similar melting temperature (T_m) from the primer extension reaction. Final wash conditions were calculated to preserve hybrids between the oligonucleotide probes and the correctly mutated DNA, but not with the wild-type DNA, which would have 1-5 mismatches depending on the oligonucleotide used. If obtained, colonies positive

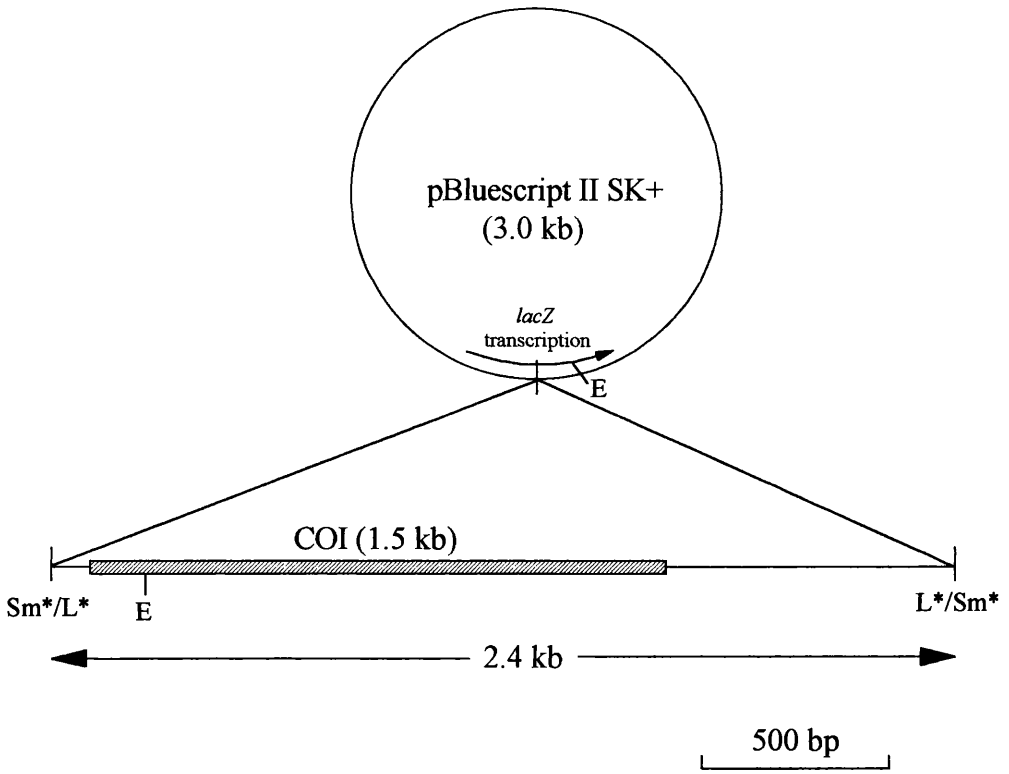
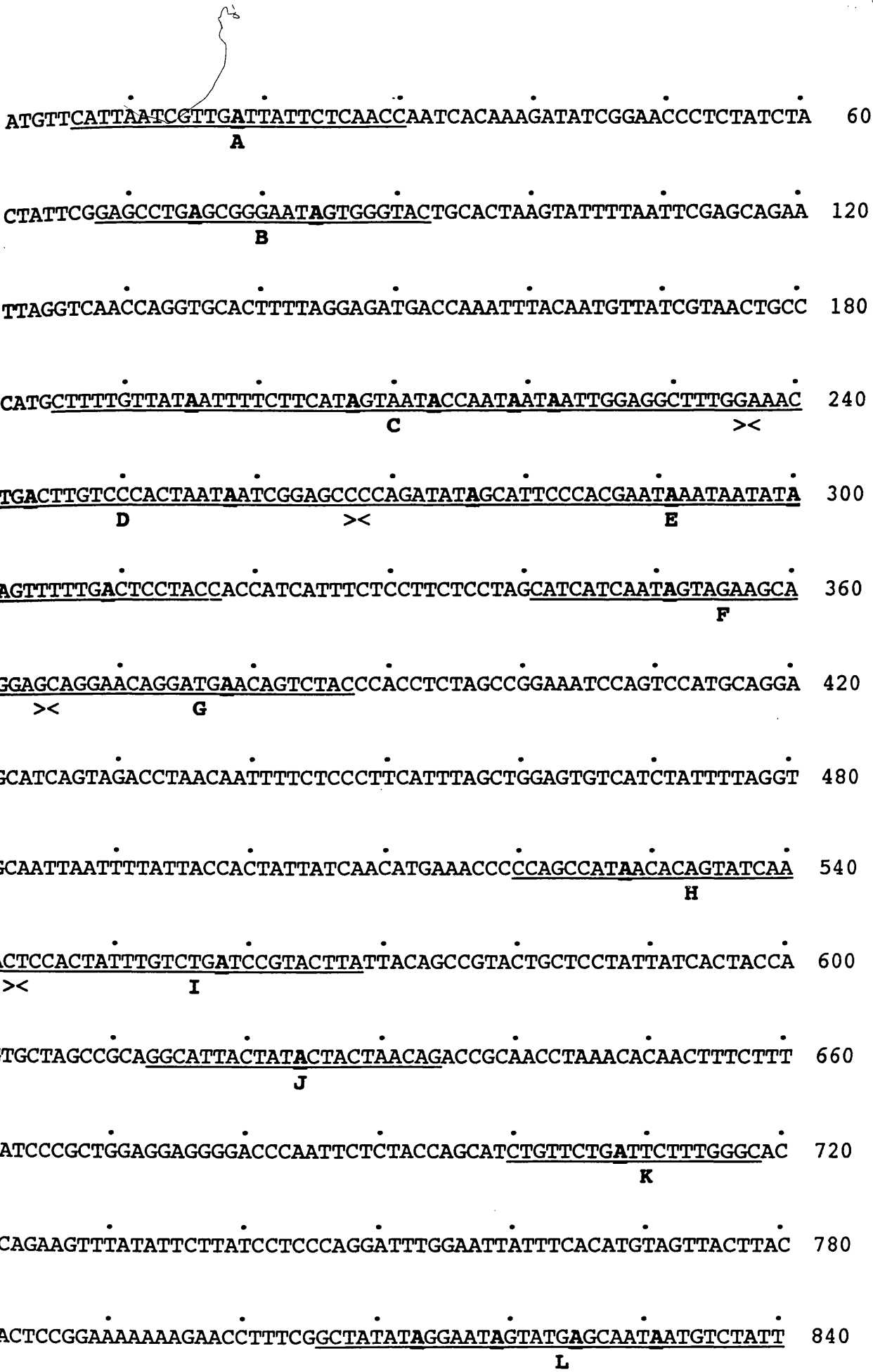


Figure 3.6 Plasmid pLR

Map of Plasmid pLR. The 2.4 kb mouse mitochondrial DNA *Lsp*I fragment containing the COXI gene is subcloned into the *Sma*I site of pBluescript II SK+. Restriction enzyme sites are as follows: E - *Eco*RV, L - *Lsp*I, Sm - *Sma*I.
* denotes site destroyed in subcloning.


ATGTTCATTAATCGTTGATTATTCTCAACCAATCACAAAGATATCGGAACCCTCTATCTA 60
A
CTATTCGGAGCCTGAGCGGGAATAGTGGGTACTGCACTAAGTATTTTAATTCGAGCAGAA 120
B
TTAGGTCAACCAGGTGCACTTTTAGGAGATGACCAAATTTACAATGTTATCGTAACTGCC 180
CATGCTTTTGTTATAATTTTCTTCATAGTAATACCAATAATAATTGGAGGCTTTGGAAAC 240
C
><
TGACTTGTCCCACTAATAATCGGAGCCCCAGATATAGCATTCCCACGAATAAATAATATA 300
D
><
E
AGTTTTTGACTCCTACCACCATCATTTCTCCTTCTCCTAGCATCATCAATAGTAGAAGCA 360
F
GGAGCAGGAACAGGATGAACAGTCTACCCACCTCTAGCCGGAAATCCAGTCCATGCAGGA 420
><
G
GCATCAGTAGACCTAACAATTTTCTCCCTTCATTTAGCTGGAGTGTCATCTATTTTAGGT 480
GCAATTAATTTTATTACCACTATTATCAACATGAAACCCCCAGCCATAACACAGTATCAA 540
H
ACTCCACTATTTGTCTGATCCGTACTTATTACAGCCGTACTGCTCCTATTATCACTACCA 600
><
I
GTGCTAGCCGCAGGCATTACTATACTACTAACAGACCGCAACCTAAACACAACTTTCTTT 660
J
GATCCCGCTGGAGGAGGGGACCCAATTCTCTACCAGCATCTGTTCTGATTCTTTGGGCAC 720
K
CCAGAAGTTTATATTCTTATCCTCCCAGGATTTGGAATTATTTCACATGTAGTTACTTAC 780
TACTCCGGAAAAAAAGAACCTTTCGGCTATATAGGAATAGTATGAGCAATAATGTCTATT 840
L

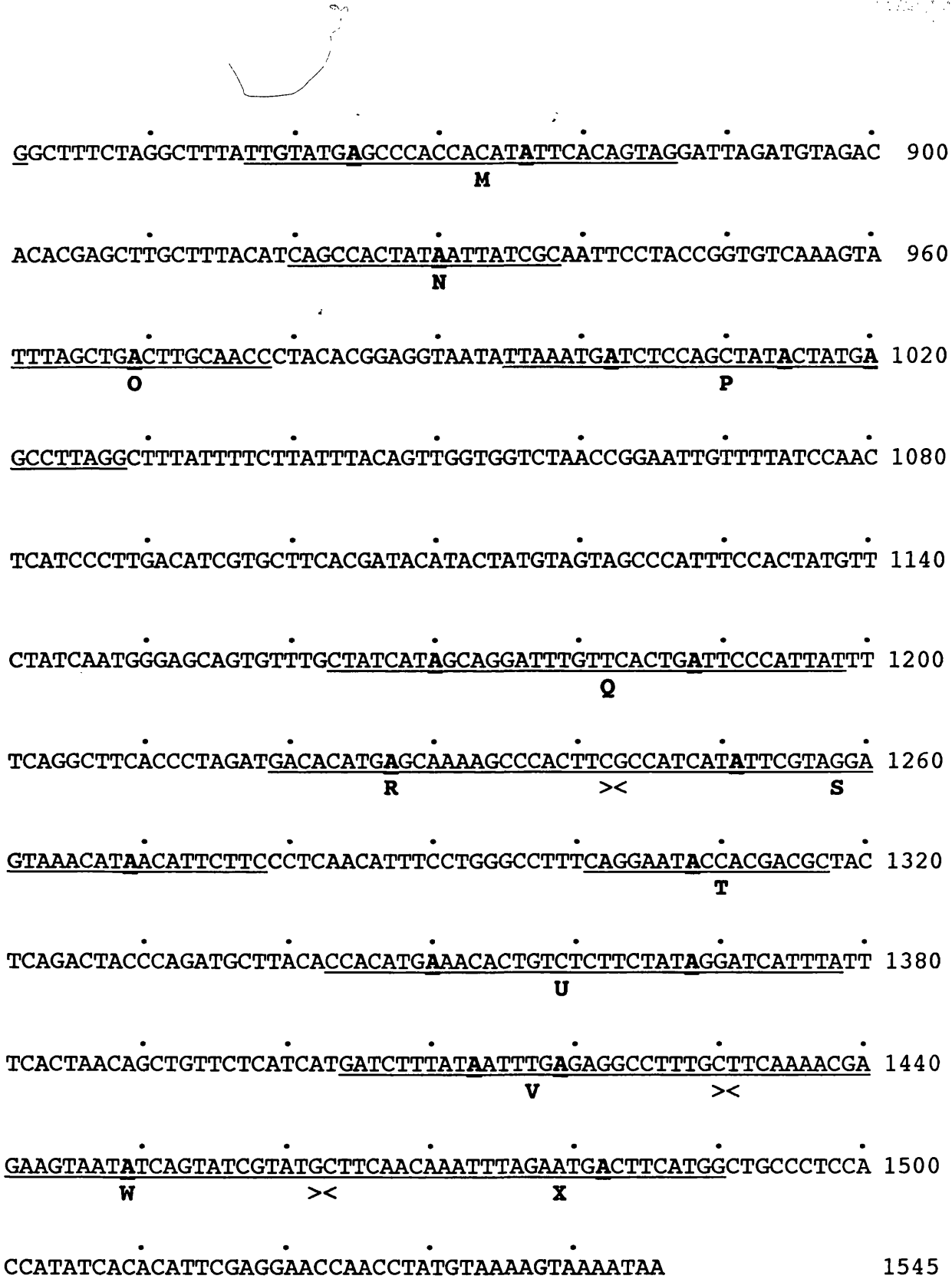


Figure 3.7.a COXI - mutagenesis strategy

The nucleotide sequence of the mouse COXI gene is shown. Bold type indicates nucleotides programmed for mutation. Underlining indicates the span of the complementary oligonucleotide to effect the A>G transition. The letter centered below this line is the reference letter for the oligonucleotide. The arrowheads ('>' and '<') are used to indicate the endpoints of the respective oligonucleotides where two mutagenic oligonucleotides are juxtaposed. The sequences of all the oligonucleotides are listed in section 2.4.1.

i) **595- 620**

```
                    L  P  V  L  A  A  G  I  T
mouse COXI          5'CTACCAGTGCTAGCCGCAGGCATTAC 3'

                    L  P  V  P  P  Q  A  L
pLR8                5'CTACCAGTACCA CCACAGGCATTAC 3'
oligo J'repair      3'GATGGTCATGATCGGCGTCCGTAATG 5'

                    L  P  V  L  A  A  G  I  T
pLR9                5'CTACCAGTACTAGCCGCAGGCATTAC 3'
```

ii) **1167-1191**

```
code-                  M  A  G  F  V  H  W  F
corrected           5'CATGGCAGGATTTGTTCACTGGTTC 3'
COXI
                       M  A  G  L  F  T  G  S
pLR10               5'CATGGCAGGAT TGTTCACTGGTTC 3'
oligo Q"repair      3'GTACCGTCCTAAACAAGTGACCAAG 5'
```

iii) **1249-1269**

```
code-                  M  F  V  G  V  N  M
corrected           5'ATGTTCGTAGGAGTAAACATG 3'
COXI
                       M  F  V  R  V  N  M
pLR10               5'ATGTTCGTAAGAGTAAACATG 3'
oligo S'repair      3'TACAAGCATCCTCATTTGTAC 5'

                       M  F  V  G  V  N  M
pLR11               5'ATGTTCGTAGGAGTAAACATG 3'
```

Figure 3.7.b Mutagenesis strategies to repair unprogrammed mutations

This figure shows the 3 unprogrammed mutations which occurred during the course of mutagenising COXI, the oligonucleotides which were designed to repair those mutations, and the product of the mutagenesis repair reaction. The nucleotide sequence is given for the region specified within the mouse COXI gene (numbered as in part (a) of this figure). The amino acid translation is shown above the nucleotide sequence. Programmed mutations (already executed) are indicated by underlining and bold font as in figure 3.7.a. The unprogrammed mutations are shown in bold font. Affected amino acid residues are also indicated in bold font. As the unprogrammed mutations shown in (i) and (ii) both involve a point deletion, both result in a frame-shifting of the encoded protein, thus all the residues encoded after the respective point deletions are shown in bold.

for both oligonucleotides were sequenced (up to 12 per primer extension reaction), and the clone with the most programmed mutations, but no unprogrammed mutations, was selected for the next round of mutagenesis.

During the initial phase of site-directed mutagenesis I attempted to use the Kunkel procedure, which was originally designed for use with bacteriophage M13 recombinants. Using pLR I observed poor growth in *E. coli* strain CJ236 (*dut-*, *ung-*) and obtained no transformants in the *ung+* or *ung-* strains with the primer extension reaction mix using CJ236-derived template DNA. However, as I was successful in obtaining clones with multiple changes from the use of a mutagenesis and screening procedure in *E. coli* strain XL1-blue, I did not pursue optimisation of the Kunkel procedure for the pBluescript derivatives with which I was working.

After four successful rounds of mutagenesis using the protocols recommended by Stratagene for use with pBluescript vectors, a clone was obtained, designated pLR4, which required only 8 further point mutations comprised in 6 oligos (see Table 3.1 for a summary of the mutagenesis procedure). The DNA polymerase Sequenase™, which cannot displace the hybridised primer from the template as easily as Klenow fragment, was then used in the subsequent primer extension reactions. The use of this engineered enzyme achieved mutations in two rounds of mutagenesis with 3 oligonucleotides which were previously unsuccessful. To carry out the remaining "problematic" mutations I then altered the protocol slightly. I hypothesised that secondary structure in the area to which the oligonucleotides were designed to hybridise could be inhibiting the annealing of primers. In addition to denaturing the template by heating (as per the original protocol), I tried alkali denaturation (as used in sequencing of double-stranded plasmid). This proved moderately successful, in that I obtained further mutations using oligonucleotides which previously had been unsuccessful (e.g. mutagenic oligonucleotide R). I also tried parallel primer extension reactions using the remaining primers singly/together, as there may have been

Template	Oligonucleotides Used	Mutations Obtained	Clone
pLR	A,B,C,D,E	A,B,C,D,E	pLR1
pLR1	F,G,H,I,J	F,G	pLR2
pLR2	H,I,J,K,L,M,N,O,P,Q,R,S,T,U	H,I,K,L*,M,O,P,U	pLR3
pLR3	J,L,N,Q,R,S,T,V,W,X	T,V,W,X	pLR4
pLR4 $	J,L,N,Q,R,S	J,N	pLR5
pLR5 $	L',Q,R,S	Q*	pLR6
pLR6 $+	L',Q',R,S	L'	pLR7
pLR7 $+	Q',R,S	R	pLR8
pLR8 $+	Q',S,J'(repair)	J'(repair)	pLR9
pLR9 #	Q',S	Q',S	pLR10
pLR10 #	Q''(repair),S'(repair)	S'(repair)	pLR11

Table 3.1 Summary of the oligonucleotide-directed mutagenesis procedure

The sequence of mutagenic events which occurred to code-correct the mouse COXI gene are presented in tabular form. Symbols used: * - a subset of the programmed mutations for this oligo was achieved (see text for details). $ - Sequenase™ used as the DNA polymerase rather than Klenow fragment. + - Alkali treatment used rather than heat to denature the template. # - Amersham oligonucleotide-directed *in vitro* mutagenesis system used. ' - indicates an oligonucleotide which was redesigned for mutagenesis, but corresponds to the general region indicated on figure 3.7.a by the letter. (repair) - indicates that the mutation encoded by the oligonucleotide is not one of the transitions originally programmed, but to correct an unprogrammed mutation which occurred during the mutagenesis procedures.

misannealing and priming by an oligonucleotide at an incorrect site, or hybridisation occurring between oligonucleotides (although no obvious sequence similarity between them is evident). However, the use of single primers produced no mutants (see section 3.1.2). During the course of the mutagenesis, I redesigned 2 oligonucleotides (L and Q) when a subset of the programmed mutations for the respective oligonucleotides was successful, but other changes remained. In the case of Q, the mutation at nt 1188 of the COXI sequence was initially successful, but the transition at nt 1170 was not (which is the 3' proximal change encoded by the oligonucleotide). For primer L it was the 5' proximal change (nt 831) encoded by the oligonucleotide which was unsuccessful with the original mutagenic oligonucleotide; it is therefore possible that 5'-3' exonuclease activity displaced part of the oligonucleotide.

After a further four mutagenesis procedures as described above I had obtained a clone (pLR8), which still required 3 programmed mutations (in oligonucleotides Q' and S) as well as a further mutation event to repair unprogrammed changes (in the region of oligonucleotide J) (fig. 3.7.b). After the next round of mutagenesis all 3 oligonucleotide primers were used to screen the progeny, but only clones positive for single mutations were found. A clone (designated pLR9) was chosen which had been correctly repaired using the mutagenic oligonucleotide J' repair. However, the region of COXI which was the target sequence for mutagenic oligonucleotides Q' and S was apparently resistant to mutagenesis. Because it seemed unlikely that these last few changes could be achieved without a selection procedure, the Amersham oligonucleotide-directed *in vitro* mutagenesis system was purchased.

The Amersham kit makes use of the Eckstein selection technique described previously, combined with a filtration step to remove single-stranded template DNA which has not undergone second strand synthesis. Using the kit, 2 clones were obtained which had both of the programmed mutations encoded by oligonucleotide S.

However, both also contained unprogrammed point mutations within the region of oligonucleotide S. Clone AmS+5 had a G-A transition at nt 1258, while clone AmS+12 had a C-G transversion at nt 1254. Both of these unprogrammed point mutations fall between the programmed mutations which were successfully corrected using the oligonucleotide. It may be that the two rare point mutations were selected for because they were able to disrupt the secondary structure in the region thus enabling the mutagenic oligonucleotide to bind, although why they were not then subsequently corrected by the same process as the programmed point mutations, is not clear. One of these 2 clones also had the code correction programmed by oligonucleotide Q', so this clone was chosen as pLR10 to undergo a final repair mutagenesis. Two repairs were necessary: (i) the repair at nt 1258, because the G-A transition caused a codon change from glycine to arginine, and (ii) correction of a single nucleotide deletion in a group of three thymine residues (1177-1179) in the region of oligonucleotide Q. The deletion (which had occurred in a previous round of mutagenesis) generates a frameshift and a premature stop codon at nt 1214. As oligo Q' did not repair this deletion, a new oligonucleotide Q" repair was designed which had ~12 nt anchor sequences to either side of the point deletion; the S' repair oligo had 10 nt anchor sequences (both repair strategies are depicted in fig. 3.7.b). Using the Amersham kit, a very rare mutagenesis event (5 ug of starting template yielded only 9 transformants) produced 2 clones which were code-corrected for the S' repair, but none of which had the thymine insertion. One of these clones with the repair at nt 1258 was designated pLR11, and was used as the template for PCR-based oligonucleotide-directed mutagenesis to repair the remaining deletion (the repair is described in section 3.2.6).

3.2.3 Creation of additional coding regions

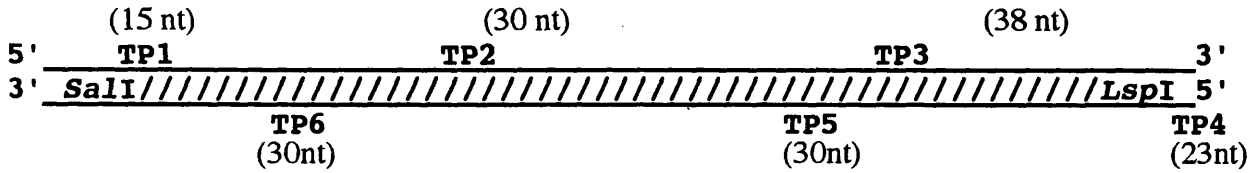
As explained in section 3.1.1, in addition to the introduction of point mutations for the purpose of code correction, it was necessary to subclone the coding sequence for a

transit peptide to target the proteins encoded by the *n-COI* based constructs to the mitochondrial compartment. It was also necessary to subclone the human *cyt b* region which encodes the 3' section of the PI construct, and to add the epitope tag described above.

3.2.3.1 Transit peptide-encoding region

Using the reported sequence of the mouse COXIV cDNA (Grossman and Akamatsu, 1990), I designed six oligonucleotides which, when annealed, were to form the complete double-stranded sequence encoding the transit peptide and restriction sites suitable for subsequent recloning (fig. 3.8). *Sal*I was chosen as the 5' restriction site because it was compatible with all 3 vectors chosen for the project (the mammalian expression vector pMam*neo* has only 3 unique sites in the MCS, limiting the choice). At the 3' end of the coding sequence *Lsp*I was chosen, because the restriction site for *Lsp*I is formed by the juxtaposition of the final 5 nt of the COXIV transit peptide sequence and the first nucleotide of the start codon of COXI, whilst maintaining the correct reading frame. The sequence of the junction is shown here - **CTT CGA /** ATG - with the *Lsp*I site emphasised in bold print. The oligonucleotides were 5' phosphorylated, annealed in equimolar amounts, ligated, and digested with *Sal*I. A sample of the annealed oligonucleotides was run on a gel at this point to check for appropriate double-stranded product formation. The remaining oligonucleotide mixture was then deproteinised with phenol/chloroform, ethanol precipitated, and subcloned into pBluescript SK+, which had been digested with *Eco*RV/*Sal*I. Twelve transformants were analysed by restriction digests; *Lsp*I to test if the site was present in the insert, *Sal*I/*Xba*I to test whether the insert was of the correct size. Eight clones met these criteria, one of which (designated pTP/cx4) contained the correct insert when sequenced. However, it had not inserted into the polylinker as designed. The restriction site of the insert fragment had not been digested, and a short sequence of DNA (which was the product of a successful *Sal*I digestion of another insert

(a)



(b)

```
     SalI                                                                LspI
5'GCGGTCGACCAGAAT/GTTGGCTTCCAGAGC GCTGAGCCTGATTGG/CAAGAGAGCCATTTC TACTTAGGTGTGCCTTCGAAGCG 3'
3'CGCCAGCTGGTCTTA CAACCGAAGGTCTCG/CGACTCGGACTAACC GTTCTCTCGGTAAAG/ATGAAGCCACACGGAAGCTTCGC 5'
```

Figure 3.8 **Creation of the COXIV transit peptide coding region**

(a) The oligonucleotides are indicated by the lines, depicting the double-stranded coding region created when the six oligonucleotides are annealed. The 6 oligonucleotides anneal to form 83 bp which encode the 22 amino acids of the COXIV mitochondrial targetting peptide, a small portion of 5' untranslated sequence, and the two restriction sites for subcloning the fragment. The designation TP1-6 indicates the oligonucleotide reference. The number of residues in each oligonucleotide are bracketed. The restriction sites encoded are also indicated.

(b) The sequences are given for oligonucleotides TP1-6, depicting the double-stranded coding region created when the six oligonucleotides are annealed. The restriction enzyme sites are indicated by underlining, breaks between oligonucleotides by a slash (/), and the first methionine codon is indicated by bold font. The amino acid sequence of the transit peptide is as already stated in figure 3.1.

fragment) had annealed to the *Sal*I site in the vector. This enabled a blunt-ended insertion event in the reverse orientation to the original design (fig. 3.9).

3.2.3.2 Patient I 3'-encoding region

The human *cyt b* coding sequence which encodes the Patient I-specific ORF was created in a similar fashion to the transit peptide-encoding sequence. It was necessary to choose suitable restriction sites in the design of the oligonucleotides in order to subclone the fragment that would be generated. *Sal*I was chosen as the 3' restriction site for the simple reason given above, whilst the choice of *Bcl*I as the enzyme for the 5' site of the fragment was more complicated. As shown in table 3.2, the sequence of the first 3 codons in the Patient I 3' region encode threonine, aspartic acid, and histidine. In bold, I have emphasised the 4 nt which are the 5' protruding end formed by digestion with *Bcl*I or *Bam*HI at an appropriate site. Alteration of the coding sequence to a *Bcl*I site would not have changed the coding sense, but there is no *Bcl*I site in pBluescript SK$^+$ to make use of during the sequential subcloning of the sections of the PI construct. Conversely, there is a *Bam*HI site in the polylinker of pBluescript SK$^+$, but alteration of the coding sequence to this restriction site would have changed the coding sense of the third codon. Therefore, I devised a subcloning strategy which utilises both of these enzymes to facilitate sucloning without changing the coding sequence (see table 3.3, and section 3.2.4 for the execution of the procedure).

Five oligonucleotides were used to encode the 20 amino acid residues, stop codon, and two restriction sites for subcloning as depicted in fig. 3.10. The oligonucleotides were treated as in section 3.2.3.1 and subcloned into pBluescript SK+ (digested with *Sal*I/*Eco*RV). Twelve transformants were analysed by double digest (*Sal*I/*Xba*I) to check the size of the insert. Clones which contained the correct size insert were analysed by single digest (*Bcl*I) for the presence of the cloning site. This required

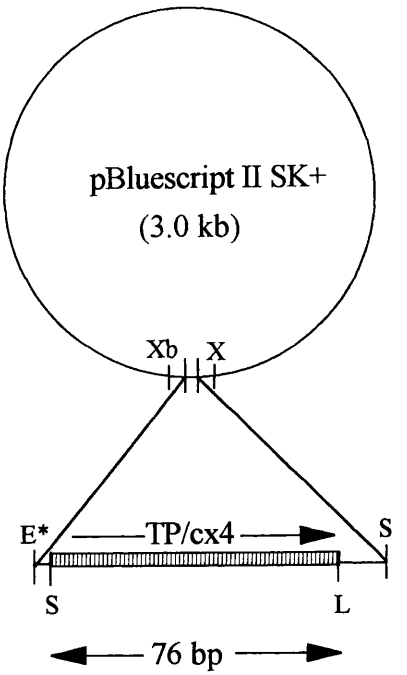


Figure 3.9 Plasmid pTP/cx4

The plasmid encoding the transit peptide of the mouse cytochrome *c* oxidase subunit IV, subcloned in pBluescript II SK+. Restriction sites are as follows: E - *Eco*RV, L - *Lsp*I, S - *Sal*I, X - *Xho*I, Xb - *Xba*I. * - denotes a site destroyed in subcloning.

	DNA sequence	Amino acid sequence	Restriction Site
a	ACG **GAT** CAT	Thr Asp His	
b	ACT **GAT** CAT	Thr Asp His	*Bcl*I
c	ACG **GAT** CCT	Thr Asp Pro	*Bam*HI

Table 3.2 The coding sequence of the first three novel amino acids of the PI3' region

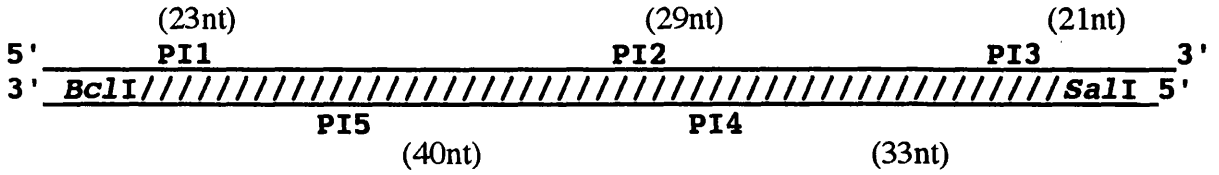
Row (a) of Table 3.2 shows the DNA sequence and the corresponding protein sequence for the first three novel residues of the Patient I 3' region.
Row (b) shows what the corresponding sequences would be if a *Bcl*I site was created.
Row (c) shows what the corresponding sequences would be if a *Bam*HI site was created.
The bases making up the restriction site are underlined. The 5' protruding end generated by digestion with either restriction enzyme is indicated by bold font.

	DNA site	Sequence	Procedure
a	Annealed PI1/PI5	5'-CGT **GAT CAT**...-3' 3'-GCA CTA GTA...-5'	digestion with *Bcl*I
b	PCR product COI/PI3'	5'-...**ACG** GAT CCC-3' 3'-...**TGC CTA GG**G-5'	digestion with *Bam*HI
c	Patient I Sequence	5'-...ACG GAT CAT...-3' 3'-...TGC CTA GTA...-5'	Ligation

Table 3.3 The strategy adopted for subcloning the PI3' region

Table 3.3 shows how both *Bcl* I and *Bam* HI were used to facilitate the subcloning of the Patient I 3' region and the COI fragment without altering the coding sequence.
Row (a) shows the double-stranded DNA sequence (of the relevant codons) from the 5' section of the PI3' region generated by the annealing of oligonucleotides PI1 and PI5. This contains a *Bcl*I site, thus maintaining the histidine codon.
Row (b) shows the double stranded DNA sequence (of the relevant codons) generated by PCR using oligonucleotide COI/PI3' as the 3' primer to amplify the region of COI found in the Patient I construct and tag it with the *cyt b* junction. This contains a *Bam*HI site.
Row (c) shows the Patient I sequence, which is generated when the products in rows (a) and (b) are digested with the appropriate restriction enzyme and ligated together. The final sequence translates as Thr Asp His, the same sequence as is found in the patient.
The bases making up the restriction sites are underlined. The complementary products created by restriction enzyme digestion which are then annealed together to create the junction found in the finished product are emphasised in bold print.

(a)



(b)

```
     BclI                                                        SalI
5' GCGTGATCATTTCTCTACTCAGA/AACCTGAAACATCGGCA TTATCCTCCTGC/TTGCAACTATAGGTCGACGCG 3'
3' CGCACTAGTAAAGAGATGAGTCT TTGGACTTTGTAGCCGT/AATAGGAGGACG AACGTTGATATCCAGCTGCGC 5'
```

Figure 3.10 Creation of the Patient I 3' region

(a) The oligonucleotides are indicated by the lines. The 5 oligonucleotides anneal to form 73 bp which encode the 20 amino acids of the Patient I 3' region, a stop codon and the two restriction sites for subcloning the fragment. The designation PI1-5 indicates the oligonucleotide reference. The number indicates the number of residues in the oligonucleotide. The restriction sites encoded are also indicated.

(b) The sequences are given for oligonucleotides PI1-5, depicting the double-stranded coding region created when the five oligonucleotides are annealed. The restriction enzyme sites are indicated by underlining, breaks between oligonucleotides by a slash (/).

transformation of the plasmid DNA into *E.coli* strain CB51, which is *dam*-, to enable digestion of prepared plasmid DNA with *BclI*, a methylation-sensitive restriction enzyme. Seven clones were chosen for sequencing, of which one (designated pPI3') had the correct sequence. Although it had inserted into the polylinker in the orientation which was planned (the insert fragment had been digested with *SalI* and successfully ligated into the *SalI* vector site), a short piece of extraneous DNA (the 3' product of *SalI*-digestion of the insert DNA) was inserted between the *Eco*RV vector site, and the blunt end of the insert DNA (fig. 3.11).

3.2.4 Subcloning PI - the Patient I construct

As the Patient I construct encodes only the first 227 nt of COXI, the requisite point mutations for the conversion of this construct to the universal code had been achieved after one successful round of mutagenesis. In order to subclone the 5' terminal portion of the code-corrected COXI, I used a PCR strategy. The 5' primer was designed to include an *Lsp*I site such that COXI could be joined to the COXIV transit peptide coding sequence whilst keeping the coding sequence in frame (see section 3.2.3.1 for the sequence at the junction). The 3' primer was engineered to contain a *Bam*HI site in the 8 nt of human cyt *b* sequence that it encoded (table 3.3). Digestion with *Bam*HI, as explained in section 3.2.3.2, generates an overhang compatible with *BclI* for joining the PCR fragment to the PI3' coding region. The PCR product was digested with *Bam*HI and subcloned into pBluescript SK+ (digested with *Eco*RV/*Bam*HI) to produce pPIpcr, a plasmid containing only the *n*-COXI PCR fragment necessary for the construction of the entire PI construct (see fig. 3.12).

To assemble the Patient I coding sequence in pBluescript SK^+ from the 3 plasmids which contained the COXIV transit peptide coding region, the relevant section of *n-COI*, and the segment of human cyt *b* coding sequence, (pTP/cx4, pPIpcr, and pPI3' respectively), the following procedures were performed (for a summary of the

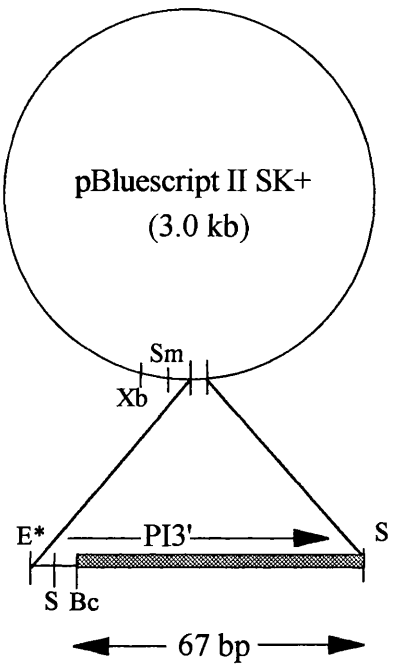
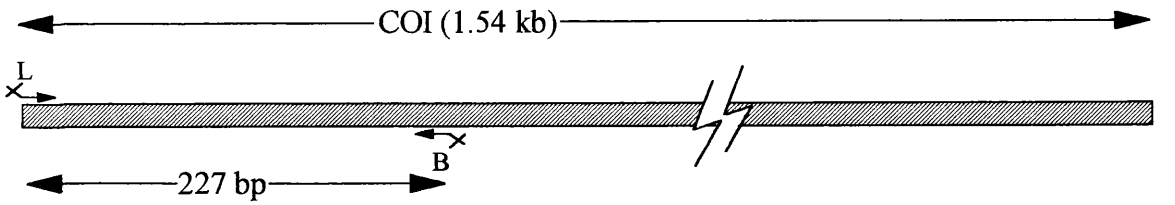


Figure 3.11 Plasmid pPI3'

The plasmid encoding the 3' region of the Patient I construct, derived from the human cyt *b* coding sequence, subcloned in pBluescript II SK+. Restriction sites are as follows: Bc - *Bcl*I, E - *Eco*RV, S - *Sal*I, Sm - *Sma*I, Xb - *Xba*I. * - denotes a site destroyed in subcloning.

(a)



(b)

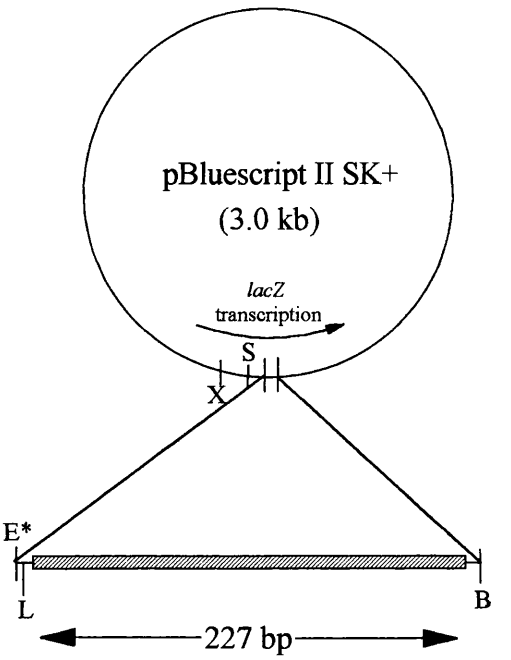


Figure 3.12 PCR of the code-corrected COXI fragment for construction of plasmid pPIpcr

(a) PCR of COXI fragment to generate sites for subcloning. The template DNA was plasmid pLR1; the PCR primers used were oligonucleotide COI/Lsp as the 5' primer, and oligonucleotide COI/PI3' as the 3' primer.

(b) The PCR product was digested with *Bam*HI, and ligated into pBluescript II SK+ which had been digested with *Eco*RV and *Bam*HI. The plasmid was named pPIpcr.

Restriction sites are as follows: B - *Bam*HI, E - *Eco*RV, L - *Lsp*I, S - *Sal*I, X - *Xho*I.
* - denotes a site destroyed in subcloning.

construction see fig. 3.13). The 72 bp *SalI/Lsp*I fragment from pTPcx4 was gel-purified using a 4% NuSieve agarose gel,and ligated into pPIpcr digested with *SalI/Lsp*I. This clone was designated pTP/PI, and contains the sequence encoding the COXIV transit peptide 5' to the *n-COI* PCR fragment. pTP/PI was digested with *Xho*I, and the 5' overhanging ends were end-filled using DNA polymerase I Klenow fragment. It was then *Bam*HI-digested and gel-purified. The isolated 300 bp fragment had an intact *Bam*HI overhang at the 3' end of the fragment, compatible with *Bcl*I, and a blunt end at the 5' end of the gene fragment, compatible with *Sma*I. pPI3', which contains the human *cyt b* fragment of the PI construct, had been digested with *Sma*I and *Bcl*I, and the 300 bp fragment encoding the transit peptide and *n-COI* 5' region was then ligated into these sites. This produced the end construct pPI/SK+, which contained the entire Patient I coding sequence with mitochondrial targetting sequence, in pBluescript SK+ (see fig. 3.14 for the final sequence).

3.2.5 Subcloning PIZ - the epitope-tagged version of the Patient I construct

It was my intention to use the Z1F11 epitope tag (described in section 3.1.1) for tagging the wild-type COXI construct, PI, and the KSS Patient II construct described in the introduction. For the nucleotide sequence encoding the seven amino acids of the epitope tag I chose the codons most commonly found in the mouse nuclear genome, whilst ensuring the new sequence did not encode any of the restriction sites used in the subcloning strategy. I planned a strategy for tagging all three constructs, using two sequential rounds of PCR (see fig. 3.15 for a depiction of the PIZ construction). The primary 3' oligonucleotide PCR primer was to be construct-specific, each having 18 nt homologous to the last 18 nt of the PCR target sequence (e.g., the primary 3' oligonucleotide for PI has 18 nt homologous to the human cyt *b* sequence which encodes the 3' region of the PI construct), and 16 nt which encoded the 5' portion of the Z1F11 tag.

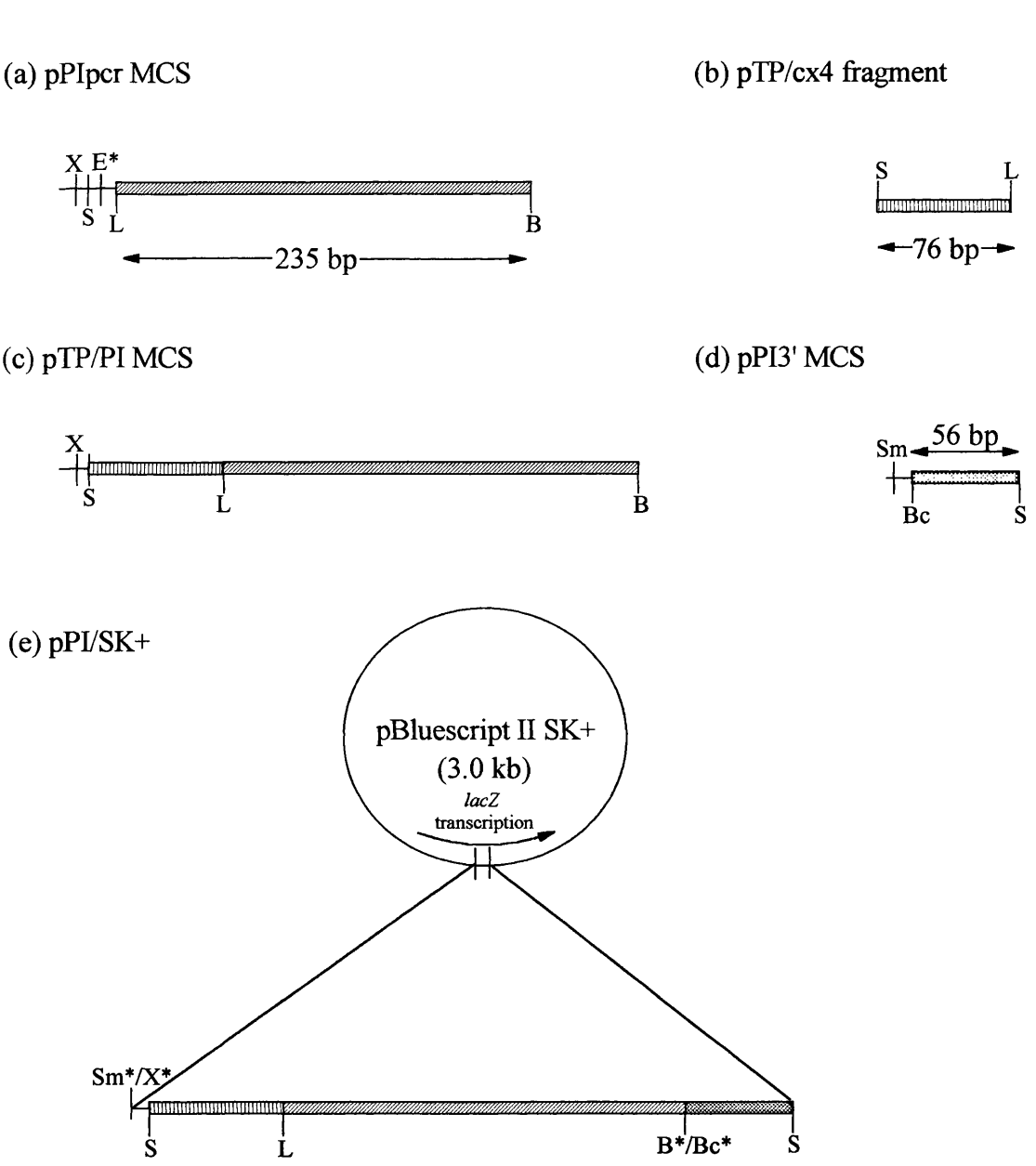


Figure 3.13 Construction of the Patient I coding sequence in pBluescript II SK+

a)The MCS of pPIpcr containing the COXI PCR fragment. b) pTPcx4 fragment for subcloning into pPIpcr. c) pTP/PI MCS containing the COXIV transit peptide-encoding sequence plus COXI PCR fragment. The plasmid is digested with *Xho*I, endfilled, then digested with *Bam*HI for ligation into plasmid pPI3'. d) pPI3' MCS containing the PI3' coding region, which is then digested with *Sma*I and *Bcl*I to permit subcloning of the pTP/PI fragment into the vector to create the entire PI coding sequence. e) The entire coding region for the Patient I construct subcloned into pBluescript II SK+. The plasmid was designated pPI/SK+.

mouse COXIV-derived sequence (transit peptide)
mouse COXI-derived sequence
human cyt *b*-derived sequence

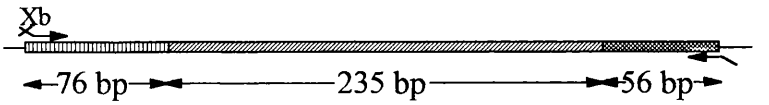
Restriction sites are as follows: B - *Bam*HI, Bc - *Bcl*I, E - *Eco*RV, L - *Lsp*I, S - *Sal*I
Sm - *Sma*I, X - *Xho*I. * - denotes a site destroyed in subcloning.

```
ATGTTGGCTTCCAGAGCGCTGAGCCTGATTGGCAAGAGAGCCATTTCTACTTCGGTGTGC
60
M  L  A  S  R  A  L  S  L  I  G  K  R  A  I  S  T  S  V  C

CTTCGAATGTTCATTAATCGTTGGTTATTCTCAACCAATCACAAAGATATCGGAACCCTC
120
L  R  M  F  I  N  R  W  L  F  S  T  N  H  K  D  I  G  T  L

TATCTACTATTCGGAGCCTGGGCGGGAATGGTGGGTACTGCACTAAGTATTTTAATTCGA
180
Y  L  L  F  G  A  W  A  G  M  V  G  T  A  L  S  I  L  I  R

GCAGAATTAGGTCAACCAGGTACACTTTTAGGAGATGACCAAATTTACAATGTTATCGTA
240
A  E  L  G  Q  P  G  A  L  L  G  D  D  Q  I  Y  N  V  I  V

ACTGCCCATGCTTTTGTTATGATTTTCTTCATGGTAATGCCAATGATGATTGGTACGGAT
300
T  A  H  A  F  V  M  I  F  F  M  V  M  P  M  M  I  G  T  D

CATTTCTCTACTCAGAAACCTGAAACATCGGCATTATCCTCCTGCTTGCAACTATAG     357
H  F  S  T  Q  K  P  E  T  S  A  L  S  S  C  L  Q  L  *
```

Figure 3.14 Patient I coding sequence

Nucleotide and corresponding amino acid sequence of the Patient I coding sequence. The *Lsp*I restriction site, at the junction of the transit peptide-encoding region and the COXI coding region, is indicated by bold font in the nucleotide sequence. The first residue of the mature Patient I peptide, if the transit peptide is proteolytically processed appropriately, is indicated by bold font in the amino acid sequence.

(a) pPI/SK+ template sequence



(b) PCR product from (a) as template for the second amplification reaction

(c) PCR product from (b) subcloned in pBluescript II SK+.

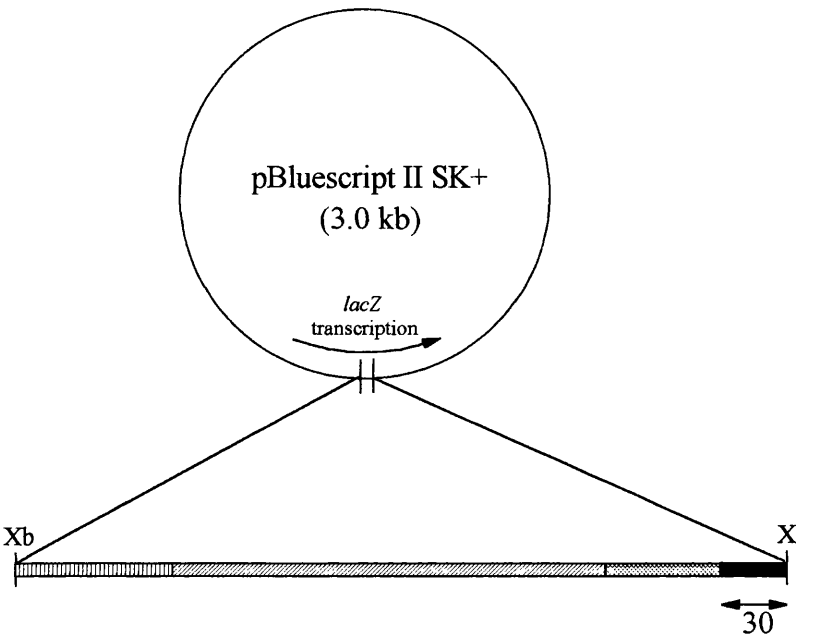


Figure 3.15 Construction of the epitope tagged version of the Patient I construct

a) The first round of PCR amplifies the target sequence and tags it with a 3' Z1F11-specific sequence, in frame. Additionally, the 5' cloning site is altered from *SalI* to *XbaI*. The template DNA is pPI/SK+; oligonucleotide 5'TP/Xba is the 5' primer, and oligonucleotide PI3'/Z1F11 is the 3' primer. b) The second round utilises a 3' primer homologous to the Z1F11 sequence, which encodes the remainder of the epitope tag, stop codon, and *XhoI* restriction site for subcloning. The template DNA is the PCR product from step (a); oligonucleotide 5'TP/Xba is the 5' PCR primer, oligonucleotide Z1F-2 is the 3' PCR primer. c) The PCR product from step (b) is digested with *XbaI* and *XhoI*, and ligated into *XbaI*/*XhoI*-digested vector. This creates plasmid pPIZ/SK+, the epitope-tagged Patient I construct subcloned into pBluescript II SK+.

mouse COXIV-derived sequence (transit peptide)

mouse COXI-derived sequence (code corrected)

human Cyt *b*-derived sequence

DNA encoding the Z1F 11 epitope tag

Restriction sites are as follows: X - *XhoI*, Xb - *XbaI*.

To construct the tagged version of the PI product, the first round of PCR included pPI/SK+ as the template. A sample of the reaction product was run on a gel to ascertain correct product formation (377 bp), and to separate product from template and primers. Both the gel-purified fragment and the unpurified PCR product were then used as templates for the second round of PCR to synthesise PIZ. The secondary 3' oligonucleotide had 16 nt homologous to the Z1F11 coding region in the new target sequence, whilst additionally encoding the remainder of the epitope tag, stop codon and an *Xho*I restriction site useful for cloning. The 5' primer used was homologous to oligonucleotide TP1, which encodes the 5' terminal portion of the transit peptide, but had an *Xba*I site rather than *Sal*I. This was to enable directional cloning of the DNA product, as *Sal*I and *Xho*I have compatible overhangs. By gel analysis, both the gel-purified and unpurified templates yielded a single product in the second PCR. DNA was extracted from the final reaction mix using Magic PCR Clean-up (supplied by Promega), digested with *Xba*I and *Xho*I, and subcloned into pBluescript SK+ digested with *Xba*I/*Xho*I, to produce pPIZ/SK+. The insert was verified by complete sequencing of both strands.

3.2.6 Subcloning *n-COI*

As the final 3 programmed mutations had been difficult to achieve using conventional oligonucleotide-directed mutagenesis, I proceeded with clone pLR11 despite the nucleotide deletion (described in section 3.2.2). I devised a strategy using PCR to insert the missing thymine at nt 1178, whilst creating an *Alw*44I restriction site proximal to the nucleotide insertion. When creating mutations by PCR, the oligonucleotides are designed such that the mismatch with the target sequence is as close as possible to the 5' end of the oligonucleotide, in order to minimise the effect a mismatch will have on the T_m of hybrid formation. The *Alw*44I site was created by making a silent mutation in the coding sequence which altered the original valine codon (GTT) to a valine codon more commonly found in the mouse nuclear genome

a)

```
                    .         .         .         .         .
Planned n-COI       TTGCTATCATGGCAGGATTTGTTCACTGGTTCCCATTATTT
sequence              A  I  M  A  G  F  V  H  W  F  P  L  F
```

b)

```
Location                          Sequence
COI/Rpr1178/B                        5' GCGGTGCACTGGTTCCCATTATTT 3'

pLR11              5'TTGCTATCATGGCAGGAT-TGTTCACTGGTTCCCATTATTT 3'

                   3'AACGATAGTACCGTCCTA-ACAAGTGACCAAGGGTAATAAA 5'

COI/Rpr1178/A       3' CGATAGTACCGTCCTAAACACGTGGCG 5'
```

c)

```
                                      Alw44I
Sequence of         TTGCTATCATGGCAGGATTTGTGCACTGGTTCCCATTATTT
pCOI/KS+              A  I  M  A  G  F  V  H  W  F  P  L  F
```

Figure 3.16 PCR strategy to repair the point deletion at nt 1178

a) The sequence of the code-corrected *n-COI* from nt 1160-1200 as it should have read, based on the original strategy (programmed changes are emphasised in bold print).

b) The sequences of the template and primers for repairing the point deletion at nt 1178 of the COXI sequence (equivalent to nt 6505 of the mouse mtDNA sequence). The double-stranded sequence of pLR11 from nt 1160-1200 is shown. Above and below pLR11 are given the sequences of the oligonucleotides used as PCR primers for the mutagenesis. COI/Rpr1178/A inserts the missing A/T base pair as well as creating a silent A-C transversion to create the *Alw*44I site. COI/Rpr1178/B encodes a T-G transversion for the creation of the *Alw*44I site. Note that the creation of the restriction site does not alter the coding sense.

(c) The sequence of the code-corrected n-COI from nt 1160-1200 as it reads in the final construct, pCOI/KS+.

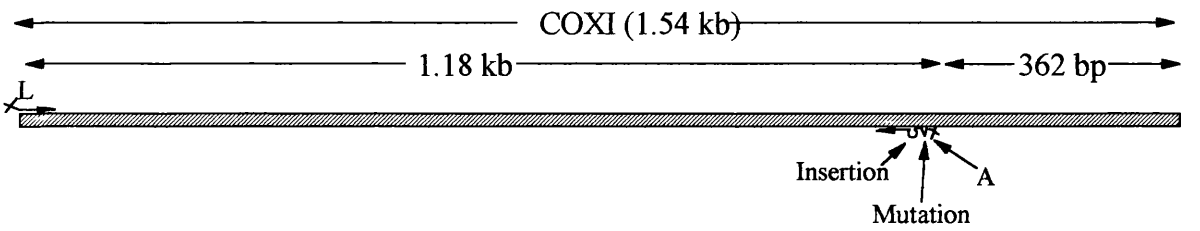
The programmed changes are emphasised in bold print. (-) indicates the missing base pair. The *Alw*44I site is indicated by underlining. Both primers have a 3 nt nonsense 5' 'tail' to provide a restriction enzyme substrate that does not have the site immediately at the end of the PCR product.

(GTG), (fig. 3.16). The design of the PCR primers allowed me to amplify *n-COI* in two segments using pLR11 as template, inserting the missing nucleotide and creating substrates for restriction enzymes to enable subcloning.

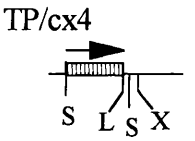
Thus, PCR was utilised for the final steps in the mutagenesis and subcloning of *n-COI*, (see fig. 3.17). Using pLR11 as template, the 5' primer created the *Lsp*I site for the junction with the transit peptide coding region (as used in subcloning the Patient I construct), whilst the 3' primer inserted the missing nucleotide at nt 1178 and engineered the creation of the *Alw*44I restriction site. The PCR product was digested with *Lsp*I and subcloned into pTPcx4 which had been digested with *Xho*I, endfilled, then *Lsp*I digested. This created pCOI5' which joins the 5' region of *n-COI* to the COXIV transit peptide sequence. This plasmid was then used as a PCR template to amplify the transit peptide/COI5' region, whilst pLR11 was used as the template for a PCR reaction to amplify the 3' region of *n-COI*, engineering the creation of the *Alw*44I site. Both products were digested with *Alw*44I, then ligated. An attempt to subclone the ligation product was unsuccessful (possibly due to low efficiency of one of the enzyme manipulations). The ligation mixture was then used as a template for PCR to amplify the intact *n-COI* coding unit.

This final PCR product was digested with *Xba*I and *Xho*I, and subcloned into pBluescript SK+ digested with *Xba*I and *Xho*I. Six transformants appeared to have the correct insert when screened by PCR. However, all of these clones grew poorly. Plasmid DNA prepared by three different variations of the alkaline lysis procedure (with solutions prepared in the lab, Magic mini-prep (Promega), and Qiagen kit) was always contaminated by degraded DNA, believed to be chromosomal, due to its large size. This may have been due to nuclease activity in dying cells. *n*-COXI had been subcloned into pBluescript SK+ such that transcription from the *lac* promoter read into the sense strand of *n-COI*, and such a transcript would potentially encode a lac Z/COXI fusion protein in frame. It is surmised that this could be toxic to cells, even

(a) Template DNA: pLR11



(b) The multiple cloning site of pTP/cx4



(c) pCOI 5'

Figure 3.17 Construction of the nuclear cytochrome *c* oxidase I gene in pBluescript II KS+

(a) PCR of *n-COI* 5' region to insert the deleted nucleotide at nt 1178 and engineer *Lsp*I and *Alw*44I restriction sites for subcloning. The template used for the PCR was plasmid pLR11. The primers used were oligonucleotide COI/Lsp as the 5' primer, and oligonucleotide COI/Rpr/A as the 3' primer.

(b) The MCS of plasmid pTP/cx4 (see figures 3.8 and 3.9), which contains the COXIV transit peptide-encoding sequence, is shown. pTP/cx4 was digested with *Xho*I, endfilled to make a blunt end, then *Lsp*I digested to prepare the plasmid for subcloning.

(c) The *n-COI* 5' PCR product subcloned into pTP/cx4. The PCR product from step(a) was digested with *Lsp*I, and ligated into vector pTP/cx4 which had been prepared as described in step (b). This plasmid was designated pCOI5'.

Restiction sites used are: A - *Alw*44I, L - *Lsp*I, S - *Sal*I, X - *Xho*I.
* - indicates a site destroyed in subcloning.

denotes mouse COXIV-derived sequence (transit peptide).

denotes mouse COXI-derived sequence.

(d) Template DNA: pCOI 5'

(e) Template DNA: pLR11

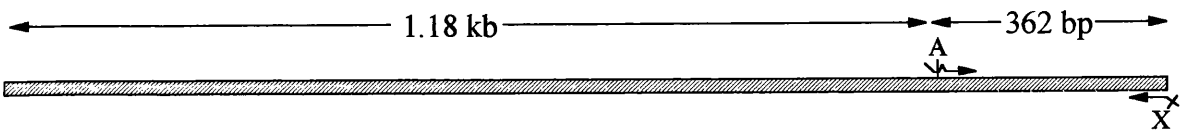


Fig. 3.17 continued

(d) PCR of the COXIV transit peptide encoding region joined to the *n-COI* 5' region. The 5' cloning site is altered from *Sal*I to *Xba*I to enable directional cloning. The template used was pCOI5'. The primers were oligonucleotide 5'TP/Xba as the 5' primer, and oligonucleotide COI/Rpr1178/A as the 3' primer.

(e) PCR of the 3' portion of *n-COI*. A one nt change is made to create the *Alw*44I restriction site for joining the 5' and 3' sections of *n-COI* together. The template used for the PCR was pLR11. The primers were oligonucleotide COI/Rpr1178/B as the 5' primer, and oligonucleotide COI/WT3'/Xho as the 3' primer.

Restiction sites used are: A - *Alw*44I, L - *Lsp*I, S - *Sal*I, X - *Xho*I, Xb - *Xba*I.

denotes mouse COXIV-derived sequence (transit peptide).

denotes mouse COXI-derived sequence.

(f) Template DNA: n-COI 5' and 3' ligation product

(g)

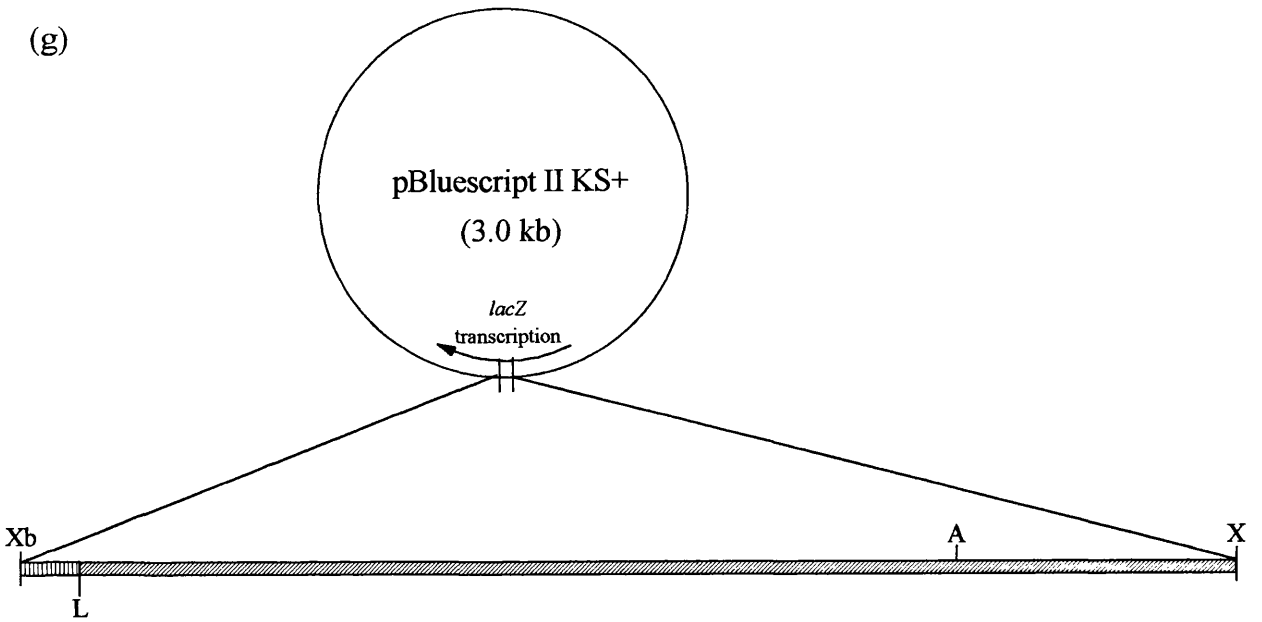


Fig. 3.17 continued

(f) The products of the PCR reactions described in steps (d) and (e) were both digested with the restriction enzyme *Alw*44I. The digestion products were ligated together. This ligation mixture was then used as a template for PCR to amplify the entire *n-COI* coding unit. The primers used were oligonucleotide 5'TP/Xba as the 5' primer, and oligonucleotide COI/WT3'/Xho as the 3' primer.

(g) The product from step (f) was digested with *Xba*I and *Xho*I, and subcloned into pBluescript II KS+ digested with *Xba*I and *Xho*I. This plasmid, which contains the entire coding region for *n-COI* is designated pCOI/KS+.

Restiction sites used are: A - *Alw*44I, L - *Lsp*I, X - *Xho*I, Xb - *Xba*I.

denotes mouse COXIV-derived sequence (transit peptide).

denotes mouse COXI-derived sequence.

at background levels of expression, i.e., from the *lac* promoter without induction. The same fragment was therefore recloned into pBluescript KS+, which has the polylinker in opposite orientation to the *lac* promoter, so that any read-through transcription would produce a transcript containing anti-sense COXI RNA. Plasmid DNA was prepared from 6 recombinant transformants and assayed by double restriction digest with *Xba*I and *Xho*I to check the size of the insert. No degradation of DNA was observed. One clone had the correct size insert. This clone was digested with *Alw*44I to ascertain that the 5' and 3' regions of *n-COI* were joined correctly. Sequence analysis detected no unprogrammed mutations. This clone is designated pCOI/KS+, and is now ready for use in expression studies. It will also now be possible to construct the PII chimeric mouse COXI gene using pCOI/KS+ as a PCR template.

3.3 Discussion

In this chapter I have described the mutagenesis and subcloning used to generate 3 synthetic genes: (i) the Patient I construct, PI, a chimeric version of mouse COXI based on a rearranged COXI gene found in the mutant mtDNA of a KSS patient, (ii) an epitope-tagged version of the Patient I construct, PIZ, and (iii) a full length universal-code version of mouse COXI. All 3 constructs have been code-corrected by site-directed mutagenesis to enable cytosolic translation, and all 3 constructs encode an amino terminal mitochondrial targetting signal from the mouse COXIV gene. The two Patient I constructs are the basis of the remaining work I shall describe in my thesis. *n-COI* was finally subcloned only at the end of the project, and can form the basis of future work in the lab.

In Sambrook *et al.*, 1989 it is stated, in a discussion of site-directed mutagenesis, that "in extremely rare cases the secondary structure [may be] so great that no mutants can be obtained." My experience with oligonucleotide-directed mutagenesis confirms that certain regions of DNA are much more resistant to mutagenesis than others. This

may be because of an inherent property of the DNA (i.e., secondary structure) or perhaps because the sequence change creates a transcription/translation product toxic to the bacterial host cell. Full-length mouse COXI would, indeed, appear to be toxic in *E. coli*, since the *n-COI* construct, when in-frame and subject to read-through transcription from the *lac* promoter in the SK+ polylinker, was associated with loss of viability of the bacterial host cells. Conversely, when the *n-COI* insert was oriented in the pBluescript KS+ polylinker such that read-through transcription would produce an antisense COXI transcript, no impairment of bacterial viability was observed.

In general, the validity of the rules stated for primer design for oligonucleotide-directed mutagenesis in Sambrook *et al.*, (1989) were supported by my own results. Where mutations proved difficult to achieve, redesign of oligonucleotides had little effect, therefore the problem seemed to be template- or mutation-dependent. The use of the various mutagenic oligonucleotides for screening putative mutants was straightforward. The empirical formula used to calculate the hypothetical Tm (see section 2.3.1.3) proved a reliable guide for most of the oligonucleotides used, apart from oligonucleotide S. This oligonucleotide, which was also involved in the only rearrangement of the template sequence during mutagenesis that I observed, was repeatedly unsuccessful as a mutagenic oligonucleotide, although it was used successfully as a sequencing primer. It also indicated many false positives during screening for mutants. When the final wash temperature was raised 10^{o}C above the calculated Tm, the problem of false positives was eliminated.

With hindsight I believe the task of mutagenising a long sequence such as COXI would have been made easier by splitting the gene into smaller fragments. Although no suitable restriction site is contained in the sequence, silent mutations which do not alter the coding sense could have been made in order to create restriction sites (as employed subsequently to repair the nucleotide deletion at nt 1178).

I would also advocate the use of PCR for site-directed mutagenesis where possible, rather than conventional techniques requiring single-stranded DNA as template. There are several reasons for this: (i) the production of good quality single-stranded DNA is dependent on many more variables than is the production of double-stranded plasmid DNA (*e.g.*, multiplicity of infection of helper phage, length of time host bacterial cells are cultured with the helper phage before harvesting). Even when the protocol has been optimised for a given clone, the yield of single-stranded DNA may be poor. The protocol recommended by Stratagene for single-stranded DNA recovery from cells containing pBluescript derivatives states "yields of single-stranded DNA can be dependent on the specific insert sequence...". The pLR series of clones had poor yields of single-stranded DNA, which hampered progress with site-directed mutagenesis. PCR is not subject to these problems. (ii) One major objection to PCR for site-directed mutagenesis given is that the relatively higher frequency of ectopic mutation makes sequencing necessary (Saiki *et al*, 1988). However, during the mutagenesis of COXI using single-stranded template, an ectopic mutation did occur in the region of mutagenic oligonucleotide J. This happened during a primer extension reaction which involved no mutagenic oligonucleotides which had a target sequence near the site in question. Thus ectopic mutation can occur with either technique, making the sequencing of the entire coding region exposed to the mutagenesis reaction essential, regardless of the mutagenesis technique used. This is another good reason for subcloning the target sequence into smaller fragments, which can be sequenced conveniently in their entirety, and can be set aside when the desired mutations are achieved for subsequent subcloning. (iii) The speed and accuracy of mutagenesis using PCR far outweigh any disadvantages the technique may have. Screening is not necessary as there can be no product if the amplification primer has not been used (unlike with single-stranded template techniques).

Having used PCR extensively for subcloning the constructs, no ectopic mutation has been detected using *Pfu* polymerase (a high temperature DNA polymerase which has

proofreading activity). In creating the epitope-tagged version of the Patient I construct, 16 nt were added to the sequence in a single round of amplification, yet a single product was observed by gel analysis and sequencing. Although PCR can generate spurious products, when using plasmid DNA as a template this is rarely a problem that cannot be solved by altering the parameters of the polymerase chain reaction. If optimal conditions for yield of the desired product had produced spurious secondary products, gel purification could have been used to separate the desired product from other amplification products. However, for a project such as this which required 43 programmed point mutations within a single coding region, it is difficult to envisage how a PCR strategy could have been employed throughout. Thus, on balance, the correct approach was probably adopted, although a switch to the PCR approach at an earlier point in time may have facilitated more rapid progress with this project.

Chapter 4

Expression studies of Patient I constructs in *E. coli* and *in vitro*

4.1 Introduction

4.1.1 Aims

There were principally two reasons for expressing the Patient I constructs in a prokaryotic system. Firstly, I wanted to test the available antisera to determine which would be most effective in analysing the mouse cell lines I was generating (discussed in chapter 5). A prokaryotic expression system seemed the most appropriate means of doing this. Secondly, if the antisera were found not to react specifically with the recombinant protein, the expressed protein could be used to generate new antisera to the recombinant protein.

An additional consideration was to observe the apparent size of the Patient I protein (PI) on SDS-PAGE. Although the molecular weight of the full-length bovine cytochrome *c* oxidase I (COXI) is 57 kDa, its apparent size by SDS-PAGE is 37 kDa, due to its extreme hydrophobicity (Hensel and Buse, 1990). The calculated molecular weight of the PI fusion peptide is ~13 kDa, and 13.75 kDa for PIZ (the epitope-tagged version). Given that these proteins are also highly hydrophobic (~50% of the residues are hydrophobic) it was possible that they would migrate with an apparent molecular weight of <13 kDa. The apparent molecular weight of the PI peptide on SDS-PAGE could be determined by use of a prokaryotic expression system, or by *in vitro* translation. Additionally, *in vitro* translation using reticulocyte lysate would confirm that the encoded RNA could be translated by eukaryotic cytosolic ribosomes.

However, the hydrophobic nature of the proteins meant that expression of the proteins might have had a toxic effect on *E. coli*. For example, it was possible that PI and PIZ could associate with the bacterial membrane, disrupting its integrity. When subcloning into the prokaryotic expression vector chosen, the manufacturer's (Qiagen) manual recommends that hydrophobic regions, particularly transmembrane

sequences, "should be removed from the recombinant protein if they are not of specific interest", to minimise this potential problem. However, this was not possible, as the entire encoded polypeptide is of a hydrophobic nature, derived as it is from COXI which is an integral membrane protein. Therefore, it was anticipated that an *in vitro* transcription/translation system might be required.

4.1.2 The antisera

An antiserum which would have been desirable in the long term is an anti-mouse COXI. No such antibody had been reported in the literature at that time. However, for the analysis of the PI polypeptide, it would have been useful only had it recognised one or more epitope(s) contained within the 76 amino-terminal residues of COXI. Even for the analysis of the full-length n-COI, an anti-mouse COXI antibody would be of limited use. It would not discriminate between the endogenous COXI and the n-COI proteins once the nuclear encoded version had been imported into the mitochondrion and the leader peptide cleaved. Thus the antibody could not be used to show conclusively whether the imported n-COI functioned normally, *i.e.*, was incorporated into the inner membrane in a functional cytochrome oxidase complex.

J. Poulton and co-workers, who had analysed the mtDNA from the model KSS patient 1, obtained a synthetic peptide based on the amino acid sequence of the putative patient 1-specific fusion protein (which is derived from the human cytochrome *b* coding sequence as explained in section 1.7.1, Poulton *et al.*, 1989b). This peptide was then used to generate antisera. Fig. 4.1 depicts the sequence of the synthetic peptide.

A peptide-KLH (keyhole limpet haemocyanin) conjugate was used to immunise 3 rabbits. The antisera were tested by ELISA and western blot by K. Morten using a BSA-peptide conjugate. 50 µl of a 10 µg/ml stock was detectable by a 1/1000

a) M I G T D H F S T Q K P E T S A L S S C L Q L

b) C Y G T D H F S T Q K P E T S A L S

Figure 4.1 Amino acid sequence of the patient 1-specific oligopeptide

a) is the amino acid sequence of the carboxy terminus (residues 74-96) of the putative KSS patient 1 polypeptide and of the analagous mouse polypeptide PI. The patient 1-specific region which is derived from cytochrome *b* coding sequence is underlined.
b) is the sequence of the synthetic peptide used to generate antipeptide antisera. An amino-terminal cysteine residue is necessary for the *in vitro* synthesis of peptides to join the peptide to the matrix by a disulphide bond. The tyrosine residue was also included to assist in the manufacture of the synthetic peptide. Residue 76 in COXI is glycine; in the hypothetical KSS patient 1 polypeptide residue 76 is encoded at the COXI/cyt *b* chimeric junction and is unchanged as a glycine.

dilution of the rabbit 3 antiserum in ELISA. Using a 1/500 dilution of the same antibody, ~1 ng of the BSA-conjugate was detectable on a western blot. On the basis of this data, the rabbit 3 pre-immune and 3rd bleed antisera were sent to me. As is evident from the westerns which I have performed using these antisera (data shown in the results section), it does not react with the 66 kDa BSA size marker. It is therefore reasonable to assume that the antiserum recognises epitope(s) contained within the peptide sequence on western blot. Later, the pre-immune and 3rd bleed antisera from rabbits 1 and 2 were also made available and tested by me. All rabbit antisera were normally used at a 1/1000 dilution for western analysis.

Preliminary western analysis of patient tissues with the rabbit 3 antiserum had been done by Dr. Morten when I began analysis of the Patient I constructs in *E. coli*. A 10% SDS-polyacrylamide gel was loaded with brain, heart, kidney and pancreas tissue extracts from the KSS patient, and control muscle tissue. This was blotted and probed with rabbit 3 immune serum. A strong band of apparent molecular weight ~58 kDa was seen in pancreas, which reportedly had a high proportion of duplicated mtDNA molecules in patient 1. No bands were reported in the range <35 kDa, the predicted size of the chimeric patient 1 protein being ~13 kDa, however, on a 10% gel the protein may have run off or been very diffuse. Additionally, the antiserum detected a number of bands non-specifically. On a 15% polyacrylamide gel a band of apparent molecular weight 12 kDa was detected in patient 1 muscle tissue, but not in control muscle. However, this band was not detected on a subsequent blot of patient tissue. It is possible that this was attributable to a lack of sensitivity in the detection system used, to limited proteolysis, or because the original positive result was, in fact, an artifact. Thus, although there was no clear evidence that the antiserum could detect a patient 1 - related fusion peptide on westerns, the antiserum was used in the following analysis because it was the only one available to me.

As explained in chapter 3, the other antiserum which was used for expression analysis was the mouse monoclonal antibody Z1F11. It had been raised against the HSV-1 65K DNA-binding protein (65KDBP), a protein essential for HSV DNA replication, which it recognised on western blots (Schenk *et al.*, 1988). Using overlapping hexapeptides, the antigenic region of 65KDBP recognised by the antibody was mapped to a 7 amino acid epitope (Murphy *et al.*, 1989). In the latter paper, Z1F11 was used to immunoprecipitate the protein, and this reaction was specifically blocked by the presence of a heptadecapeptide (corresponding to amino acids 357 to 373 of 65KDBP) which contained the 7 amino acid epitope. I created an epitope-tag coding region for the PIZ constructs based on the defined amino acid sequence. The antibody, of immunoglobulin subtype G1, was provided as ascites fluid and used at a 1/1000 - 1/3000 dilution for westerns.

For western analysis, the ECL detection system (supplied by Amersham) was used. This system makes use of the enzyme horseradish peroxidase (HRP) for chemiluminescent detection. The primary antibody binds to the denatured protein which is fixed to a membrane. The secondary antibody, e.g., goat anti-mouse, then binds to the primary antibody. The secondary antibody is conjugated to HRP, so that when the HRP substrate is added, light is emitted. The reaction can be very sensitive, and exposure times usually range from seconds to a few minutes.

4.1.3 The prokaryotic expression vector pQE32

For expression of PI in *E. coli*, I chose the QIA*express* (pQE) system supplied by Qiagen. There were 3 main reasons for the choice: 1) the tight regulation of transcription, 2) the easy and reputedly efficient procedure for protein purification, 3) other researchers in the department who could be consulted were using the system routinely.

Transcription of the recombinant gene in the pQE vectors is initiated from an engineered promoter/operator element designed to enable a high level of protein expression. The element is composed of the *E. coli* phage T5 promoter and two copies of the *lac* operator sequence. The *lacI* gene, which encodes the lac repressor protein, is present on a second high-copy number plasmid pRep4. The high level of repressor expression this produces is necessary to ensure repression of transcription from the engineered promoter element. Expression is induced by the addition of IPTG, which inactivates the lac repressor protein, displacing it from the operator elements. The pQE vectors also contain a synthetic ribosome binding site for optimal mRNA recognition and binding, and translational stop codons in all 3 reading frames immediately following the multiple cloning site.

The pQE vectors encode a tag of 6 histidine residues (either 5' or 3' with respect to the cloning site) to enable affinity purification of the recombinant protein using the Ni-NTA (Ni^{2+}-nitrilotriacetic acid) resin, supplied by Qiagen. The stability of the 6 x His/Ni-NTA interaction is maintained in the presence of non-ionic detergents such as Triton X-100 (0.1-1%), enabling their use for purification of membrane-associated proteins. Protein denaturants such as 8M urea can also be used during purification, because binding of the tagged protein to the resin is not dependent on protein structure. Elution is achieved by the addition of a chelating agent (EDTA), lowering of pH (causing protonation of the histidine residues), or competition with imidazole (which binds to the Ni-NTA and displaces the tagged protein). The 6 x His tag is reportedly non-immunogenic (apart from in some species of monkey) and therefore need not be removed when raising antisera to the recombinant protein. The tag is also reported usually not to interfere with the structure or function of the purified protein. Lastly, the QIA*express* product information reports that this system has been used with membrane proteins; a paper has since been published citing the use of the QIA*express* system for purifying a transmembrane protein with 8 membrane-spanning domains (Waeber *et al.*, 1993).

The specific vector chosen was pQE32. The pQE-30 series has the 6 x His tag encoded at the amino-terminus of the recombinant protein. Reportedly, placing the histidine tag-encoding sequence at the 5' end of the gene yields higher expression levels than 3' tagged constructs (Qiagen manual, S. LeGrice, personal communication). Because PI is a low molecular weight protein, I considered obtaining the Type II vector which encodes mouse DHFR (dihydrofolate reductase) as a carrier protein between the amino-terminal histidine tag and the recombinant protein. However, the manufacturer's information defined a "small" protein (requiring the carrier protein for stable expression) as <10 kDa, so I made use of the general purpose vector. pQE32 is the vector in the pQE-30 series which allowed subcloning of the PI constructs into the same reading frame as the 6 x His affinity tag.

4.2 Results

In section 4.1.2 it was explained that the testing of the anti-patient 1 peptide antiserum on western blots of patient tissues, carried out by Dr. Morten, was inconclusive, in that it was unclear as to whether the rabbit 3 antiserum was reacting with a patient-specific protein. In order that the available antisera could be tested specifically for their reactivity to the chimeric protein, the Patient I-analagous construct was subcloned into the prokaryotic expression vector pQE32. The epitope-tagged version of the Patient I construct was also subcloned into pQE32 to test the reactivity of the monoclonal antibody Z1F11 with the PIZ protein.

4.2.1 Subcloning of Patient I constructs into pQE32

Sub-cloning of the PI and PIZ constructs into the pQE vector was done in *E. coli* strain XL1-blue, which contains the *lacI*^{q} gene. This gene has a mutated promoter causing constitutive expression of the lac repressor protein; the high levels of lacI

protein should be sufficient for blocking expression of the recombinant protein during propagation.

Plasmid pPI/SK+ (the Patient I construct in pBluescript SK+, described in section 3.2.4) was digested with *Sal*I, and the 370 bp Patient I insert sequence was gel-purified. The fragment was subcloned into pQE32 which had been digested with *Sal*I and dephosphorylated with calf intestinal alkaline phosphatase. Transformants were mini-prepped and screened by restriction digest with *Sal*I, and 2 clones were chosen for sequencing. Of these, the plasmid designated pPI/Q contained the correct insert sequence in the appropriate orientation for protein expression (fig. 4.2.a).

Plasmid pPIZ/SK+ (the epitope-tagged Patient I construct in pBluescript SK+, described in section 3.2.5) was digested with *Xba*I, and the 5' overhanging end was endfilled using DNA polymerase Klenow fragment. The plasmid was then *Xho*I digested, and the fragment gel-purified. The isolated 390 bp fragment had an intact *Xho*I site overhang at the 3' end of the fragment, compatible with a *Sal*I overhang, and a blunt end at the 5' end of the gene fragment, compatible with *Sma*I. pQE32 had been digested with *Sma*I and *Sal*I, and the fragment was ligated into the vector multiple cloning site in frame for correct translation. Transformants were mini-prepped and screened by restriction digest with *Lsp*I (a single site is present in the insert sequence, none in the vector, see fig. 3.15), and a clone, pPIZ/Q, was selected for sequencing to confirm that it contained the correct insert sequence (fig. 4.2.b).

It should be noted that the prokaryotic expression vector pQE32 encodes an additional 20 and 19 amino acid residues respectively at the amino terminus of the PI and PIZ proteins (fig. 4.2). As these residues are much less hydrophobic than the PI protein, in addition to adding ~2 kDa to the molecular weight, they may have a significant effect on the mobility of the recombinant protein.

4.2.a pPI/Q

```
ATGAGAGGATCT   //   GGGATCCGCATGCGAGCTCGGTACCCCGGGTCGACCAGAATG
M  R  G  S (6 x H)  G  I  R  M  R  A  R  Y  P  G  S  T  R  M
```

4.2.b pPIZ/Q

```
ATGAGAGGATCT   //   GGGATCCGCATGCGAGCTCGGTACCCCTAGACAGAATG
M  R  G  S (6 x H)  G  I  R  M  R  A  R  Y  P  L  N  R  M
```

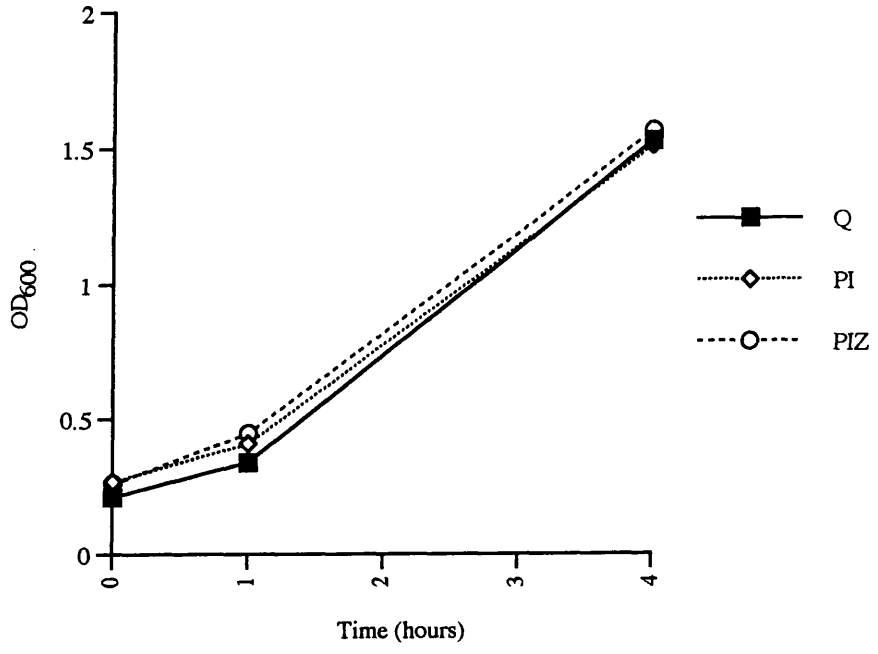
Figure 4.2 The nucleotide and amino acid sequences of the amino-terminal region of the PI constructs in pQE32

These are the amino-terminal sequences of plasmids pPI/Q and pPIZ/Q. The sequence shown spans the initiation codon of the prokaryotic expression vector to the first codon of the PI constructs. The region of the multiple cloning site involved in the subcloning procedure is underlined in each case. As described in the text, the subcloning of pPI/Q required only the ligation of a *Sal*I end into a *Sal*I site (GTCGAC). The subcloning of pPIZ/Q required the ligation of a blunt-ended *Xba*I end (CTAGA) into a digested *Sma*I site (CCC). The vector-derived sequence is in plain font, the insert-derived sequence in bold font.

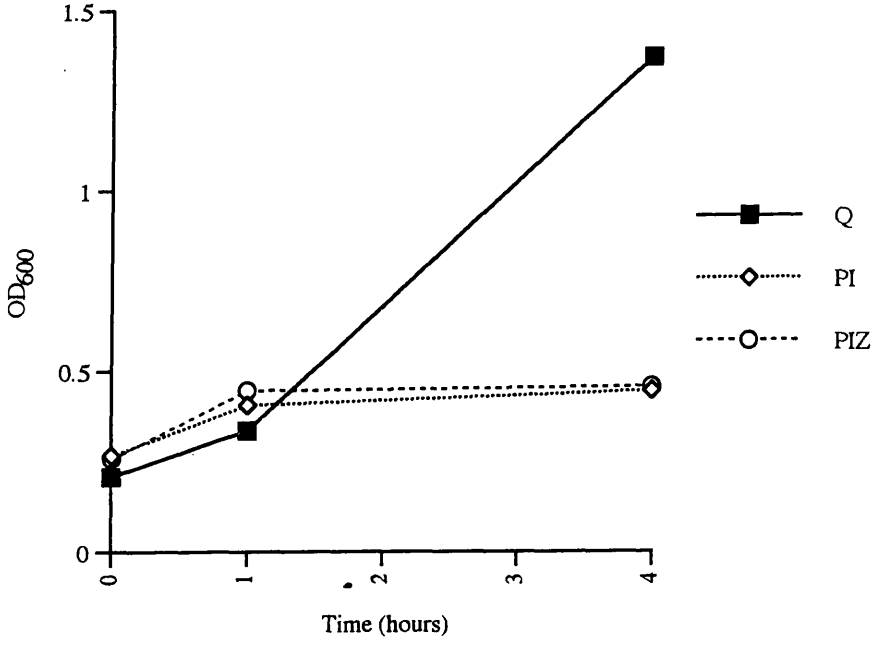
4.2.2 Time-course and growth analysis of *E. coli* transformants

Both constructs pPI/Q and pPIZ/Q were transformed into the Qiagen recommended *E. coli* host strain M15. Small proteins can be more susceptible to cellular proteases, because of problems with folding. Suggestions for minimising degradation in strains which are not deficient in a cellular protease include reducing the growth temperature and shortening the induction time prior to harvesting. A time-course of growth was taken, to determine whether shortening the induction period was advisable (fig. 4.3). A 1/10 dilution of overnight cultures of pQE32, pPI/Q and pPIZ/Q were grown for 1 hour. At this point the cultures were split in half, and expression was induced in one aliquot of each isolate by the addition of IPTG to a final concentration of 2 mM. The cultures were then incubated for a further 3 hours. The uninduced cultures displayed no difference in growth rate over the span of 4 hours, having reached stationary phase by this time. However, the cultures to which IPTG had been added displayed significant differences in growth. The vector control culture showed a small reduction in OD_{600} at the final time point, when compared to the uninduced control. Both pPI/Q and pPIZ/Q cultures effectively ceased growing upon the addition of IPTG to induce expression of the recombinant proteins, and showed no growth 1 hour post-induction. The third graph in figure 4.4 shows another time-course of growth of the 3 different transformants. In this case a 1/4 dilution of overnight culture was induced with IPTG after 1 hour of growth. OD_{600} readings were taken at hourly intervals. Although vector-transformed *E. coli* continued to grow (as in graph 2), the pPI/Q- and pPIZ/Q-transformed cultures grew minimally over the first hour post-induction, then ceased growing. Other researchers in the department using the Qiaex vectors did not observe a cessation of growth upon induction of the recombinant proteins with which they were working. However, the proteins in question were not expected to associate with membranes (personal communication, G. Glasssmith).

Graph a



Graph b



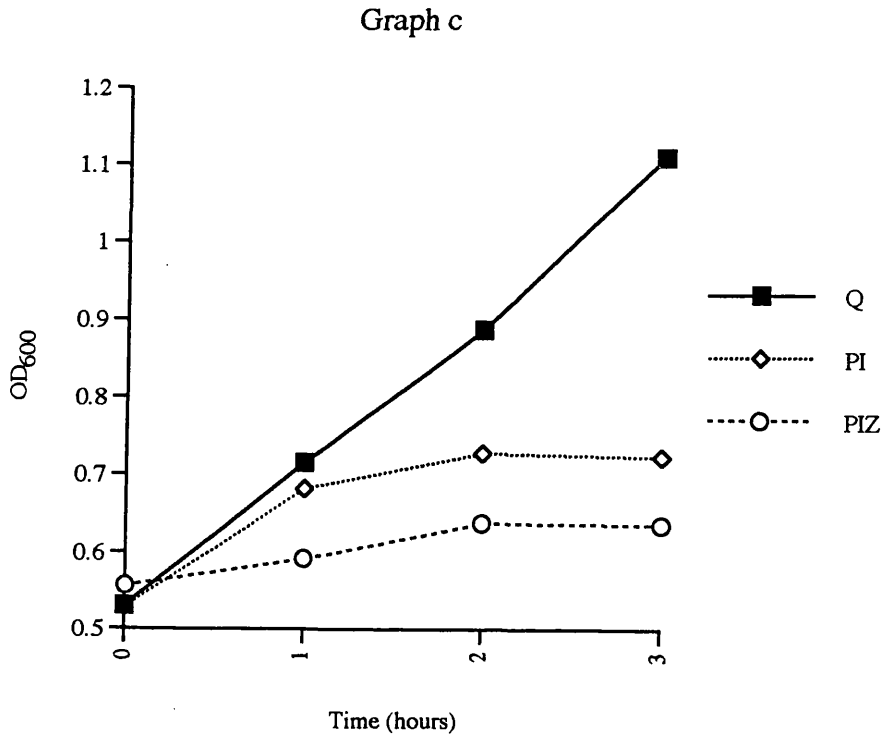


Figure 4.4 Time-course assays for growth

Overnight cultures of *E. coli* were used to inoculate fresh medium which was supplemented with appropriate antibiotic, and grown with agitation at 37°C. Readings were taken on a spectrophotometer at OD_{600} of samples from these cultures at the time points specified to assay bacterial growth. The 3 graphs presented in this figure plot the timepoint of the sample against the OD_{600} reading.
Graph 1 and 2 display the data for overnight cultures of *E. coli* which were diluted 1/10. After incubating for 1 hour the cultures were split into two aliquots, and IPTG was added to one aliquot of each. Graph 1 presents the data for the uninduced samples, graph 2 for the induced samples. Graph 3 presents the data for overnight cultures of *E. coli* which were diluted 1/4, grown for 1 hour, then induced with IPTG.

Each of the isolates had also been streaked on agar plates +/- glucose. After overnight incubation pPI/Q and pPIZ/Q colonies on the glucose plates (the supplement of 2% glucose is to maximise repression of expression by the lacI protein) appeared healthy, while colonies on the agar plates not supplemented with glucose had grown poorly. Glucose supplementation made no apparent difference to the growth of vector-transformed *E. coli*. When pPI/Q and pPIZ/Q post-induction cultures were plated out, colonies were observed rather than confluent growth, which was observed with post-induction pQE32 culture. This suggests that expression of the PI and PIZ proteins was toxic to *E. coli* rather than merely inhibiting growth for a temporary period.

These results are consistent with expression of the recombinant protein upon IPTG induction, and that expression of the protein was highly toxic to the cells. Indeed, even basal levels of transcription of the recombinant genes appears to be inhibitory to growth of the cells. Plasmid DNA was prepared from the isolates, and restriction digests were performed. No gross rearrangement of the recombinant plasmids could be detected (constructs containing genes encoding toxic proteins may give rise to deletions). However, for the western analysis which follows, Carbenicillin (a more stable analogue of ampicillin) was used in the culture medium and in agar plates to ensure maintenance of the recombinant plasmids in the host strain. The isolates were maintained on agar plates and grown in overnight cultures containing 2% glucose to maximise repression of expression. Finally, the induction period was reduced to only 1 hour prior to harvesting.

4.2.3 Western blot analysis of *E. coli* expressing PI transgenes

The purpose of the western analysis using the prokaryotic expression constructs, as explained previously, was to assess the mobility of the encoded polypeptides on SDS-PAGE gels, and to evaluate the usefulness of the various antisera available. This section is therefore subdivided into two sub-sections. The first discusses the western

blots which used as primary antibody the rabbit 3 antiserum raised against the patient 1 peptide (described in section 4.1.2) . The second sub-section presents western blots for which the primary antibody was the monoclonal antibody Z1F11, which should recognise the epitope tag at the carboxy terminus of the recombinant protein encoded by the pPIZ/Q construct.

4.2.3.1 Antiserum rabbit 3

Western blots of both 12% and 15% polyacrylamide gels on which were loaded samples of *E. coli* induced with IPTG were prepared. Figure 4.4 is an example. A blot of total bacterial extracts of pPI/Q, pPIZ/Q, and pQE32 was probed with R3P and R3I revealing a reactive band of ~40 kDa in cells transformed with the vector pQE32 (fig. 4.4). The ladder of bands at ~55-66 kDa is apparently specific to pPI/Q and pPIZ/Q transformants (lanes 2 and 3). These bands could represent multimers of the recombinant protein, aggregates of the recombinant protein with native *E. coli* protein(s), or induction of stress-response proteins that for some reason react with the antiserum. The pPI- and pPIZ-encoded proteins each contain 2 cysteine residues (1 in the transit peptide, 1 in the patient I 3' region), sufficient for the formation of disulphide bonds, potentially enabling the formation of multimers. However, the presence of β-mercaptoethanol in the loading buffer should prevent multimer formation. Preincubation of R3I antiserum with the patient 1 peptide did not selectively inhibit any of the bands observed by western blotting, suggesting that none of the proteins detected are PI or PIZ. Because of the large number of cross-reacting bands I obtained with the rabbit 3 antiserum, I also tested whether the secondary antibody or the HRP detection system was responsible for any of the bands, but this was negative (data not shown).

In conclusion for this sub-section, no polypeptides were identified as specific to the pPI/Q or pPIZ/Q transformed *E. coli*, as recognised by the rabbit 3 antiserum on

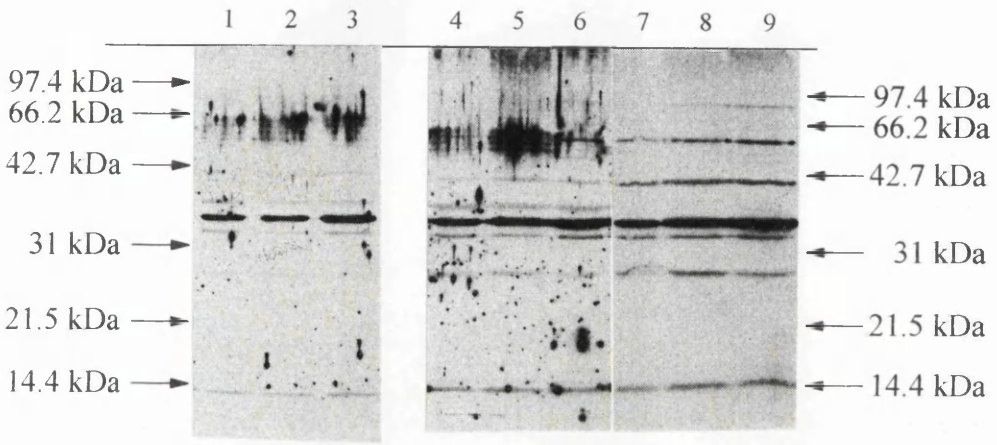


Figure 4.4 Western blots of 12% SDS-PAGE gels loaded with *E. coli* isolates as specified below

Lanes 1, 4 and 7 - pQE32, Lanes 2, 5 and 8 - pPI/Q, Lanes 3, 6 and 9 - pPIZ/Q.
The western blot of lanes 1-3 was probed with rabbit 3 immune antiserum (R3I), which had been preincubated with the patient 1-specific peptide (1 μg/ml for 2 hours in blocking solution at room temperature), the blot of lanes 4-6 was probed with R3I, and the blot of lanes 7-9 was probed with R3P. Primary antibody concentration was 1/1000; secondary antibody concentration was 1/2000. The size markers on the left of the figure refer to lanes 1-3, the size markers on the right of the figure refer to lanes 4-9.

12/15% polyacrylamide gels. Although it is possible that the fusion proteins would have run off a 12% SDS-polyacrylamide gel, it is unlikely that the histidine-tagged PI or PIZ would not be present on a 15% SDS-polyacrylamide gel. One possible interpretation of the data is that the antiserum does not, in fact, recognise the epitope against which it was supposedly raised. Another is that the fusion protein encoded by these constructs is either too unstable, too insoluble, or too toxic to be detected.

4.2.3.2 Western blot analysis with the monoclonal antibody Z1F11

Fig 4.5 shows a western blot of a 12% polyacrylamide gel, on which was loaded unfractionated *E. coli*, probed with Z1F11, the monoclonal antibody which should recognise the epitope tag region of the pPIZ/Q recombinant protein. Z1F11 failed to react with any bands of ~55-66 kDa apparent size, further evidence that these are not multimers of the recombinant protein. The monoclonal antibody also failed to react with any band specific to the pPIZ lane (lane 2) on this 12% polyacrylamide gel. However, strong (non-specific) signal in the 14 kDa range may obscure a meaningful band. Neither 15% nor 17.5% polyacrylamide gels detected any bands of low molecular weight specific to pPIZ (data not shown).

SDS PAGE analysis was carried out on protein fractions prepared as before (Ni-NTA resin purified, supernatant thereof, and pellet) from pPIZ/Q isolates grown at 30 °C and 37 °C. Both uninduced and induced fractions were analysed by probing western blots with Z1F11. No band specific to pPIZ/Q (in comparison to the vector transformed *E. coli*) was detected on 15% polyacrylamide gels (data not shown). As a control, I obtained an aliquot of 65KDBP (the protein against which Z1F11 was raised, sect.4.1.2). I confirmed that the aliquot of antibody in my possession recognised the denatured protein on a western blot and that my technique was not at fault.

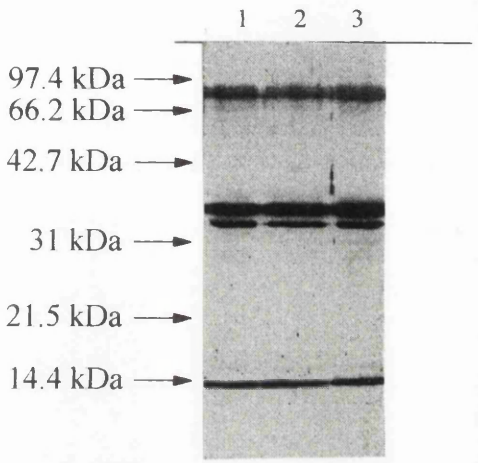


Figure 4.5 Western blot of a 12% SDS-PAGE gel loaded with *E. coli* isolates as specified below

Lane 1 - pQE32, Lane 2 - pPI/Q, Lane 3 - pPIZ/Q. The primary antibody was Z1F11, the secondary antibody was HRP-conjugated anti-mouse; both were used at a concentration of 1/2000.

In conclusion for this sub-section, Z1F11 did not react with the polypeptides indicated as potential multimers of the recombinant protein by the rabbit 3 antiserum. Nor did the monoclonal antibody clearly indicate any recombinant protein on western blots. However, if a recombinant protein of apparent molecular weight <14 kDa was produced, which remained in the pelleted fraction, it would not have been detected above background using this procedure.

4.2.4 *In vitro* transcription/translation of the PI constructs

As no positive results were obtained from the western analysis of bacterially-expressed PI/PIZ proteins using the available antisera, I decided to translate the recombinant proteins *in vitro* so that their apparent sizes on SDS-PAGE could be defined. The templates used were pPI/SK+ and pPIZ/SK+, as the pBluescript vectors contain phage T3 and T7 promoters, one to either side of the multiple cloning site. I used the Promega TNT™ coupled reticulocyte lysate system for *in vitro* transcription/translation with T3 RNA polymerase, according to the manufacturer's instructions. ^{14}C-leucine was chosen for labelling the proteins as the patient I-analagous polypeptide has twice as many leucine residues as methionine.

Products of the *in vitro* reactions were separated in a 17.5% polyacrylamide gel (fig. 4.6). Lane 1 shows the positive control (DNA template encoding Luciferase which was provided with the kit) which produced a visible band by autoradiography after overnight exposure, whereas the pPIZ/SK+ *in vitro* reaction, lane 4, produced a barely visible band of <14 kDa after 5 days. After 12 days exposure a faint band appeared, in all 3 lanes other than the "no template" negative control (lane 2), of about the same size as the putative PIZ band. I repeated the *in vitro* reactions, but still observed a faint band in all 3 reactions using template DNA. This band made it uncertain as to whether the pPI/SK+ and pPIZ/SK+ constructs were being translated, although the PIZ lane does appear to show a doublet.

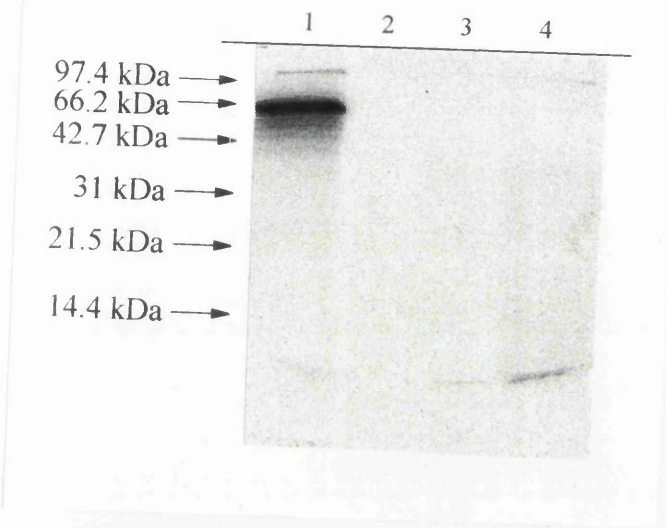


Figure 4.6 ***In vitro*** **translation**

In vitro translation was performed using the Promega TNT™ coupled reticulocyte lysate system for *in vitro* transcription/translation with T3 RNA polymerase, according to the manufacturer's instructions. The templates used were as follows: Lane 1 - Luciferase control template, Lane 2 - no template DNA, Lane 3 - pPI/SK+, Lane 4 - pPIZ/SK+.

In conclusion, the *in vitro* translation reaction using pPIZ/SK+ as a template gives a product of apparent molecular weight of <14 kDa on SDS-PAGE. The fusion PIZ may be displaced by the haemoglobin, giving the *in vitro* synthesised protein a smaller apparent size than it would have in extracts from prokaryotic or eukaryotic expression systems. Because of the faint non-specific band produced by all 3 *in vitro* reactions containing template, it is unclear whether the pPI/SK+ reaction was successful. One must assume that if it were, the PI protein has a similar apparent size, running just below the PIZ protein. The poor yield of product for both reactions may be due to the hydrophobic nature of the polypeptides. Finally, all of the available antisera cross-reacted to other low molecular weight proteins, so that no reaction to the PI or PIZ proteins could be confirmed on western blots.

4.3 Discussion

The experiments described in this chapter set out to characterise the proteins encoded by the synthetic genes PI and PIZ, and to determine the utility of various available antisera to monitor their expression. Three simple conclusions can be drawn: the PI and PIZ proteins are poorly translated *in vitro*, poorly expressed in *E. coli* where they appear to be toxic, and the proteins were not detected above background in these conditions by any of several different antisera supposedly reactive against epitopes they contain. These findings each have a series of ramifications and possible explanations that are now considered in turn.

The *in vitro* translation of pPIZ/SK+ demonstrated that the chimeric gene can be transcribed and that the message can be translated by eukaryotic cytosolic ribosomes, albeit at a low level in the rabbit reticulocyte lysate. Further, it showed that the apparent size of the PIZ protein is <14 kDa, detectable on a 15% SDS-polyacrylamide gel. It is not clear whether the pPI/SK+ construct was transcribed/translated, although there is no obvious reason to account for any difference in the expression levels of the

2 constructs *in vitro*. The pPI/SK+ mRNA has a slightly different nucleotide sequence in the polylinker region (fig. 4.2), but the three codons which differ from pPIZ/SK+ are all found elsewhere in the coding sequence of both constructs and therefore should not be a hindrance to translation. However, the PI peptide lacks the epitope-tag, which is a highly polar region in a hydrophobic peptide and hence may aid solubilisation of PIZ or affect mobility on SDS polyacrylamide gels. As the PIZ coding region was amplified by PCR from the same plasmid DNA prep of pPI/SK+ as was used for the *in vitro* translation reaction, and the insert in pPIZ/SK+ was sequenced, the insert in this DNA prep of pPI/SK+ cannot contain a point mutation creating a premature stop codon. If the PI polypeptide were synthesised in the coupled transcription/ translation reaction, its predicted molecular weight would be ~770 Da less than PIZ. This predicted mobility difference may be increased somewhat by the highly charged epitope tag. Because an apparently non-specific protein of this size was produced in all 3 *in vitro* reactions with DNA template, it can only be concluded that if the PI protein was synthesised and present on 15% gels, it comigrates with the non-specific product.

Nevertheless, a product was observed, specific to the pPIZ/SK+ lane (fig. 4.6), of an apparent mobility on SDS-PAGE consistent with the predicted size of PIZ. The *in vitro* translation reactions did not yield a large amount of product, suggesting that in this system the PI/PIZ proteins are poorly translated, unstable or insoluble. If the *in vitro* translation were to be repeated, I would try using a system which incorporates phospholipid vesicles or some other artificial membranes in order to stimulate/stabilise the expression of the hydrophobic polypeptides. In order to remove non-specific products one could linearise the constructs and remove unnecessary vector sequence, or amplify the transgene portion with the vector-encoded transcriptional promoter by PCR.

No protein specific to the pPI/Q isolates was detected above background using the prokaryotic expression system, although the rabbit 3 antiserum appeared to recognise the KLH-conjugated peptide on a western blot (carried out by K. Morten). It is possible that the two amino acids included in the patient 1-analagous peptide which are not specified by the mtDNA nucleotide sequence (fig. 4.1) are somehow critical to epitope detection by this antiserum on western blots. No protein specific to the pPIZ/Q isolates was detected above background using the monoclonal antibody Z1F11. As discussed in section 4.1.2, Z1F11 had been shown to be effective on western blots at detecting the protein against which it was raised. However, although the epitope had been mapped to two sequential overlapping hexapeptides by ELISA, the blocking experiment which was performed to confirm the mapping was performed using a heptadecapeptide. Therefore, it cannot be ruled out that other residues in the environment of the 7 amino acid epitope are influential in western analysis. It is also possible that a post-translational modification occurs in the eukaryotic system that is required for epitope detection by western blotting.

The time-course growth analysis of the isolates discussed in section 4.2.3 suggests that both pPI/Q and pPIZ/Q constructs are being expressed in the bacterial system. Possibly the recombinant protein was degraded very rapidly in *E. coli*, and was not present in sufficient quantity to be detected on a western by either antibody. It is possible that the protein was insoluble in the conditions used. Due to its extreme hydrophobicity, the full-length COXI forms aggregates, even in the presence of SDS (Nijtmans *et al.*, 1998). Another possibility is that the protein was being secreted and was therefore not in the preparations loaded onto the acrylamide gels. It is possible that the mitochondrial targetting peptide could have functioned as a signal sequence for protein export.

Mitochondrial targetting signals share some properties with prokaryotic plasma membrane targetting signals (Roise & Schatz, 1988). In a membrane environment

they have an alpha-helical structure, although bacterial sequences tend to be less positively charged (the mouse COXIV transit peptide has 4 basic residues out of 22). Because of N-terminal processing of secreted proteins, Qiagen recommend the use of vectors with a carboxy-terminal histidine-tag for purifying extracellular proteins. Although the PI constructs had an amino-terminal histidine-tag, it may have been worth trying to purify recombinant protein from the medium. However, as extracellular targetting is one approach for enabling the expression of toxic proteins in *E. coli*, and expression of both PI constructs is highly toxic to *E. coli*, it would seem unlikely that secretion of the recombinant protein was the primary problem with the western analysis.

As the growth analysis indicated, expression of the constructs appears to be highly toxic. This may be due to the presence of the transit peptide sequence at the amino terminus of the recombinant protein, the hydrophobic nature of the polypeptides, or the combination of both features. Association of the 25 amino acid yeast COXIV transit peptide with the outer membrane of mitochondria occurs by partitioning of the presequence directly into the lipid bilayer (Roise, 1992). The association of a synthetic peptide (in this study the 45 amino-terminal residues of the bovine cytochrome P-450 precursor) with the mitochondrial outer membrane is independent of cytosolic factors and mitochondrial surface protein components sensitive to trypsin (Furuya *et al.*, 1991). In a study using 3 peptides based on the amino-terminus of the yeast COXIV precursor which were 15, 25, and 33 residues in length, both the 25- and 33-residue peptides uncoupled respiration in isolated yeast mitochondria (Roise *et al.*, 1986). Another study tested amphipathic peptides for effects on the structural and functional properties of isolated rat liver mitochondria, also using the yeast COXIV transit peptide as the model peptide in the study. Leakage of the matrix enzyme adenylate kinase was measured as a function of outer membrane lysis, and this showed that the integrity of the membrane was increasingly compromised as the amount of peptide increased. The peptide also caused uncoupling of respiration, and

dissipation of the membrane potential across the inner membrane (Nicolay *et al.*, 1994).

Therefore, it is not unreasonable to assume that overexpression of a protein such as PI or PIZ which included an amino terminal transit peptide could have caused similar effects on bacterial membrane structure and properties. In prokaryotes, the respiratory chain enzymes are situated in the cytoplasmic membrane, the inner of a double membrane structure, while the outer membrane is semi-permeable like the outer mitochondrial membrane (Stryer, 1988). It is possible that the recombinant protein was associating with the cytoplasmic membrane, and effectively perforating it. The fragment of COXI included in the chimeric peptides PI and PIZ include the entire first transmembrane domain and half of the second transmembrane domain of COXI (Tsukihara *et al.*, 1996). As noted, even background levels of expression of the full-length, universal-code version of the mouse COXI gene were apparently detrimental to *E. coli* (section 3.2.6). It is possible that PI and PIZ may be toxic due to interference with the assembly or activity of a bacterial cytochrome oxidase, but not likely. A heme aa_3-type cytochrome *c* oxidase which is equivalent to the mitochondrial enzyme is not found in *E. coli*. The 2 predominant forms of terminal oxidase which are found in *E. coli* are cytochrome *o* and cytochrome *d*, both of which have ubiquinol-8 as electron donor (Ludwig, 1987). Neither of these oxidases have a subunit with significant homology to the first 70 amino acids of the mouse COXI subunit. Therefore it is unlikely that the PI and PIZ proteins were toxic due to interaction with other subunits of the *E. coli* terminal oxidases. As the first 70 amino acids of COXI are not involved in metal binding sites (Tsukihara *et al.*, 1996), it is also unlikely that the proteins were toxic due to sequestration of cofactors. Nor could this small fragment of one COX subunit have acted independently as a proton pump.

To clarify exactly what was toxic to *E. coli*, possible experiments would include the expression of the COXIV transit peptide alone, and the COXI fragment alone. This

would allow one to identify whether either of these peptides individually was responsible for the drastic penotype observed in *E. coli* cultures. The universal equivalent of rat COXII has been expressed in *E. coli* (Cao *et al.*, 1991). Although only low levels of expression were achieved, the authors do not report that expression of the protein was toxic to *E. coli*. As a different core subunit of a mammalian cytochrome *c* oxidase was not found to be toxic to *E. coli*, it may be that the inclusion of the transit peptide in the prokaryotic constructs was responsible for the phenotype observed.

In retrospect, removal of the targetting sequence coding region from the constructs to enable testing in bacterial expression systems would have been a good idea. It is possible this would have made the recombinant protein less toxic to the cells. Additionally, if I were to repeat this analysis, I would try using the prokaryotic expression vector which encodes a carrier protein such as DHFR. It is possible that this could improve the stability of the recombinant protein as well as reducing its toxicity. As DHFR is reportedly poorly immunogenic in mice and rats, the fusion protein could also be used to generate new antisera, for further analysis. Another possibility would be to express PI in an alternative expression system, such as Baculovirus infection of insect cells, or in yeast.

The expression and characterisation of an unknown peptide in the prokaryotic system was hindered by the use of antisera which were not well documented. If the project is to be pursued, it will be necessary to obtain an effective antiserum with which to assess Patient I transgene expression. There are four options available: obtain an anti-COXI antibody which recognises an epitope within the truncated COXI region that PI contains, again try to raise an anti-peptide antisera, perhaps including residues either side of the formerly chosen sequence, pursue expression and purification of PI in order to generate an anti-PI antisera, or to obtain a more reliable epitope-tagging system.

Chapter 5

Expression studies of Patient I constructs in mouse cells

5.1 Introduction

5.1.1 Aims

The aim of this project was to test the hypothesis that abnormal proteins, encoded in affected individuals by chimeric mitochondrial genes, contribute to mitochondrial dysfunction and pathogenesis. The evidence for the presence of a fusion protein in cases of rearranged mtDNA in humans is summarised in section 1.5.7. Model systems, including plants and yeast, in which an abnormal protein in mitochondria have been shown to cause dysfunction have been discussed. In this chapter I describe the generation and analysis of mouse cell-lines transfected with two patient-analagous constructs. Mouse mtDNA and mouse cell-lines were used in this work because the long-term aim of the overall project was to create a transgenic mouse model for diseases such as Kearns-Sayre syndrome, which are associated with gross rearrangements of mtDNA.

The specific aim of this PhD project was to generate mouse cell-lines expressing chimeric mitochondrial peptides and to assess the phenotype of the cell-lines produced. In chapter 3 I have described the work which was necessary to produce the synthetic genes PI and PIZ ("Patient I", encoding a patient-analagous chimeric mitochondrial peptide, and the Z1F11 epitope-tagged version, respectively). These genes are capable of cytosolic translation (as they have been mutated to read in the universal code) and the expressed proteins are capable of mitochondrial import (as they contain a mitochondrial transit peptide). In chapter 4 I have described the expression of these constructs in a prokaryotic system, and the *in vitro* translation of the genes. Both PI and PIZ would appear to be highly toxic in *E. coli*, and poorly expressed in the *in vitro* system used. Additionally, I was unable to detect expression of the recombinant proteins above background using the antisera available.

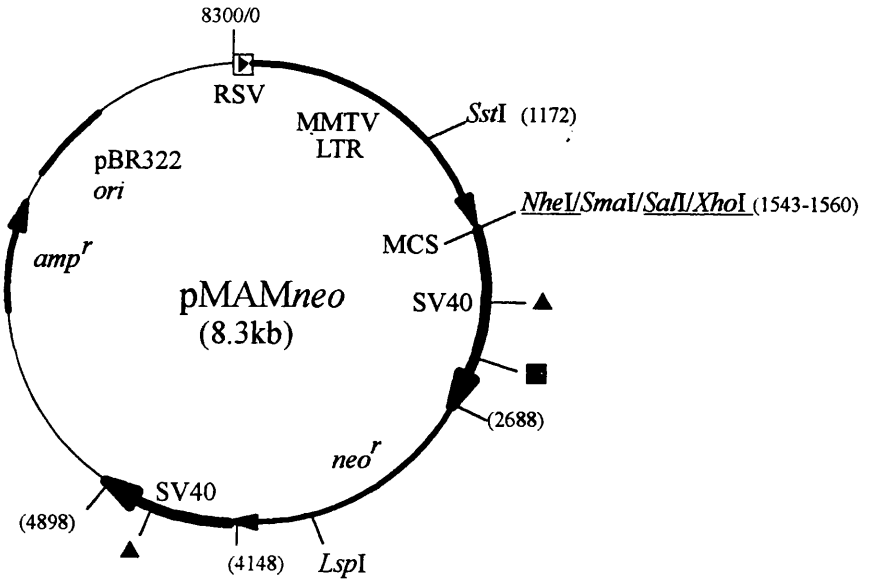
The work described in this chapter begins with the generation of mouse cell-lines carrying the Patient I constructs under the control of an inducible promoter. Northern blotting is then described, aimed at establishing whether these cell-lines were expressing the transgene. Furthermore, using the antisera described in section 4.1.2, western blotting was performed to assay whether the transcripts were being correctly translated. Finally, a preliminary analysis of the cellular phenotype generated by expression of the abnormal protein PIZ was performed.

5.1.2 The expression vector pMam*neo*

The expression vector chosen for the analysis was pMam*neo* (supplied by Clontech). This is a mammalian expression vector which can be used for controlled expression in hormonally responsive cell-lines. A gene inserted at the polycloning site is placed under the transcriptional regulation of the dexamethasone-inducible MMTV-LTR promoter, linked to the RSV-LTR enhancer element. The manufacturer reports that this construction enables a 10-fold increase in expression via induction with dexamethasone. RNA processing of the transcript is facilitated by the SV40 early splicing region and polyadenylation site. The neomycin-resistance marker allows selection of transformants growing in media supplemented with the antibiotic G418. The plasmid also contains the ampicillin resistance marker and replication origin for propagation in *E. coli* (fig. 5.1).

5.1.3 Cell-lines

The cell-line used initially in this work was the mouse LTK- cell line, derived from connective tissue with fibroblast-like morphology. By treatment with ethidium bromide (50 ng/ml) I generated a derivative of the line which, on the basis of Southern blotting, PCR and a requirement for uridine supplementation in the medium, appeared to be ρ^o. My intention was to compare the phenotype of LTK- Patient I



▲ SV40 early splicing region and polyadenylation

■ SV40 *ori* and early promoter

RSV-LTR enhancer element

Arrow showing direction of transcription

Figure 5.1 The eukaryotic expression vector pMam*neo*

This figure depicts the vector used for the expression of both the PI and PIZ constructs in the mouse fibroblast cell-line 3T3. All restriction enzyme sites used for cloning and generating restriction fragments for templates of random-primed probes are displayed. Vector-encoded sequences pertinent to the work described are labelled. The *E. coli* origin and ampicillin-resistance marker are required for propagation of the vector in *E. coli*. The Multiple Cloning Site (MCS) is a region containing several restriction enzyme sites which are unique in the vector, enabling subcloning of the gene to be expressed. The MCS is located downstream of the Rous Sarcoma Virus enhancer element, and the Mouse Mammary Tumour Virus LTR which contain regions requisite for transcription under the control of the inducing agent dexamethasone. The SV40 early splicing region and polyadenylation sequence is necessary for appropriate processing of the transcript. Note that the neomycin-resistance marker also contains these elements.

clones with LTK-ρ° Patient I clones in order to assess whether the chimeric protein caused mitochondrial dysfunction in a context other than interference with aerobic respiration. However, this line of inquiry was not pursued for two reasons. Although the LTK-ρ° cell line demonstrated the same degree of sensitivity to G418 as the parental line (800 μg/ml to ensure no resistant colonies), several attempts to transfect the cell line with the pMam/PI construct failed. Control transfections indicated that the reagent used in the lab at that time was defective. I was also unable to ascertain whether the LTK- cell line had appropriate receptors for dexamethasone. However, the manufacturers of pMam*neo* (Clontech) reported that the vector had been successfully used with another mouse fibroblast cell line, 3T3, which is commonly used in research. By using this line any failure to detect a transcribed product would not be attributable to lack of responsiveness to the inducing agent. Therefore, the experiments discussed subsequently in this chapter are all with the 3T3-derived clones. There was insufficient time to generate a ρ° derivative of the 3T3 cell line.

5.1.4 The ratio of nuclear to mitochondrial DNA

As discussed in section 1.5.4, many studies have now been carried out to assess the relative levels of wild-type and mutant mtDNA molecules in affected patient tissues. Techniques used include quantitative amplification of mtDNA from single muscle fibres and *in situ* hybridisation of muscle sections. All studies have shown a large increase in the copy number of deleted mtDNA molecules in RRFs, associated with mitochondrial proliferation. The studies are contradictory as to whether there is a depletion of normal mtDNA in COX- fibers, and in RRF. Thus, another question which I addressed was whether the presence of the abnormal polypeptide PIZ would have a discernible effect on mtDNA copy number.

As no deleted mtDNA was present in this study, the proportion of mtDNA was quantitated simply with respect to nuclear DNA. I chose to analyse the effect that

growth in the two extremes of respiratory stress would have on the relative proportion of mtDNA to nuclear DNA; minimal stress, where the media contains glucose and the transgene is not expressed, compared to induction of transgene expression in a medium which requires cellular respiration for survival. DNA was prepared from cell-lines after 4 days growth in the respective media, the same time point at which cells were assessed for respiratory competence.

5.1.5 Assay of respiratory chain function

Cells can generate energy in the form of ATP by at least two basic mechanisms, either by using the glycolytic pathway or using the respiratory chain. In glycolysis, glucose is converted into pyruvate and in the process generates net ATP. The entry of galactose into the glycolytic pathway requires 4 additional enzymatic steps to convert it to glucose 1-phosphate. Under aerobic conditions pyruvate normally enters the citric acid cycle; however, when the amount of oxygen is limiting (such as in actively contracting muscle) pyruvate is converted into lactate (Stryer, 1988). In cultured fibroblasts, >97% of the glucose utilised has been shown to be converted to lactic acid, thus the pyruvate generated does not enter the citric acid cycle (Donnelly & Scheffler, 1976). For cells in culture, the main source of aerobic energy is glutamine, which feeds into the citric acid cycle later in the cycle (Reitzer *et al*, 1979). The latter study demonstrated that cultured HeLa cells growing in media containing >1 mM glucose derive 35% of their energy from the glycolytic pathway, and 65% from oxidative phosphorylation. However, if the media provided other hexose sugars (such as galactose) for glycolysis, >98% of cellular energy was obtained from the aerobic respiratory pathway utilising glutamine as the metabolic source. The hexose sugars were primarily found to be metabolised for biosynthetic precursors rather than for the generation of ATP. Chu *et al* (1972, 1974) successfully used galactose medium to select for respiration-deficient mutants from a Chinese hamster somatic cell-line. Galactose medium has since been used to assay for a respiratory deficient phenotype

in cells heterozygous for deleted mtDNA (Hayashi *et al*, 1991). This study found that a clear threshold existed whereby cells grown in glucose medium reached an equilibrium state of 75-85% deleted mtDNA, above which they grew poorly. However, when clones were grown in galactose medium, the threshold for growth decreased to 50% deleted mtDNA, and galactose medium was lethal to cells with >72% deleted mtDNA (as well as to ρ^o cells).

On the basis of the above, I have investigated respiratory function in cells transfected with the PIZ construct, by growing them in galactose-containing ("gal") medium. The PIZ clones were also grown in a medium referred to as "superglu", which contains all supplements necessary for supporting the growth of ρ^o cells. "Superglu" was chosen as the control condition for the experiment because the underlying hypothesis was that the presence of the chimeric protein might be detrimental to respiration even at very low levels (given the difficulty of detecting a chimeric protein *in vivo*), and it is possible that some transcription of the transgene would occur even in the absence of the inducing agent dexamethasone. The cells were thus incubated in four media; "superglu" +/- dexamethasone, and "gal" +/- dexamethasone. The presence or absence of dexamethasone was to test the effect of enhanced transgene expression in the respective media.

5.1.6 Assays of mitochondrial proliferation

Although the distribution of the wild-type and mutant mtDNA in diseased muscle fibres is controversial, with reference to the total copy number of molecules per muscle fibre, the data suggests that muscle fibres of affected individuals which are displaying some respiratory deficiency do not yet have an increase in mitochondrial genome copy number, and possess only a small proportion of mutant mtDNA molecules. The identification of such fibres supports the hypothesis that mitochondrial proliferation in RRFs is an ineffective response to biochemical

deficiencies as opposed to a causal event. Based on this idea, I assayed for mitochondrial proliferation in cells expressing PIZ by confocal microscopy. The cells were again grown in two conditions (superglu *vs.* gal + dexamethasone), stained with a mitochondrial-specific dye, fixed, then double-labeled with the monoclonal antibody Z1F11 (previously described in section 4.1.2). Two forms of the mitochondrial-specific dye were used, one which detects all mitochondria present in the cell, and the reduced form of the dye, which must be oxidised by functional mitochondria in order to fluoresce. Comparison of the two staining patterns enabled a preliminary assessment of mitochondrial activity in the cells.

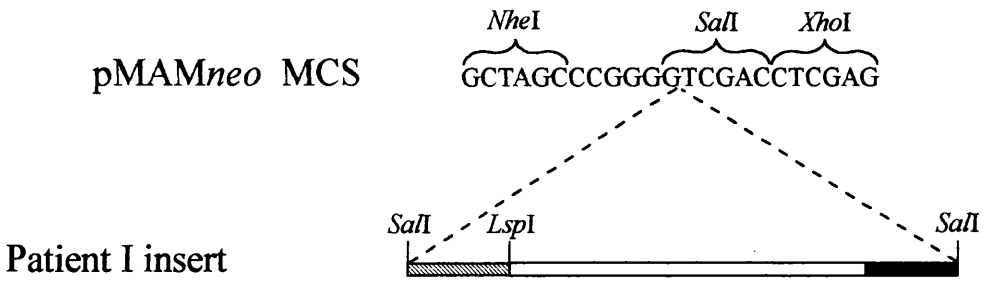
5.2 Results

5.2.1 Subcloning of Patient I constructs into pMam*neo*

The PI and PIZ constructs were subcloned into the pMam*neo* vector (fig. 5.2). The subcloning was performed in *E. coli* strain XL1-Blue. Plasmid pPI/SK+ (the Patient I construct in pBluescript SK+, described in section 3.2.4, depicted in fig. 3.13) was digested with *Sal*I, and the 370 bp Patient I insert sequence was gel-purified. The fragment was subcloned into pMam*neo,* which had been digested with *Sal*I and dephosphorylated with calf intestinal alkaline phosphatase. Transformants were screened by restriction digest with *Sal*I, and one clone (designated pM/PI) was chosen for sequencing. This showed that pM/PI contained the correct insert sequence in the appropriate orientation for protein expression.

Plasmid pPIZ/SK+ (the epitope-tagged Patient I construct in pBluescript SK+, described in section 3.2.5, depicted in fig. 3.15) was digested with *Xba*I and *Xho*I , and the insert fragment gel-purified. The isolated 390 bp fragment had an intact *Xho*I site overhang at the 3' end of the fragment, and an intact *Xba*I site (compatible with *Nhe*I) at the 5' end of the gene fragment. pMam*neo* was digested with *Nhe*I and *Xho*I,

a) pM/PI



b) pM/PIZ

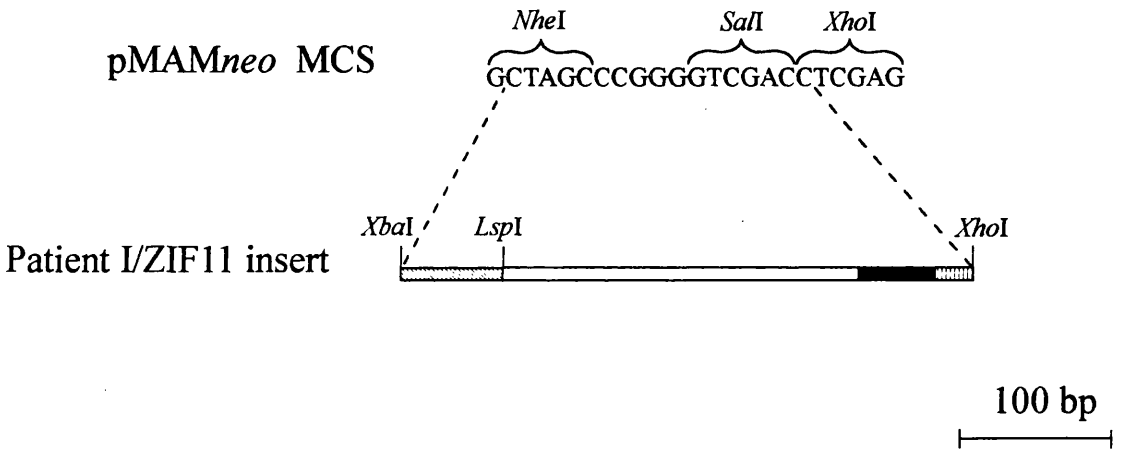
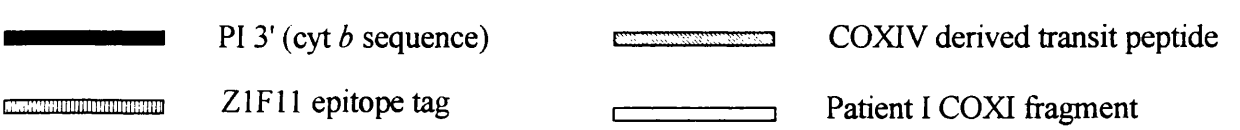


Figure 5.2 Subcloning of the Patient I gene and the epitope-tagged version into pMam*neo*

This figure depicts the orientation of the two genes when subcloned into the pMam*neo* MCS.
a) depicts the Patient I insert (subcloned from plasmid pPI/SK+).
b) depicts the Z1F11 epitope tagged Patient I insert (subcloned from plasmid pPIZ/SK+).
The key explains the derivation of the various segments of the final constructs.



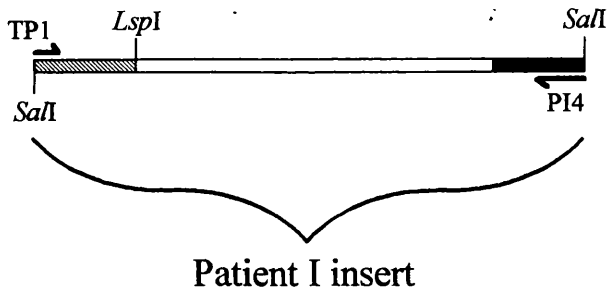
and the fragment was ligated into the vector multiple cloning site. Transformants were screened by restriction digest with *Lsp*I (one site is present in the insert sequence, one in the vector). Two clones displayed the appropriate restriction pattern, and were selected for sequencing to confirm that they contained the correct insert sequence (designated pM/PIZ8 and pM/PIZ10).

5.2.2 Generation and selection of cell clones

Mouse 3T3 cells were transfected with plasmid DNA by liposome mediation, using DOTAP (Boehringer Mannheim) according to the manufacturer's protocol (described in sect. 2.6.4). Six vector-transformed clones, and twelve each of the pM/PI- and pM/PIZ-transfected clones were plated in 24-well tissue culture dishes. Six colonies each from pM/PI, pM/PIZ8 and pM/PIZ10 transfections were chosen for screening by PCR, to ascertain that the transgene had integrated in a non-disrupted manner (fig. 5.3). Genomic DNA was prepared from these 18 cell lines, plus 2 colonies transfected with vector only. For pM/PI transfected lines the 5' PCR primer used was TP1, the oligonucleotide encoding the amino terminus of the COXIV transit peptide, sense strand. In this case the 3' PCR primer used was PI4, the oligonucleotide encoding the carboxy terminus of the Patient I chimeric gene (cytochrome *b*-derived), antisense strand. This pair of primers should amplify a product of 375 bp. For pM/PIZ transfected cell lines, the 5' PCR primer used was 5'TP/Xba, the oligonucleotide which encodes the amino terminus of the COXIV transit peptide on the sense strand, but contains an *Xba*I site for cloning, rather than the *Sal*I site. In the latter case, the 3' PCR primer used was PIZ1, the oligonucleotide encoding the junction between the cytochrome *b* (cyt *b*) region and the Z1F11 epitope tag, on the antisense strand. This pair of primers should amplify a product of 377 bp.

Figure 5.4 shows the ethidium bromide-stained gel containing the PCR products from the PI cell lines. As expected, these PCR reactions produced no product for the

a) Patient I insert



b) Epitope-tagged Patient I insert

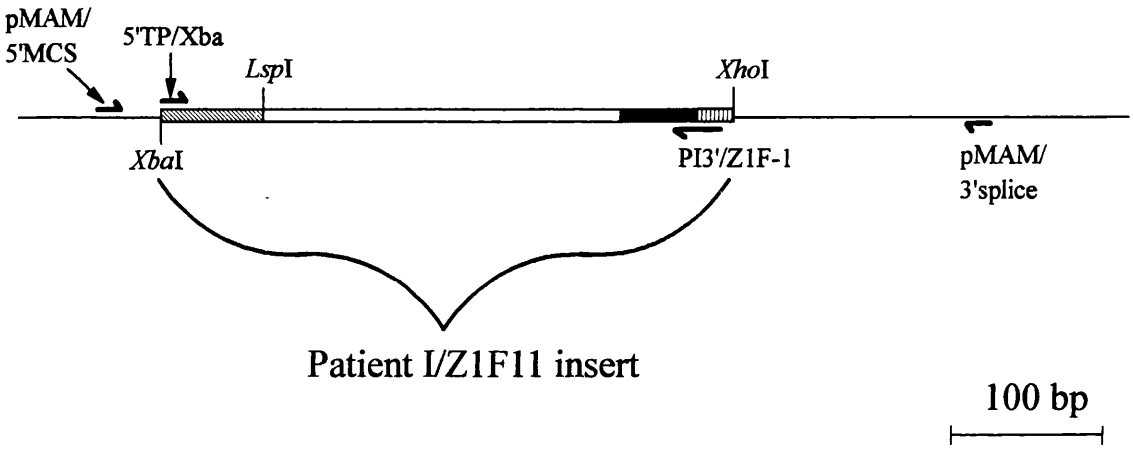
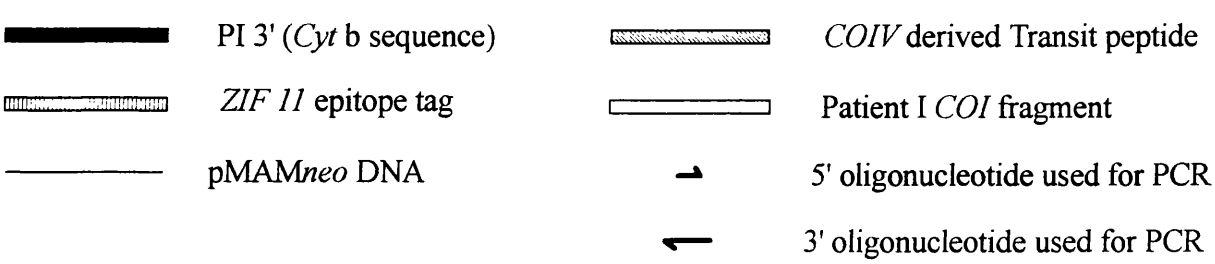


Figure 5.3 The generation of PCR products to ascertain integration of the transgenes

This figure depicts where the primers used in the PCR reactions described in the text (and shown in fig. 5.4 and 5.5) anneal to the target templates. The sequences for the oligonucleotides are given in section 2.4. The oligonucleotides used as primers are designated as follows:

TP1	5' section of the sense strand of the COXIV transit peptide
PI4	3' section of the antisense strand of the cyt *b*-derived region
5'TP/Xba	as TP1, but 27 nt long, with an *Xba*I site substituted for the *Sal*I site
PIZ1	junction between the cyt *b* region and the Z1F11 tag, antisense strand
pMam/5'MCS	anneals ~40 nt upstream of the vector MCS, sense strand
pMam/3'splice	anneals ~170 nt downstream of the vector MCS, antisense strand



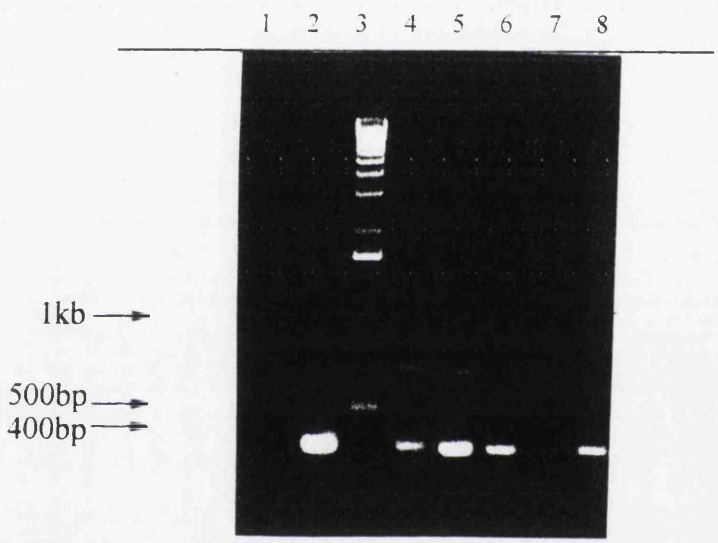
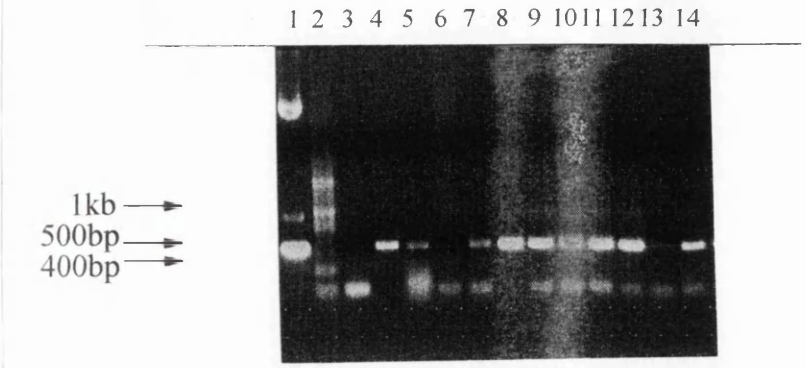


Figure 5.4 PCR of 3T3 clones to demonstrate incorporation of the PI construct

PCR was performed on genomic DNA prepared from G418-resistant clones, as well as positive and negative controls. The 5' PCR primer used was oligonucleotide TP1; the 3' PCR primer used was oligonucleotide PI4 (see text and fig. 5.3 for description, or sections 2.4.5 and 2.4.6 for sequences). The products were run on a 1.65% TAE agarose gel. Lane 1 - no DNA template, Lane 2 - Sal I-digested plasmid pM/PI, Lane 3 - marker, Lane 4 - PI.1, Lane 5 - 3T3 PI.2, Lane 6 - 3T3 PI.3, Lane 7 - 3T3 PI.4, Lane 8 - 3T3 PI.6.

pMam*neo*-transfected control cell-lines. Four of the six pM/PI-transfected cell lines contained, based on this assay, an insert of the correct size to be the full-length coding sequence of the PI transgene. These four clones were designated PI.1, PI.2, PI.3, and PI.6 respectively. Figure 5.5 shows the ethidium bromide-stained gel containing the PCR products from the PIZ cell-lines obtained in two separate transfections with clones pM/PIZ8 and pM/PIZ10. Of the pM/PIZ8-transfected cell lines, five of six contained an insert the correct size to be the full length coding sequence of the respective transgene. These clones were designated PIZ8.1, PIZ8.2, PIZ8.3, PIZ8.5, and PIZ8.6. Of the pM/PIZ10-transfected cell lines, all five contained an insert the correct size to be the full-length coding sequence of the respective transgene. These clones were designated PIZ10.1, PIZ10.2, PIZ10.3, PIZ10.4, and PIZ10.5. Subsequently, genomic DNA from the set of 3T3-derived clones shown to have been successfully transfected with PIZ8 were used as templates in PCR reactions primed by vector-specific oligonucleotides situated 5' of the multiple cloning site, and 3' of the SV40 splice acceptor site (see fig. 5.3). The predicted size of the correctly amplified insert is ~600 bp. Again, for those cell-lines shown in fig. 5.5.a to contain the transgene, these reactions produced DNA fragments of an appropriate size to contain the intact transgene situated within the vector-encoded sequences necessary for gene expression. The vector-transformed cell-line (designated 3T3pM) also produced a band of appropriate size (no insert, 227 bp), and no band was observed for PIZ8.4 (previously negative) (fig. 5.5.b).

In repeated PCR reactions using both sets of primers clone PIZ8.5 yielded very little product compared to the other cell lines. The observed product is unlikely to have been the result of PCR contamination, because no band was observed in the negative control (no template) lanes by gel analysis, nor was a band of the predicted size for the transgene observed in the 3T3pM samples. Nor is the faint product likely to have been the result of contaminated template, as genomic DNA was prepared from PIZ8.5



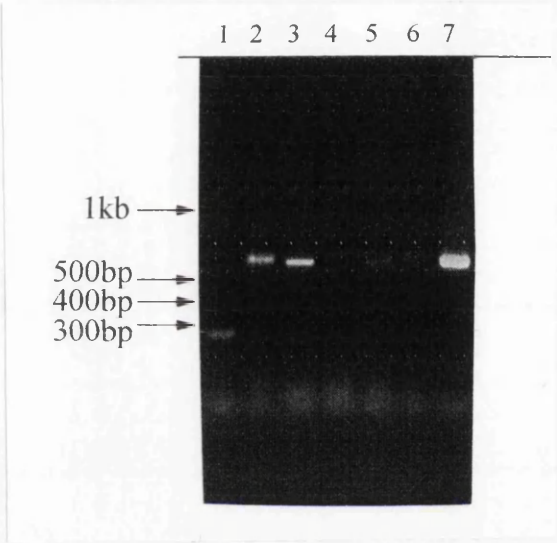


Figure 5.5 PCR of 3T3 clones to demonstrate incorporation of the PIZ construct

Figure 5.5.a PCR was performed on genomic DNA prepared from G418-resistant clones, as well as positive and negative controls. The 5' PCR primer used was oligonucleotide 5'TP/Xba; the 3' PCR primer used was oligonucleotide PI3'/ZIF-1 (see text and fig. 5.3 for description, or section 2.4.7 for sequences). The products were run on a 1.5% TAE agarose gel. Lane 1 - plasmid pM/PIZ8, Lane 2 - LTK- cells, Lane 3 - 3T3pM, Lane 4 - PIZ8.3, Lane 5 - PIZ10.2, Lane 6 - PIZ8.4, Lane 7 - PIZ8.6, Lane 8 - PIZ10.1, Lane 9 - PIZ10.4, Lane 10 - PIZ10.5, Lane 11 - PIZ8.1, Lane 12 - PIZ8.2, Lane 13 - PIZ8.5, Lane 14 - PIZ10.3.

Figure 5.5.b PCR of 3T3 clones to demonstrate incorporation of the PIZ construct with pMam*neo*-encoded expression signals intact

PCR was performed on genomic DNA prepared from 3T3-derived G418-resistant clones, as well as positive and negative conrols. The 5' PCR primer used was oligonucleotide pMam/5'MCS; the 3' PCR primer used was oligonucleotide pMam/3'splice (see figure 5.3 for description or section 2.4.8 for sequences). The products were run on a 1.4% TAE agarose gel. Lane 1 - 3T3pM, Lane 2 - PIZ8.1, Lane 3 - PIZ8.3, Lane 4 - PIZ8.4, Lane 5 - PIZ8.5, Lane 6 - PIZ8.6, Lane 7 - plasmid pM/PIZ8.

on multiple occasions. It is possible that the transgene was less stable in this clone, or that the "clone" is a mixed population.

5.2.3 Slot blot analysis of mtDNA copy number

As discussed in section 5.1.4, one question which interested me was whether expression of PIZ would have any discernible effect on mtDNA copy number. To address this question I prepared a slot blot of total DNA from three transfected cell lines, incubated in two different media, and probed it with three different random-primed probes. Figure 5.6 depicts this slot blot. The cell lines from which DNA was prepared were 3T3pM, PIZ8.3 and PIZ8.5. The cells from which DNA was to be prepared were plated out and incubated in the two media considered to be the two extremes of low and high respiratory stress conditions, either "superglu" or galactose + dexamethasone, for four days. 5 μg of DNA from each aliquot were loaded in triplicate onto the membrane to enable hybridisation with the three different probes. The three probes were chosen to detect either mtDNA, a single copy mouse nuclear gene, or the transgene. The random-primed probes were prepared using cytochrome *c* oxidase II as the template DNA for the mitochondrial probe, the template DNA for the nuclear gene was Etl-1 (kindly donated by Dr. P.Shields) and the transgene template DNA was the *Sst*I/*Xho*I 800bp fragment from pM/PIZ. The transgene probe included the PIZ-coding region and ~400 bp of the MMTV LTR region of the plasmid, which encompasses the transcriptional start site of the transgene. Thus, the transgene probe should hybridise to the 3T3pM DNA preparation as well, as it contains approximately 400 bp of vector sequence.

Although the experiment gave no clear indication with regard to changes in mitochondrial DNA copy number in the two different media conditions, it did produce relevant data with regard to the transgene. The probe derived from the single copy nuclear gene should act as an internal loading control. In lane B it would appear

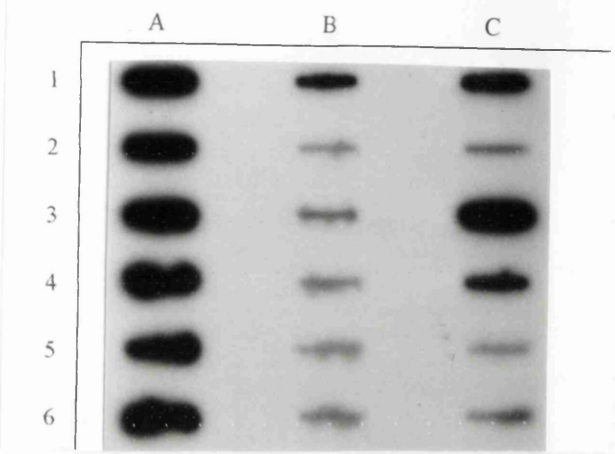


Figure 5.6 Slot blot analysis of total DNA extracted from selected 3T3 clones

DNA was prepared from 3 cell-lines (3T3pM, PIZ8.3 and PIZ8.5) each grown in two different media conditions ("superglu" or "gal" + dexamethasone). 5 μg of these 6 DNA preparations were then blotted onto the membrane in triplicate. The membrane was hybridised with random-primed probes prepared from 3 different templates - *Etl*-1, a single-copy mouse nuclear gene, COXII, a mitochondrial gene, or the *Sst*I/*Xho*I fragment from pM/PIZ. Hybridisation was performed overnight at 65 ^{0}C. Wash conditions were at high stringency as specified in section 2.11.2.

Probes:	Lane A -	COXII
	Lane B -	*Etl*-1
	Lane C -	pM/PIZ
Samples:	Rows 1	3T3pM grown in "superglu"
	Rows 2	3T3pM grown in galactose + dexamethasone
	Rows 3	PIZ8.3 grown in "superglu"
	Rows 4	PIZ8.3 grown in galactose + dexamethasone
	Rows 5	PIZ8.5 grown in "superglu"
	Rows 6	PIZ8.5 grown in galactose + dexamethasone

that the 5 μg sample for 3T3pM grown in "superglu" is overloaded with respect to the other five samples. One would expect that the relatively strong hybridisation of the transgene probe to this DNA preparation (lane C, row 1) is due to overloading as well. In rows 1, 2, 5, and 6, the intensity of the bands in lanes B and C are roughly equivalent. However, in rows 3 and 4, the bands in lane C are much more intense than in lane B. This suggests that clone PIZ8.3 has incorporated multiple copies of the transgene. Although the intensity of the signal in lane B is roughly equivalent between rows 3 and 4 (the PIZ8.3 grown in "superglu" versus "gal" + dexamethasone), the intensity of the signal is much less in row 4, lane C than in row 3, lane C. This suggests that under conditions of respiratory stress (growth in galactose + dexamethasone), this particular cell-line may be losing copies of the transgene. This is consistent with the hypothesis that expression of PIZ is detrimental to cellular respiration.

5.2.4 Western analysis

The western analysis of the 3T3-derived clones was performed in parallel with the work described in chapter 4. As discussed in chapter 4, the western analysis on *E. coli* transformants proved inconclusive. If the antisera tested recognised the PI or PIZ proteins, the reaction was obscured by the cross reactions with other proteins. For the western analysis of the mouse cell-lines the same antisera as described in Chapter 4 were used. These were the anti-Patient I-specific-peptide rabbit polyclonal antisera, and the mouse monoclonal antibody Z1F11 which should recognise the carboxy-terminal epitope tag of PIZ.

Initially, western analysis was performed on the PI transgenic clones. The rabbit 3 immune antiserum (R3I) was used to probe blots of total protein from 3T3 clones shown to have integrated an intact PI gene by PCR (data not shown). The encoded PI protein is calculated to be 13.0 kDa in molecular weight, but no bands were detected

less than 21 kDa in size, and no bands specific to the PI clones as compared to 3T3pM were apparent. The rabbit 1 and 2 preimmune and immune antisera were also tested, but no band specific to the 3T3 PI clones could be detected (data not shown).

Although the polyclonal antiserum was unsuccessful in detecting the PI protein, analysis of the PIZ clones with the monoclonal antisera revealed evidence that supports transgene expression at the protein level. The calculated size of PIZ, including the amino terminal transit-peptide, is 13.75 kDa; if the mitochondrial transit-peptide is cleaved correctly the calculated size of the imported polypeptide is 11.4 kDa. Fig. 5.7 shows a western blot of a 17.5% polyacrylamide gel, loaded with crude cell extracts from 3T3pM (lane 1) and PIZ clonal cell lines (lanes 2-5 and 7-10), which was probed with Z1F11. Migrating at an apparent molecular weight of ~14 kDa in lanes 2, 4, and 8 (clones 3T3/PIZ8.1, PIZ8.3, and PIZ10.3) there is a doublet. Lane 10 (clone 3T3/PIZ10.5) also appears to show a band migrating at this apparent molecular weight This doublet/band is not apparent in lane 1, even on long exposure. Comparisons of the intensity of cross-reacting higher molecular weight proteins suggest that lane 1 is underloaded compared to the other lanes. However, lane 5 would appear to have been loaded with at least an equivalent amount of protein as lane 2 (note the 31 kDa cross-reacting band), yet no band in the size range of ~14 kDa is evident in lane 5. This is consistent with clone PIZ8.1 expressing the PIZ protein, whilst clone PIZ8.6 is either not expressing the protein, or is expressing it at a very low level. This correlates with the northern data (fig. 5.8.d) which indicates that clone PIZ8.6 is not expressing the transcript for the transgene. It is possible that the doublet observed represents the proteolytically processed and unprocessed forms of PIZ. This would suggest that the protein is not only being expressed, but that it is being imported into mitochondria and that the transit-peptide is being cleaved appropriately.

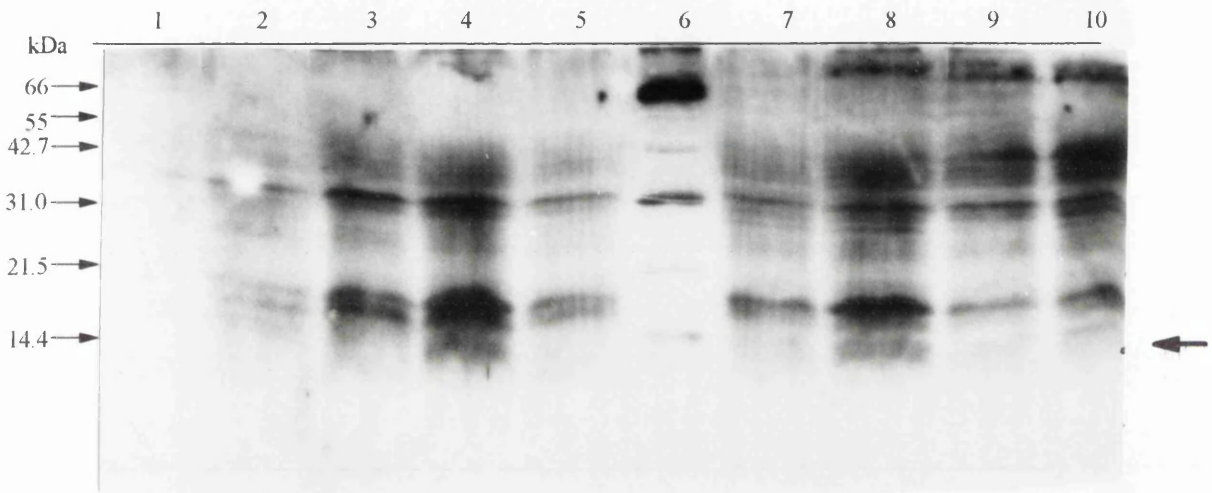


Figure 5.7 Western blot of selected PIZ clones probed with the Z1F11 monoclonal antibody

A 17.5% SDS polyacrylamide gel was loaded with denatured samples of selected 3T3 clones, run at 150 V, and blotted overnight. The blot was incubated with the Z1F11 monoclonal antibody, followed by the HRP-conjugated anti-mouse secondary antibody, both at a concentration of 1/2000. The Amersham ECL detection system was used. Lane 1 - 3T3pM, Lane 2 - PIZ8.1, Lane 3 - PIZ8.2, Lane 4 - PIZ8.3, Lane 5 - PIZ8.6, Lane 6 - marker, Lane 7 - PIZ10.1, Lane 8 - PIZ10.3, Lane 9 - PIZ10.4, Lane 10 - PIZ10.5. The arrow indicates the putative PIZ protein doublet.

In order to eliminate some of the background from non-specific cross-reacting bands, I attempted immunoprecipitation of PIZ (see section 2.14 for details), although Z1F11 was reportedly inefficient for this purpose (Dr.A. Cross, personal communication). Both the monoclonal Z1F11 and the rabbit polyclonal R3I were used to immunoprecipitate protein from clones 3T3pM, PIZ8.3 or PIZ8.5 (the first as a negative control, and PIZ8.3 because it appeared from the previous western blot to be expressing detectable levels of PIZ). Blots of gels containing these samples were probed with both antibodies (data not shown). R3I detected no bands smaller than 31 kDa, and none specific to the PIZ clones. No definitive bands were detected in the western probed with Z1F11.

5.2.5 Effects of transgene expression on mitochondrial density and respiration

As discussed in section 5.1.6, I wished to assess the effect transgene expression might have on mitochondrial proliferation using immunofluorescence labeling with the Z1F11 antibody. I had also hoped to show mitochondrial import of the processed form of PIZ. However, as is apparent from the data presented in section 5.2.4, the monoclonal antibody cross-reacts with many other proteins synthesised in the 3T3 cell-lines. The background levels of fluorescence from these non-specific reactions in the control cell line was of such a degree that, although the cell-line PIZ8.3 appeared to fluoresce more brightly, no conclusions could be drawn from these images.

However, the use of mitochondrial-specific dyes did enable a preliminary assessment of mitochondrial respiratory activity. Figure 5.8 shows images of cells scanned using a confocal microscope. The cell-lines shown are 3T3pM and PIZ8.3, cultured in galactose medium, with dexamethasone. Two forms of the mitochondrial-specific dye (MitoTracker, obtained from Molecular Probes, Inc.) were utilised; the CMX-rosamine form, which detects all mitochondria present in the cell, and CXXRos-H_2, the reduced form of the dye, which must be oxidised by functional mitochondria in

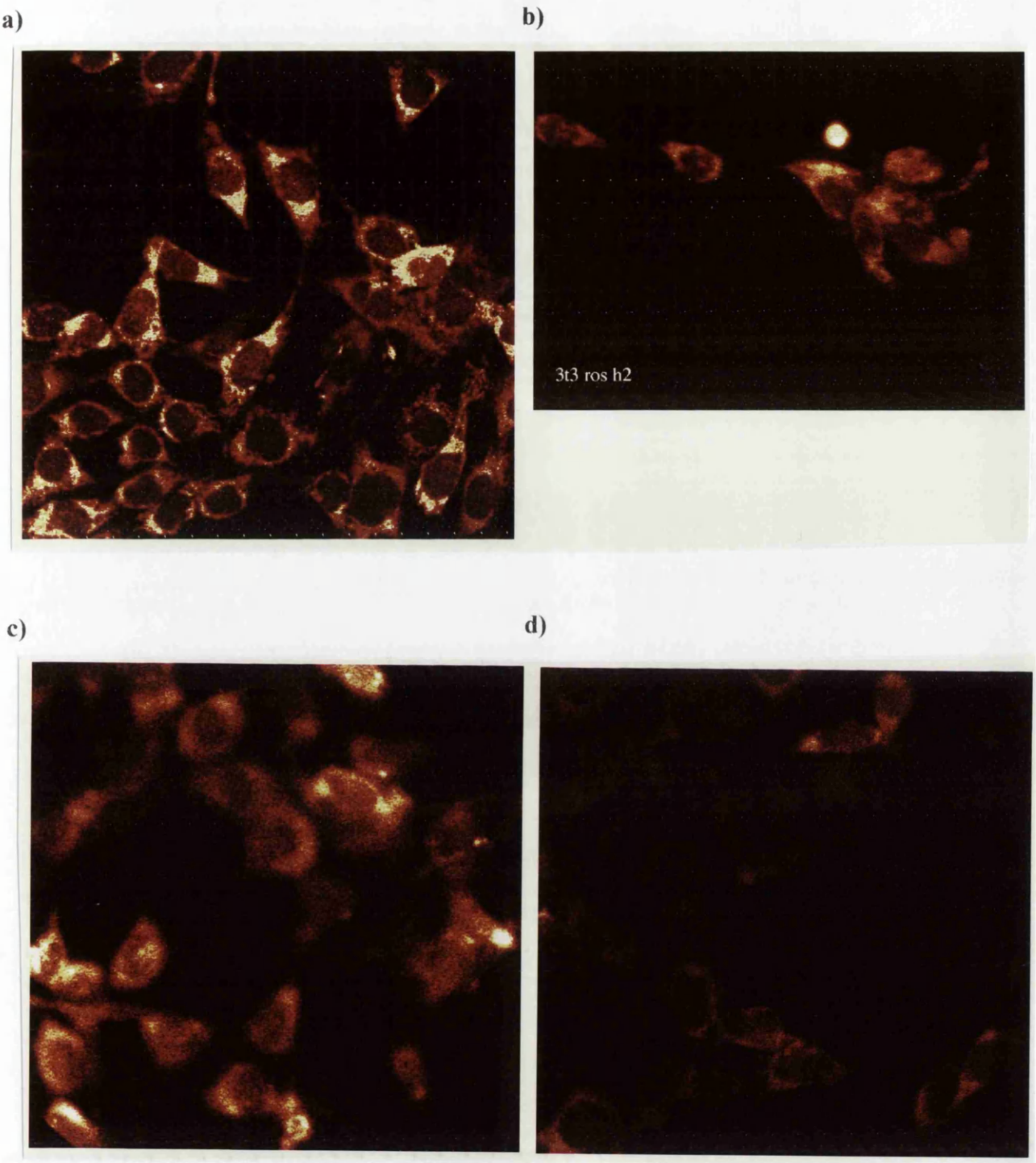


Figure 5.8 Labeling of 3T3 and PIZ8.3 cells with mitochondrial-specific dyes

Cells were cultured in galactose medium with dexamethasone. Panels a and b show 3T3 cells, panels c and d show PIZ8.3 cells. Panels a and c were labeled with CMX-rosamine, the oxidised form of the mitochondrial-specific dye, which detects all mitochondria present in the cell. Panels b and d were labeled with CXXRos-H_2, the reduced form of the mitochondrial-specific dye, which detects only functionally respiring mitochondria.

order to fluoresce. A comparison of fig. 5.8.a (3T3pM cells stained with the oxidised form of the dye) and fig. 5.8.b (3T3pM cells stained with the reduced form of the dye) suggests that there is little difference in the degree of fluorescence emitted from the cells. This indicates that in the control cell-line the mitochondria are fully functional. The degree of fluorescence in fig. 5.8.c (PIZ8.3 cells stained with CMX-rosamine) indicates that mitochondria are present at a level at least equivalent to that of the control cell line. However, a comparison of fig. 5.8.c and fig. 5.8.d (PIZ8.3 cells stained with CXXRos-H_2) indicates that there is a substantial difference in the degree of fluorescence emitted from the cells using the two forms of the dye. This indicates that respiration in the PIZ8.3 cell-line is not functioning at the same capacity as in the control cell-line. Due to the different intensities of signal emanating from different cells in panel 5.8.d, it would appear that not all cells are equally affected by PIZ expression. Data presented in section 5.2.3 suggested that PIZ8.3 cells cultured in galactose + dexamethasone (a condition designed to induce respiratory stress) may be losing copies of the transgene. It is possible that this occurs in some cells, but not all; the lower level of transgenic protein produced in the cells which decrease the copy number of the transgene may enable these cells to maintain a higher level of respiratory activity, thus explaining the differing degrees of fluorescence observed.

5.2.6 Galactose assay for respiratory competence

As described in section 5.1.4, galactose can be used as the sugar source in tissue-culture media to assay for the respiratory competence of mammalian cells. The four media conditions used in these assays were "superglu" without dexamethasone, "superglu" with dexamethasone, galactose without dexamethasone, and galactose with dexamethasone. These four conditions were intended to test the effect of transgene expression in low and high respiratory stress conditions. 1 x 10^5 cells were plated in 25 cm^2 flasks, for each of the four media conditions to be tested. The cells were harvested and counted after 4 days of growth. The data presented in Table 5.1

displays the number of assays performed for each cell line, and the average number of cells harvested in each condition. Appendix 1 lists the raw data for each cell line.

	3T3	3T3/pM	PIZ8.1	PIZ8.2	PIZ8.3	PIZ8.5	PIZ8.6
n	2	5	3	2	3	3	1
s/g	4.15+/-0.16	3.23+/-0.45	5.00+/-0.31	5.41+/-0.05	2.75+/-0.17	2.48+/-0.10	3.94
s/g+dex	4.35+/-0.16	3.16+/-0.54	4.64+/-0.38	4.91+/-0.51	2.32+/-0.29	2.28+/-0.10	3.76
gal	1.86+/-0.07	1.61+/-0.59	1.98+/-0.18	2.38+/-0.01	1.27+/-0.31	1.26+/-0.10	1.99
gal+dex	2.06+/-0.07	1.62+/-0.36	1.45+/-0.30	1.97+/-0.16	0.88+/-0.22	1.03+/-0.12	1.60

Table 5.1 Growth assays performed on 3T3-derived cell lines

n denotes the number of assays performed on the respective cell line. The following four rows denote the mean number of cells x 10^6 harvested when grown in the media condition specified for four days, +/- standard deviation of the sample. As clone PIZ8.6 was only tested on one occasion, there is no s.d. for these samples.

Glucocorticoids are reported to inhibit the proliferation of fibroblasts (Ramalingam *et al.*, 1997, Stewart *et al.*, 1995); however, this was not observed in the samples taken from either the 3T3 or 3T3pM control cell lines. Although the rate of growth of the vector-transfected control cell line 3T3pM is not altered by the presence of dexamethasone, it is noted that the growth rate in three of the medium conditions tested is slower than that of three PIZ clones tested (PIZ8.1, PIZ8.2 and PIZ8.6). It is possible that the observed difference in growth rate in the minimal stress medium ("superglu" without dexamethasone) is due to some positional effect of the transgenic insertion in this clone. For this reason, comparison of cell numbers harvested between clones is less informative than comparing the relative rates of growth in differing media conditions for individual clones. Thus, a *t*-test statistical analysis of the data presented in table 5.1 was performed, in which the effect of dexamethasone (which induces transgene expression in PIZ-transfected clones) on the rate of cell growth was assessed.

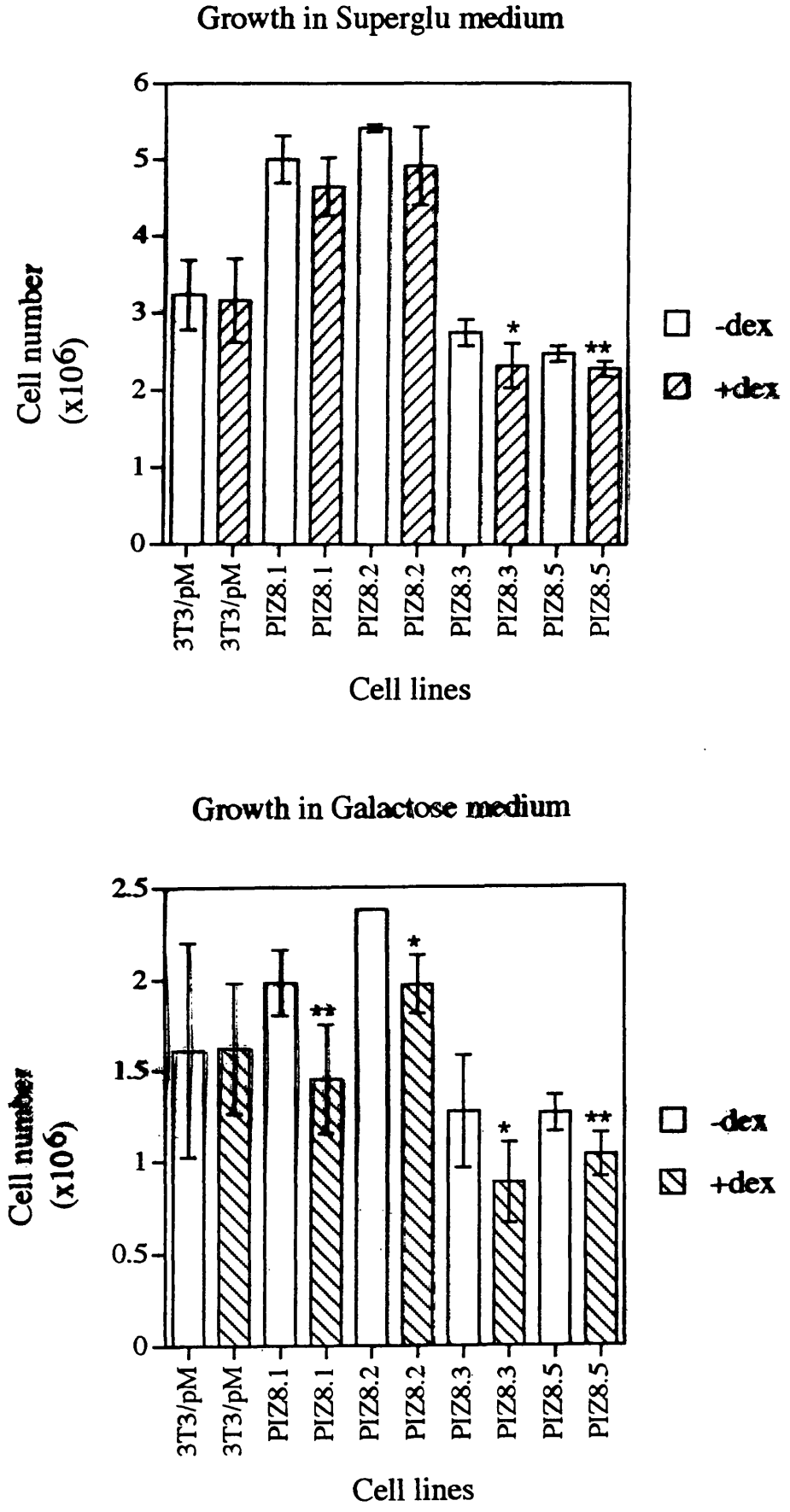
A *t*-test is the standard statistical method for analysing data from two independent samples with respect to one variable. The assumptions made in choosing a *t*-test are that the samples have been drawn from normal populations with equal variance and with measurements on an interval scale. The *t*-test is applicable when the sample size is small, because *t* takes the number of observations in the sample into account. The test is executed with respect to the null hypothesis, i.e., the variable tested has no effect on the populations tested. In this case, the null hypothesis is that induction of transgene expression will have no effect on the rate of growth of the cells in culture. The value for *t* obtained by the test is a deviation from the mean expressed in units of standard deviation. By taking the degrees of freedom into account (which is determined by the two sample sizes) one can determine the statistical probability of whether the two samples could have been obtained from the same population. In general, if the probability (p) obtained is less than or equal to 5%, then the null hypothesis can be rejected in favour of the experimental hypothesis that the variable tested does indeed have a statistically significant effect. A further consideration is whether to apply a one-tailed or two-tailed criterion to the value of *t* obtained for the samples compared. A one-tailed test can be used where the experimental hypothesis is directional. In this case the prediction is directional, because the expectation is that expression of the PIZ protein will have a detrimental effect on respiratory function, thereby decreasing the rate of cell growth.

As can be seen from table 5.2, untransfected 3T3 cells and the vector-transformed cell line, 3T3pM, displayed no significant alteration in growth rate, as measured by the t-test, upon the addition of dexamethasone to the culture medium, whether the medium contained glucose or galactose as the carbon source. For clones PIZ8.1 and PIZ8.2 (two of the PIZ transfected clones which grew rapidly in "superglu" medium as compared to the control cell lines) there is no significant reduction in growth rate when transgene expression is induced in this low respiratory stress condition (glucose

	Media	n	x	s	t	df	p
3T3	s/g	2	4.15	0.16			
	s/g+dex	2	4.35	0.16	0.50	2	N/S
	gal	2	1.86	0.07			
	gal+dex	2	2.06	0.07	2.00	2	N/S
3T3/pM	s/g	5	3.23	0.45			
	s/g+dex	5	3.16	0.54	0.18	8	N/S
	gal	5	1.61	0.59			
	gal+dex	5	1.62	0.36	0.03	8	N/S
PIZ8.1	s/g	3	5.00	0.31			
	s/g+dex	3	4.64	0.38	1.04	4	N/S
	gal	3	1.98	0.18			
	gal+dex	3	1.45	0.30	2.16	4	$p<0.02$
PIZ8.2	s/g	2	5.41	0.05			
	s/g+dex	2	4.91	0.51	0.97	2	N/S
	gal	2	2.38	0.01			
	gal+dex	2	1.97	0.16	2.67	2	$p<0.05$
PIZ8.3	s/g	3	2.75	0.17			
	s/g+dex	3	2.32	0.29	1.80	4	$p<0.05$
	gal	3	1.27	0.31			
	gal+dex	3	0.88	0.22	1.44	4	$p<0.05$
PIZ8.5	s/g	3	2.48	0.10			
	s/g+dex	3	2.28	0.10	1.97	4	$p<0.02$
	gal	3	1.26	0.10			
	gal+dex	3	1.03	0.12	2.03	4	$p<0.02$

Table 5.2 t-test analysis of the effect of transgene expression on cell growth

This t-test tests the null hypothesis that transgene expression has no effect on cell growth. Cell lines are as described previously. Media conditions are: s/g ("superglu"), s/g+dex ("superglu" with dexamethasone), gal (galactose), gal+dex (galactose with dexamethasone). n denotes the number of assays performed on the respective cell line. x denotes the mean number of cells x 10^6 harvested when grown in those media conditions for four days. s indicates standard deviation. The value for t has been calculated to compare the growth of a cell line in the respective media with or without dexamethasone. "df" indicates the degrees of freedom for the respective calculation. The probability (p) is given for the premise that the 2 respective samples have arisen from the same population, using values for a 1-tailed test. N/S indicates not significant.



Graph 5.1 Graphical presentation of data in Table 5.2

medium). The value for *t* obtained for these two cell lines in galactose medium indicates that transgene expression does significantly decrease the rate of growth in high respiratory stress conditions. This would suggest that the PIZ protein is detrimental to respiration. However, the standard deviations for the samples which were compared for clone PIZ8.2 appear to be disparate, so a variance ratio test is performed below to assess the validity of applying a *t*-test to this data. For clones PIZ8.3 and PIZ8.5 there is a statistically significant difference in the samples obtained for the two conditions tested (with or without dexamethasone) in both media. Thus the expression of PIZ appears to be detrimental to cell growth in these two cell lines whether the cells are in a low or high respiratory stress environment. A possible explanation of why two clones are affected by transgene expression in a low respiratory stress environment, whilst the other two are not, is the level of expression of the transgene. Whether because of a positional effect of the transgene insertion site, or due to copy number if multiple integrations of the transgene have occurred, it is likely that the clones PIZ8.3 and PIZ8.5 are expressing the gene product at a higher level than clones PIZ8.1 and PIZ8.2.

The variance ratio test is used in conjunction with the *t*-test. It is used to compare the standard deviations of samples which contain fewer than 30 observations, in order to assess whether the samples are so different in variance that the t-test cannot be used reliably with the sampled data. As with the t-test a probability (p) of less than or equal to 5% is considered significant. There was no need to test the samples for the 3T3 cell line because the standard deviations for the respective samples were equal. The variance ratio test results indicate that all samples compared, apart from clone PIZ8.2 in galactose medium, are similar enough in dispersion for the *t*-test results to be accepted within the standard limits of probability, i.e., the two samples compared have a 95% probability of having been sampled from populations of equal or very similar variance. The F-test of clone PIZ8.2 in galactose medium indicates there is a 5% probability that the two samples are from populations heterogeneous in

	Media	Variance	F-value	df	p=0.05
3T3/pM	s/g	0.2007			
	s/g+dex	0.2927	1.5	4x4	N/S
	gal	0.3422			
	gal+dex	0.1318	2.6	4x4	N/S
PIZ8.1	s/g	0.0973			
	s/g+dex	0.1467	1.5	2x2	N/S
	gal	0.0313			
	gal+dex	0.0876	2.8	2x2	N/S
PIZ8.2	s/g	0.0020			
	s/g+dex	0.2601	130.1	1x1	N/S
	gal	0.0001			
	gal+dex	0.0241	240.5	1x1	significant
PIZ8.3	s/g	0.0285			
	s/g+dex	0.0853	3.0	2x2	N/S
	gal	0.0955			
	gal+dex	0.0488	2.0	2x2	N/S
PIZ8.5	s/g	0.0098			
	s/g+dex	0.0097	1.0	2x2	N/S
	gal	0.0104			
	gal+dex	0.0153	1.5	2x2	N/S

Table 5.3 Variance ratio test for the data presented in table 5.2

The variance ratio test (or F-test) tests the assumption of homogeneity of variance of the 2 sets of data compared in the t-test. The comparison is made between samples for a cell line in the respective media, with or without dexamethasone, the hormone which induces transgene expression in transfected cell lines. Cell lines are as described previously. Media conditions are: s/g ("superglu"), s/g+dex ("superglu" with dexamethasone), gal (galactose), gal+dex (galactose with dexamethasone). The F-value has been calculated to compare the variances (s^2) obtained for the respective samples. "df" indicates the degrees of freedom for the respective calculation. The variance ratio is considered with respect to a probability (p) that there is a less than or equal to 5% chance that the 2 respective samples were drawn from populations heterogeneous in variance. (N/S indicates not significant, i.e., the populations considered are 95% probable to be homogeneous for variance).

dispersion. However, the value calculated for *t* when comparing these two samples is more than one unit of standard deviation above the tabulated value for *t* which gives p=0.05. Thus, the results of the *t*-test for PIZ8.2 should be considered with caution, rather than rejected outright; additional data should be collected for this clone.

All cell lines containing an intact transgene have displayed a reduction in growth rate in galactose medium in the presence of dexamethasone, whether an encoded transcript/protein has been detected or not. This suggests that the presence of the chimeric gene product (whether RNA or protein), even at levels below the detection threshold of the techniques used, may be detrimental to cell growth when growth is dependent upon mitochondrial respiratory pathways.

As discussed in section 5.1.4, it is estimated that HeLa cells obtain about 65% of their energy in normal media conditions from oxidative phosphorylation, and only 35% from glycolysis. Therefore, even in highly supplemented media it is likely that cells derive a proportion of their energy from aerobic respiration. Likewise, it is probable that 3T3 cells will also derive a proportion of their energy from aerobic respiration even in "superglu" medium, and it is therefore possible that were expression of PIZ significantly impairing respiration an effect on cell growth rate would be observed in the supplemented medium. Thus, it seems probable from the *t*-test that the expression of the transgene in clones PIZ8.3 and PIZ8.5 is having a detrimental effect on respiration even in the glucose medium. The rates of growth in galactose compared to "superglu" medium may reflect the relative contribution of aerobic respiration to cell viability

In order to perform a *t*-test analysis comparing growth of the cell-lines in galactose medium with growth in "superglu" medium, it was not possible to do a direct comparison, as the growth rate of the cells is so disparate in the two different media that even the control cell lines display a statistically significant slowing of the growth

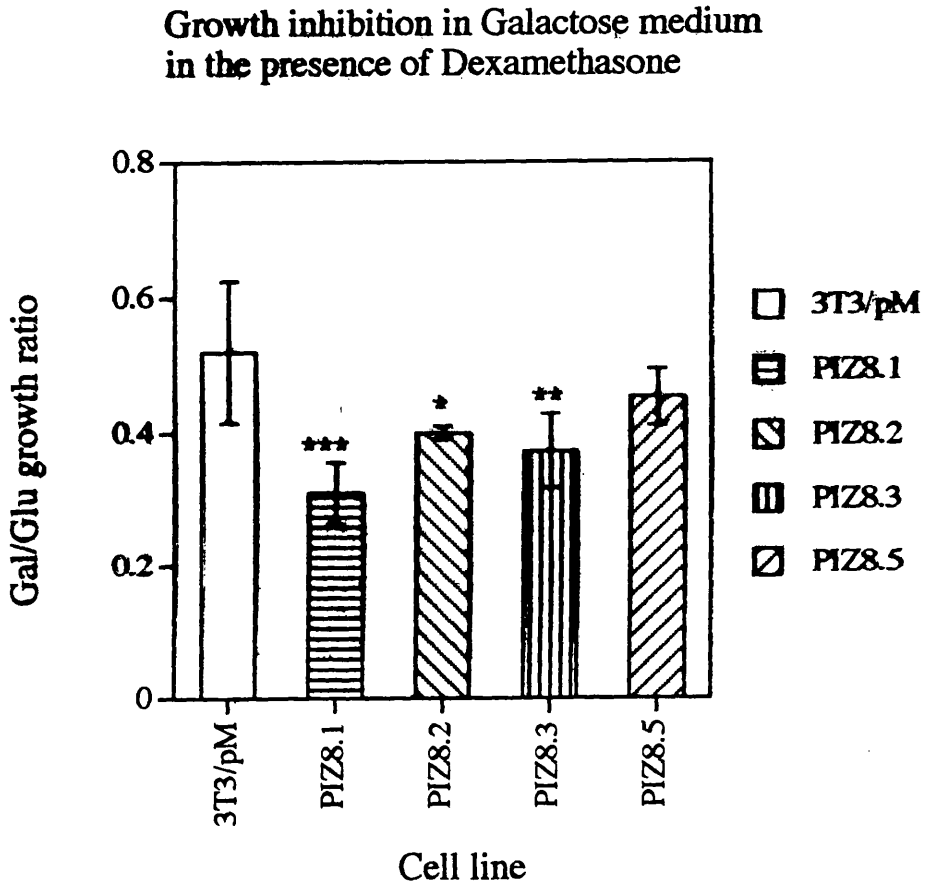
rate when galactose is substituted for glucose in the medium. Given that each time a cell growth assay was performed on a clone the initial step was to set up four identical flasks (prior to changing the medium to one of the four test media), it is appropriate to consider flasks in pairs. This enables the calculation of a value for growth rate which represents the proportion of cells growing under conditions subject to one test variable, the carbon source (galactose versus "superglu"). Thus, for each assay performed with each clone, the number of cells which had grown in galactose medium (without dexamethasone) was divided by the number of cells which had grown in "superglu" medium (without dexamethasone) to attain a ratio of cell growth for the two test conditions. These calculations were also performed to find the ratio for growth in the presence of dexamethasone. The ratio values could then be utilised to perform a *t*-test comparing a clone's relative rate of growth (either in the presence or absence of dexamethasone) to that of the control cell line. If the relative rate of growth of the PIZ clones differs significantly from that of the 3T3pM control cell line in the presence of dexamethasone, but not in the absence of dexamethasone, this would imply that PIZ expression inhibits growth to a greater degree in the high respiratory stress medium than in the low respiratory stress medium. This would suggest that PIZ expression is directly affecting respiration in the patient analagous cell lines.

Table 5.4 displays the results of the *t*-test analysis performed on the data as described above. For all four PIZ clones there is no statistically significant difference in the relative rate of growth of the clones when grown in the absence of dexamethasone when compared to 3T3pM. For three of the clones (PIZ8.1, PIZ8.2, and PIZ8.3) there is a significant difference in the ratio of growth in the presence of dexamethasone. This supports the hypothesis that PIZ expression inhibits growth to a greater degree in the high respiratory stress medium than in the low respiratory stress medium. The statistic for clone PIZ8.2 also indicates that expression of the chimeric peptide at a level below the detection threshold of the western analysis (fig. 5.11.a) is detrimental

	Dex	n	x	s	t	df	p
3T3/pM	without	5	0.492	0.134			
PIZ8.1	without	3	0.400	0.059	0.977	6	N/S
3T3/pM	with	5	0.520	0.105			
PIZ8.1	with	3	0.310	0.046	2.852	6	$p<0.002$
3T3/pM	without	5	0.492	0.134			
PIZ8.2	without	2	0.440	0.010	0.459	5	N/S
3T3/pM	with	5	0.520	0.105			
PIZ8.2	with	2	0.400	0.010	1.368	5	$p<0.05$
3T3/pM	without	5	0.492	0.134			
PIZ8.3	without	3	0.457	0.098	0.342	6	N/S
3T3/pM	with	5	0.520	0.105			
PIZ8.3	with	3	0.373	0.055	1.951	6	$p<0.01$
3T3/pM	without	5	0.492	0.134			
PIZ8.5	without	3	0.510	0.022	0.201	6	N/S
3T3/pM	with	5	0.520	0.105			
PIZ8.5	with	3	0.453	0.042	0.918	6	N/S

Table 5.4 ***t*-test comparing the proportional growth rates of the PIZ clones in two media to the control cell line 3T3pM, either with or without dexamethasone**

The statistical measure of *t* is described previously. In this test the analysis considers whether incubation in galactose medium inhibits growth of the PIZ clones to a greater extent when expression of the transgene is induced than when it is not. A direct comparison of the cell counts in galactose medium to "superglu" medium is not possible, as even the control cell line grows significantly slower in galactose medium. Therefore, for each sample taken in each cell line the cell count in galactose medium was divided by the cell count in "superglu" medium to arrive at a proportional rate of growth. This was also done comparing the cell count in galactose medium with dexamethasone to the cell count in "superglu" with dexamethasone in the medium. The *t*-test above compares this proportional rate of growth of an individual PIZ clone to the control cell line 3T3pM in order to assess whether there is a significant difference in how dexamethasone affects the rate of growth. Cell lines are as described previously. The growth conditions are either with or without dexamethasone (Dex). n denotes the number of assays performed on the respective cell line. x denotes the mean of the proportional counts for the various cell lines in the respective media. s indicates standard deviation. The value for *t* has been calculated to compare the proportional rate of growth of a PIZ clone to that of the control cell line 3T3pM in media either containing dexamethasone (the hormone which induces transgene expression) or without. "df" indicates the degrees of freedom for the respective calculation. The probability (p) is given for the premise that the 2 respective samples have arisen from the same population, using values for a 1-tailed test. A 1-tailed test is valid because the prediction for the effect of dexamethasone on cell growth was directional, i.e., that the induction of transgene expression would be detrimental to cell growth. A probability of less than or equal to 5% is considered significant, indicating that the null hypothesis can be rejected. (N/S indicates not significant).



Graph 5.2 Graphical presentation of data in Table 5.4

* indicates degree of significance: * = $p<0.05$; **=$p<0.01$; ***=$p<0.002$

to mitochondrial respiration. Although the *t*-test analysis of the data for the fourth clone, PIZ8.5, indicates that the results are not statistically significant, PIZ8.5 does display an apparent decrease in the ratio [rate of growth in galactose + dexamethasone over "superglu" + dexamethasone] when compared to the ratio [rate of growth in galactose - dexamethasone over "superglu" - dexamethasone]. Thus, this clone follows the trend set by the other three PIZ clones.

The variance ratio test is described previously (see Table 5.3). In order to assure the validity of the data presented in Table 5.4, an F-test was performed. The results are displayed in Table 5.5. The variance ratio test results indicate that all samples compared, apart from the proportional rate of growth for clone PIZ8.5 grown in the absence of dexamethasone, are similar enough in dispersion for the *t*-test results to be accepted within the standard limits of probability, i.e., the two samples compared have a 95% probability of having been sampled from populations of equal or very similar variance. The F-test of clone PIZ8.5 grown without dexamethasone in the medium compared to the control cell line 3T3pM indicates there is a 5% probability that the two samples are from populations heterogeneous in dispersion. Thus, the *t*-test data for those samples is not reliable. However, the *t*-test data for clones PIZ8.1, PIZ8.2, and PIZ8.3 is reliable within the limits of the statistical analysis.

In the studies performed by Chu *et al.,* (1972, 1974*)* mutants incapable of growth by respiration were selected, using the galactose medium. The phenotype observed here is much less severe, as the transgenic clones can continue to grow and divide, but is nevertheless suggestive of a deficiency in aerobic respiration, associated with the expression of the transgene. I would suggest that the data presented in this section support the conclusion that there is a detrimental effect caused by PIZ expression, and that it is mediated by inhibiting aerobic respiration.

	Dex	Variance	F-value	df	p=0.05
3T3/pM	without	0.0179			
PIZ8.1	without	0.0035	4.29	4x2	N/S
3T3/pM	with	0.0110			
PIZ8.1	with	0.0021	4.41	4x2	N/S
3T3/pM	without	0.0179			
PIZ8.2	without	0.0001	111.60	4x1	N/S
3T3/pM	with	0.0110			
PIZ8.2	with	0.0001	68.50	4x1	N/S
3T3/pM	without	0.0179			
PIZ8.3	without	0.0095	1.57	4x2	N/S
3T3/pM	with	0.0110			
PIZ8.3	with	0.0030	3.01	4x2	N/S
3T3/pM	without	0.0179			
PIZ8.5	without	0.0005	31.88	4x2	significant
3T3/pM	with	0.0110			
PIZ8.5	with	0.0017	5.30	4x2	N/S

Table 5.5 Variance ratio test for the data presented in table 5.4

The purpose of the variance ratio test is described previously. The comparison is made between the specified PIZ cell line and the 3T3pM control line. Cell lines are as described previously. Data is for the proportional rates of growth in galactose medium/"superglu" medium, either with or without dexamethasone as specified. The F-value has been calculated to compare the variances (s^2) obtained for the respective samples. "df" indicates the degrees of freedom for the respective calculation. The variance ratio is considered with respect to a probability (p) that there is a less than or equal to 5% chance that the 2 respective samples were drawn from populations heterogeneous in variance. (N/S indicates not significant, i.e., the populations considered are 95% probable to be homogeneous for variance).

5.3 Discussion

The work described in this chapter presents the evidence that the Patient I constructs, nuclear versions of a chimeric mitochondrial gene, were successfully expressed in mammalian cells. It goes on to analyse the effect that the expression of these chimeric mitochondrial proteins had on cell phenotype.

Using PCR it was demonstrated that selected G418-resistant clones had incorporated the PI construct DNA. Northern analysis of these clones was inconclusive. The putative PI transcript was obscured by the endogenous COXI transcript due to upregulation of mitochondrial transcription caused by the transgene induction agent dexamethasone, and the choice of a COXI fragment as template for the synthesis of the probe. Western analysis of these clones using rabbit polyclonal antisera raised against a peptide specific to the Patient I polypeptide yielded no positive data. As the rabbit antisera had also been unsuccessful in detecting the PI polypeptide in a bacterial expression system, (see chapter 4), the remainder of the project focussed on the PIZ clones.

Using PCR it was demonstrated that selected G418-resistant clones had incorporated the PIZ constructs with appropriate vector-encoded expression signals intact. Northern analysis of the cell lines suggests that the transgene was being expressed in clones PIZ8.2, PIZ8.3 and possibly PIZ8.5. It would also appear that the use of the glucocorticoid dexamethasone as the induction agent for expression of the transgene has increased the rate of mitochondrial transcription in the cells, leading to an increased level of endogenous mitochondrial transcripts and partially processed transcripts which cross-hybridise to the probes used to detect the transgene transcript. To confirm the identity of the putative transgene transcript, and to clarify whether the other PIZ-transfected cell-lines are expressing the transgene, the northern analysis

would need to be repeated using an oligonucleotide probe which was transgene-specific.

Analysis of total DNA prepared from cell lines 3T3pM, PIZ8.3 and PIZ8.5 suggest that the clone PIZ8.3 harbours multiple copies of the transgene PIZ. A multiple integration event is supported by the observation that clone PIZ8.3 exhibited a band of greater intensity than the other three PIZ clones for the putative neomycin phosphotransferase transcript when the northern blot was hybridised to a probe containing the SV40 early splicing region which detected the 3' untranslated RNA of this transcript. The analysis by slot blot also indicates that under growth conditions which increase the requirement for respiration (galactose as the sugar source), clone PIZ8.3 is apparently deleting some copies of the transgene. This would support the conclusion that expression of the transgene is detrimental to respiration and cell survival or growth. Although no apparent change in the ratio of mitochondrial to nuclear DNA was observed when the expression of PIZ was induced, several possibilities are still consistent with the expression of a chimeric peptide, encoded by mutant mtDNA, being pathogenic. It is possible that additional factors, perhaps tissue-specific, are required to signal to the nucleus that mtDNA replication is an appropriate response to respirational deficiency, or, that the threshold of respiratory insufficiency has not been reached which will stimulate the proliferation response. Perhaps the proliferation of mutant mtDNA molecules escalates in proportion to the initial depletion of wildtype mtDNA molecules (the hypothetical replicative advantage of the smaller genome) and, as only one form of the mitochondrial genome is present in this study, no change will occur in copy number. It is also probable that insufficient time was allowed to observe a significant change. If this experiment were to be repeated, one would include all PIZ clones used in the statistical analysis. One would also use all 4 media conditions, rather than the 2 extremes only. Finally, it would be best to have access to a phosphoimager, or similar device, for analysis of the results.

Western analysis of the PIZ clones using the monoclonal antibody Z1F11, which recognises the carboxy terminal epitope tag of the PIZ protein, detects what appears to be a doublet of less than 14 kDa in clones PIZ8.1, PIZ8.3 and PIZ10.3 (fig. 5.11). The appearance of two bands in the approximate size range for PIZ suggests that not only is the protein being expressed, but that the amino-terminal peptide is being cleaved - indicating that PIZ is being imported into the mitochondria. The doublet is not apparent in the control cell line 3T3pM, but it is not apparent in some of the other PIZ clones either (e.g., PIZ8.2, which does express the putative transgene mRNA). It is formally possible that the proteins represented by the two bands are below the detection threshhold in all cell-lines tested other than PIZ8.1 and PIZ8.3, including 3T3pM. In order to test whether 3T3pM was expressing proteins in this size range, and to try to isolate PIZ from cross-reacting proteins, immunoprecipitation utilising the Z1F11 monoclonal antibody was performed on clones 3T3pM, PIZ8.3 and PIZ8.5 (fig. 5.12). Although the signal to noise ratio of the blot is far from optimal, it appears that there is a diffuse band of less than 14 kDa in size, specific to the lanes containing PIZ8.3 and PIZ8.5 cellular proteins. This supports the conclusion that the doublet apparent in fig. 5.11 is specific to the PIZ clones.

To confirm the identity of the putative PIZ protein bands, the western analysis would need to be repeated, using additional techniques to attempt to reduce background signal. Such techniques could include a gel-system which yields better resolution of low molecular weight proteins, alternative detergents, or altering the pH at which incubation with the antiserum was performed. Alternatively, it would be necessary to obtain a better antibody, as discussed in section 4.3. Although the western blots presented do not independently confirm the expression of PIZ, the data discussed below supports the interpretation that the transgene protein is being expressed in the PIZ clones.

Fluorescence microscopy of transfected 3T3 cells stained with mitochondrial-specific dyes (section 5.2.5) supports the conclusion that PIZ was being expressed in cell-line PIZ8.3, and that expression of the protein inhibits respiratory activity. The apparent variance in respiration between individual PIZ8.3 cells could be due to loss of copies of the transgene. Although these results are preliminary, they are intriguing, and warrant further investigation. An additional control could be added by using a ρ^0 derivative of the cell line (if it were available) to ensure that cells known not to be functionally respiring exhibited no staining with the reduced form of the dye.

In order to test whether the chimeric peptide conveyed a respiratory phenotype on the 3T3 cell line, assays of growth in galactose medium were performed. The cell lines were grown in four different media conditions, which should create high or low stress environments for the cells depending on the carbon source and the presence or absence of the inducing agent for expression of the transgene. Two *t*-tests were performed on the data obtained; the first *t*-test utilised cell numbers from individual clones grown in either glucose or galactose medium and compare them for the presence/absence of dexamethasone as the independent variable. The presence of dexamethasone in the medium had no significant effect on cell numbers for the control cell line 3T3pM in either galactose or glucose medium. Clones PIZ8.1 and PIZ8.2 showed no significant effect of dexamethasone induction of transgene expression in glucose medium, but in galactose medium (which requires a greater proprtion of cellular energy to be derived from respiration) the induction of PIZ expression did reduce the growth rate significantly. However, a variance ratio test indicated that the results for clone PIZ8.2 are questionable. Clones PIZ8.3 and PIZ8.5 demonstrated a significant reduction in growth rate in both glucose and galactose media in the presence of dexamethasone. Thus, expression of the transgene does appear to inhibit growth, but it could not be attributed specifically to inhibition of respiratory activity.

The second *t*-test was intended to analyse for inhibition of respiratory activity directly. None of the four clones tested differed significantly in the proportional rate of growth (galactose/"superglu") from the control cell line when dexamethasone was absent from the growth media. However, when expression of PIZ was induced, the proportional rate of growth for clones PIZ8.1, PIZ8.2 and PIZ8.3 was significantly reduced in comparison to 3T3pM. Because the numerator in this function (growth in galactose medium) relies to a greater degree on respiratory activity than the denominator (growth in glucose medium), this suggests that PIZ is specifically inhibiting growth via the inhibition of respiration.

However, a possible difficulty with the analysis of the PIZ clones has become apparent in the course of the work. In the introduction to this chapter I stated that the advantage of the approach chosen for analysis of PIZ was that by expressing the protein transgenically in a controlled fashion any observed phenotype could be attributed to a single variable. It is now apparent that the agent which enabled the controlled expression of the transgene has a direct effect on mitochondrial transcription. Experiments have shown that dexamethasone treatment of a rat hepatoma cell line led to a 3- to 4-fold increase in mitochondrial RNA levels (including COXI mRNA specifically), without changing the levels of mitochondrial DNA in the cells. An increase in the levels of incompletely processed precursors was also detected. These observations were attributed to increased levels of transcription (Van Itallie, 1990 and 1992). Steroid hormones may act directly on mitochondrial gene transcription via steroid receptors which bind to hormone response elements. The sequences of hormone response elements from human and mouse mitochondrial genomes confered dexamethasone-inducibility on hybrid reporter consructs (Demonacos *et al.*, 1996). Gel retardation assays have shown that glucocorticoid response elements from cytochrome *c* oxidase I and III genes, and from within the D-loop region, all bind purified glucocorticoid receptor, as well as protein from

mitochondrial extracts which is apparently the glucocorticoid receptor (Demonacos *et al.*, 1995).

Thus, it is possible that the increased rate of mitochondrial transcription induced by the presence of dexamethasone is placing additional demands on the resources of the mitochondria, and on the expression mechanism. If, for instance, the presence of an abnormal polypeptide such as PIZ interferes with mitochondrial activity by interfering with a component of the translation mechanism (e.g., binding available chaperonins) then the effects of the two factors - dexamethasone and PIZ - cannot be disentangled. However, as discussed in the previous two paragraphs, because the PIZ clones demonstrate a statistically significant reduction in growth in galactose +dexamethasone/"superglu" + dexamethasone when compared to the 3T3pM control cell line, and the level of staining with CXXRos-H_2 differs dramatically between the two cell lines, it suggests that the presence of the abnormal polypeptide is inhibiting growth via the inhibition of respiration directly, whatever the precise mechanism.

Another possible objection to the interpretation of the data is the presence of the carboxy terminal epitope tag. The statistical analysis of the cell growth assays indicates that PIZ expression is detrimental to respiration. Although epitope-tagging is a commonly used technique, PIZ is an amended version of the Patient I protein. To be certain that the Z1F11 epitope tag is not contributing to the phenotype of respiratory deficiency, the cell growth assays should preferably be repeated utilising PI-transfected clones which have been shown to express the transgene by northern analysis.

A third potential objection involves the transit peptide. The mitochondrial targeting peptide from *Neurospora crassa* COXIV increases permeability of the inner membrane from isolated rat liver mitochondria (Sokolove and Kinnally, 1996). The authors suggest that this is attributable to the creation of a pore in the inner

membrane. It is possible that overexpression of a targeted protein to mitochondria would produce a similar effect. However, as a precaution because of the possible detrimental effects of overexpression of amphipathic molecules, in the induction of transgene expression, the lowest concentration of the inducing agent recommended by the suppliers of the expression vector was used for the galactose assays. Additionally, in the cellular environment, cytosolic chaperones would be available to escort the transgenic protein to the mitochondral import apparatus, making random insertion into the mitochondrial membranes less likely. Because a doublet was detected by western analysis, at least some of the transgenic protein appears to have been processed, and therefore this proportion of the transit peptide has entered the matrix. In order to control for a detrimental effect of (overexpression of) the transit peptide, one could simply use the COXIV protein as a passenger, presuming it to be "nontoxic". Although the targeting peptide from another nuclear-encoded mitochondrial protein could be used to target PIZ (as it is essential to target nuclear-encoded proteins), there is no reason to presume that another transit peptide would have an effect which differed from the COXIV transit peptide.

In summary, data have been presented that are consistent with expression of a mitochondrial fusion gene in several clonal cell lines transfected with construct PIZ. Preliminary fluorescence data suggested that mitochondrial membrane potential was decreased in the one PIZ clone examined, compared to a control. The statistical analysis of the cell growth assays using alternative carbon sources as respiratory substrates implied that PIZ expression inhibited respiratory function. This negative growth phenotype represents good, albeit indirect, evidence that the inserted mitochondrial transgene was transcribed, translated, and targeted to mitochondria.

Chapter 6

Concluding remarks

6.1 The approach adopted for this work

Based on the arguments presented in section 1.5.6, it was proposed that a novel protein encoded by a chimeric mitochondrial gene created by a rearrangement event in mitochondrial DNA could contribute to respiratory deficiency in affected patient tissues. The aim of this project was to create a model system in cultured mouse fibroblast cells which could be used to investigate the effect such a chimeric mitochondrial polypeptide might have on respiration. The strategy adopted required the relocation of a chimeric mitochondrial gene to the nucleus. In this way any observed phenotype could be attributed specifically to the presence of the protein, as opposed to the rearranged mtDNA or chimeric transcripts, in the mitochondria.

In my analysis of the phenotype attributable to the novel protein, the constructs were placed under the transcriptional control of an inducible promoter. This is an advantage over alternative approaches such as the culturing of patient-derived cell lines, or the creation of cybrids harbouring patient-derived mitochondria, in which the mitochondrial protein will be constitutively expressed. In some cases researchers have reported difficulty in generating stable cell lines harbouring mtDNA with partial duplications, using the two techniques described (Poulton *et al.*, 1993, Holt *et al.*, 1997). The ability to inhibit expression whilst a cell-line is being established enables one to clearly establish whether expression of the chimeric protein is associated with any observed phenotype. Transfection into a standard cultured cell-line reduces the possibility of an unknown mitochondrial defect confusing the interpretation of the phenotype, nor is there the risk of incompatibility between the nuclear and mitochondrial genomes. Lastly, there is no restriction on the recipient cell lines which can be used for further analysis, unlike the other two approaches which are restricted to patient-derived cell lines which can be cultured from primary tissue, or to those ρ^0 cell lines that are available as recipients for cybrid formation.

6.2 A summary of the work presented

I have described in chapter 3 the mutagenesis and subcloning procedures required to convert the mouse COXI gene into a nuclear version; 43 point mutations for the full-length gene, synthesis and subcloning of a transit-peptide encoding region. Also described are the synthesis and subcloning of construct-specific coding regions for PI and PIZ which encode a peptide, in untagged and epitope-tagged forms respectively, which is analagous to a putative patient fusion peptide encoded by rearranged mtDNA in a specific patient.

In chapter 4 I have described the analysis of the PI and PIZ constructs in a prokaryotic expression vector using the antisera available. The time-course growth analysis suggested that both pPI/Q and pPIZ/Q constructs were being expressed in the bacterial system, and that expression of both PI and PIZ was highly toxic to *E. coli.* This may have been due to interference with assembly/activity of the bacterial cytochrome *c* oxidase, and/or detrimental effects of the amphipathic transit peptide on membrane integrity. Neither PI nor PIZ, when expressed in *E. coli,* was detected by any of the available antisera above background levels. Possibly the recombinant protein was degraded very rapidly in *E. coli*, and was not present in sufficient quantity to be detected on a western by either antibody. It is also possible that the proteins were insoluble in the conditions used. *In vitro* translation suggested that PIZ was synthesised at low levels by eukaryotic cytosolic ribosomes, whilst evidence for the translation of PI was inconclusive. All of the available antisera cross-reacted to other low molecular weight proteins, so that no reaction to the PI or PIZ proteins could be confirmed on western blots.

In chapter 5 I have presented data showing that the PIZ construct was successfully transfected into 3T3 cells, generating multiple, clonal cell-lines. Northern and western analysis suggested that the PIZ constructs were expressed in most of these

clones. Staining of cells with mitochondrial-specific dyes indicated that respiration in the PIZ8.3 cell-line was not functioning at the same capacity as in the control cell-line. The assays of growth in galactose medium indicated that expression of the PIZ protein generates a phenotype indicative of respiration deficiency. The analysis of all the available data indicates that PIZ has been successfully expressed in several clones, and that expression of this protein produces a moderate respiratory deficiency.

6.3 Retrospective comments

My analysis of the cell-lines generated was frustratingly hindered by the lack of a specific antibody. The rabbit polyclonal antisera had been raised against a peptide derived from a novel region of the patient 1 chimeric mitochondrial gene. The level of cross-reactivity of the antisera was a problem, as was the apparent inability to detect the fusion protein. My anticipation of this possibility, by constructing an epitope-tagged version, did enable detection of the fusion protein in 3T3 cells. However, the high level of cross-reactivity was, once again, frustrating. In order to pursue any further research with these cell lines, and to validate the conclusions drawn from this work, a suitable antibody would be essential. A PI-specific antibody would be preferred, but the expression of the constructs in a prokaryotic expression vector was apparently highly toxic to the cells. As stated in section 4.3, possible approaches would be the removal of the transit peptide, and/or using a carrier protein such as DHFR. However, another possibility would be to express PI in an alternative expression system, such as Baculovirus infection of insect cells. This option might be more costly, but it could prove faster to optimise, given that expression in eukaryotic cells produced a much more subtle phenotype than in prokaryotic cells. A COXI antibody which recognises an epitope in the amino-terminal region would be sufficient for the PI- and PIZ-transfected cell-lines. If the PII construct is generated in the future, an anti-COXI antibody may not be sufficient for the analysis of these cell lines as the PII protein may have an apparent molecular weight similar to the native

COXI by SDS-PAGE. Alternatively, the PI construct could be tagged with a new epitope, and new cell lines generated for analysis. However, the level of cross-reactivity I experienced would not be acceptable. If I were to repeat this work, I would choose a commercially available antibody, with several publications to support the efficacy of the epitope-tag, preferably in a mitochondrial/hydrophobic membrane protein.

If another model chimeric mitochondrial gene was chosen, it may be simpler to synthesise it *de novo* from overlapping oligonucleotides, rather than subclone and mutagenise the mitochondrial version, although all eight mutations required to express the PI portion of COXI in the nucleus were achieved in one successful round of mutagenesis. The original rationale for using site-directed mutagenesis was 3-fold; firstly, the full-length COXI would be useful as a control, secondly, it could answer the academic point of whether COXI is capable of being synthesised in the cytosol and imported into mitochondria, and, thirdly, the PII model gene was also desired. If I were to begin the project anew, I would choose direct synthesis of the chimeric gene, although this approach also has its potential problems. Unpredicted alignments of oligonucleotides were observed in the construction of the transit peptide- and cytochrome *b*-encoding regions.

If more time had been available, it would have been desirable to repeat the microscopy experiments to assay respiratory activity using the Mitotracker dyes with other clones. Preliminary data suggested that expression of PIZ was detrimental to respiratory function, i.e., in the presence of dexamethasone, clone 3T3 PIZ8.3 exhibited staining comparable to the control cell line with the general mitochondrial dye, but a less intense staining with the mitochondrial dye which required respiratory activity to activate fluorescence. If a better antibody were available, it would be desirable to test for colocalisation of the chimeric protein with mitochondria by immunofluorescence labeling of the PIZ (or PI) clones. However, because of the high

level of cross-reactivity of the Z1F11 monoclonal antibody with other proteins expressed by the 3T3 cells in the presence of dexamethasone, it was impossible to ascertain the cellular location of PIZ by confocal microscopy.

6.4 What mechanism is responsible for the observed respiratory phenotype?

In order to test whether the respiratory deficiency observed is attributable to PIZ expression specifically, to chimeric mitochondrial proteins in general, or the result of the presence of an exogenous protein (any non-mitochondrial protein) inside mitochondria, further analysis is necessary. As mentioned in section 5.3, the galactose assays could be repeated using the PI-transfected cell lines to ascertain that the Z1F11 epitope tag is not contributing to the phenotype. The PII constructs, which can now be completed because the full-length nuclear COXI gene has been engineered, are potentially available to test whether another chimeric mitochondrial gene would produce the same phenotype. Alternatively, another model chimeric gene could be chosen which does not involve COXI in order to test that the mechanism of pathogenesis is not specifically due to an attribute of cytochrome *c* oxidase I. For example, one could repeat these experiments using an ND subunit fusion peptide. It would be interesting to observe whether such a cell line would develop a biochemical defect limited to complex I, as can happen *in vivo* (Morgan-Hughes *et al.*, 1990; Hammans *et al.*, 1992a). This would address the question of whether the fusion protein acts by a specific mechanism, disrupting the assembly/activity of the respiratory complex in which the non-mutated gene product would have inserted (one interpretation of the results in the papers previously referenced), or by a more general mechanism, such as insertion into the inner membrane or sequestration of mitochondrial chaperones. The mechanism responsible for the respiratory deficiency observed in the PIZ cell lines could also be addressed by targeting random peptides (initially of a hydrophobicity similar to mitochondrially-encoded proteins) into mitochondria. If random proteins were found to cause mitochondrial dysfunction, it

would support the hypothesis that fusion proteins are detrimental, but that they act by some mechanism which is probably not specific to the respiratory complexes themselves. It is also possible that the mechanism of pathogenesis is not universal for all chimeric proteins.

Another experiment which should be performed is to transfect cell lines with the PI and PII constructs which have had the transit-peptide encoding region deleted. The constructs were designed such that there is a restriction enzyme site between the transit-peptide coding region and the coding region for COXI, with the intention that this could be removed if the COXIV transit peptide did not function correctly. By removing the transit peptide, one could test whether the expression of non-mitochondrially targeted PIZ produced a wide-ranging deleterious phenotype by interacting with membranes in general, as opposed to a respiratory phenotype due to mitochondrial dysfunction.

It is possible that the COXIV transit peptide itself is deleterious to mitochondrial function by creating a pore in the inner membrane (Sokolove and Kinnally, 1996). In order to test whether the induction of PIZ expression causes an increase in the permeability of the mitochondrial inner membrane in the PIZ cell lines, one could use microscopy to inspect the mitochondria for swelling. However, swollen mitochondria are observed in RRFs in patients of mitochondrial diseases, and conceivably (if swollen mitochondria were observed) this could equally be the result of transgene expression, if PIZ is causing mitochondrial dysfunction in an analagous manner to the disease state. Thus, one would also have to control in such an experiment by creating a cell line which used the COXIV transit peptide to target a protein that should have no adverse effect on mitochondrial function, such as a matrix protein, or DHFR, which has been used as a passenger protein previously (Hurt *et al.*, 1985). Another experiment to test for pore formation by the COXIV transit peptide would be to fractionate the mitochondria; if a significant amount of unprocessed PIZ were isolated

in association with the inner membrane, this would suggest that the transit peptide is inserted into the inner membrane, but has not entered the matrix for processing. One could then treat isolated mitoplasts from PIZ cell lines with protease to digest exposed proteins, to see whether a significant amount of COXIV transit peptide is then found inserted into the inner membrane. Cross-linking of import intermediates to the mitochondrial import apparatus would also distinguish between PIZ which was being imported, and PIZ inserted into the membrane in a non-specific fashion (Cyr *et al.*, 1995).

In order to see exactly where inside mitochondria the PIZ protein is localising, one could perform mitochondrial fractionation on the PIZ clones. A good antibody would be necessary for this work. PIZ is likely to be found in either the matrix or inner membrane fraction, because processing of the transit peptide was inferred to have taken place. One possibility is that PIZ is interfering with the activity of complex IV, and therefore one might expect to find it in association with other subunits of the cytochrome oxidase complex. Use of two-dimensional blue native-SDS PAGE gels could be used to test for incorporation of the chimeric protein into the complex (Nijtmans *et al.*, 1995). Alternatively, PIZ may be disrupting assembly of complex IV. This could be tested by immunoprecipitation of assembled complex (Spelbrink *et al.*, 1994) and comparison to a control cell line. One could also assay the levels of the subunits of the complex, to see whether lack of assembly has led to an increased turnover rate for the respective proteins, as in the yeast *QCR6* mutant (Schmitt and Trumpower, 1991). This could be accomplished by western analysis with the appropriate antibodies, or by ^{35}S-Met labelling of mitochondrial translation products. Another possibility is that PIZ is causing respiratory dysfunction by inserting into the inner membrane in a non-specific fashion; it might then be isolated in the inner membrane fraction, but not specifically associated with complex IV subunits. If the phenotype is due to sequestration of mitochondrial chaperones it would be predicted that PIZ would be isolated in the matrix fraction.

6.5 Further studies

The PI constructs have been subcloned into the pMam*neo* vector, and can now be used to analyse expression in any nuclear background which is compatible with steroid induction. Because the nuclear constructs were designed in cassette fashion, with restriction enzyme sites at either end of the PI and PIZ genes, the constructs can be easily subcloned into other vectors which would allow their analysis in cell lines which are not compatible with steroid induction. In the study by Holt *et al.*, (1997) in which cybrids were created using patient-derived mitochondria harbouring mtDNA with a partial duplication, two different recipient cell lines were used (discussed in section 1.5.5). The researchers found a subtle respiratory phenotype in one nuclear background, and mtDNA depletion in the other. This intriguing result suggests that my constructs should be tested in other cell lines. Obvious candidates for further cell lines in which to test expression are muscle and neural cell lines, given the clinical presentations of the syndromes associated with rearrangements of mtDNA. Promoters are also available which enable expression at varying levels, as opposed to the on/off expression of the vector which I have used. This could address the question of what level of protein is necessary to observe a respiratory phenotype. However, from a physiological point-of-view, the question of threshold is probably better addressed by the use of cybrids/patient-derived cell lines in which the proportions of wild-type to mutant mtDNA can be assessed.

An additional advantage of the approach adopted is the possibility of creating transgenic mice, as discussed in section 1.6. The chimeric gene could be placed under the control of a tissue-specific promoter; this would enable investigation of the effect the presence of the chimeric protein would have on a particular tissue *in vivo*, in isolation. Again, muscle tissue would be an obvious choice. Given the frequent occurrence of diabetes in association with the presence of partially-duplicated

mtDNA molecules, another possibility would be to use a promoter specific to the pancreas, e.g., to see if a diabetic phenotype is elicited.

6.6 Clinical implications

The research reported here has important potential implications for treatment and therapy of the individuals which harbour rearranged mtDNA molecules. Lightowlers *et al.*, (1997), have reviewed recent research into altering the proportions of mtDNA heteroplasmy *in vivo*. Using a peptidyl-nucleic acid (PNA) construct specific to a mutant template differing at a single base from the wild-type sequence, Taylor *et al.*, (1997) demonstrated selective binding at physiological conditions. The PNA was shown to inhibit replication of the mutant mtDNA, but not wild-type mtDNA, even at a 1000-fold molar excess. Furthermore, a mitochondrial transit peptide has already been shown to import covalently coupled double-stranded DNA molecules into the mitochondrial matrix (Seibel *et al.*, 1995). Lightowlers *et al.*, report that they have coupled a transit peptide to the PNA construct and achieved mitochondrial targeting within cultured muscle cells. If the chimeric peptide is the primary step in pathogenesis, then treatment which will prevent development of the associated syndromes is possible. In familial cases, infants at risk could be treated from birth, because the rearrangement would be known in advance. Using the targetted PNA, both replication of the rearranged mtDNA molecules and translation of any chimeric transcripts present could be blocked. By blocking replication at an early stage, it may be possible to effectively 'cure' the individual. In cases which are sporadic, an early diagnosis would be essential to prevent further deterioration of the child. In such cases, lifelong treatment with the blocking peptide may be necessary to prevent translation of the chimeric peptide.

6.7 Conclusions

The expression of the chimeric protein PIZ in isolation from other potential variables produced a subtle, but statistically significant respiratory phenotype in the fibroblast cell-line tested. Chimeric mitochondrial genes formed by rearrangements of mtDNA are transcribed and processed *in vivo*, and there is no obvious reason why chimeric transcripts in particular would not be translated. Failure to detect a fusion peptide *in vivo* may indicate that the protein is usually too unstable. It does not negate a role for such a protein (as discussed with reference to CMS in *Petunia*). In patient muscle, biochemical deficiency precedes mitochondrial proliferation and the formation of RRFs. Therefore, the expression of a chimeric mitochondrial protein *in vivo* may be a contributory factor to the biochemical deficiency observed. In fact, the observation of a respiratory deficiency in cell culture, attributable solely to the presence of the chimeric protein, suggests that expression of a chimeric mitochondrial protein may be the primary step in pathogenesis.

Appendix 1

	SUPERGLU	SG+DEX	GALACTOSE	GAL+DEX
3T3	4.300	4.500	1.810	2.020
	4.000	4.200	1.900	2.100
3T3pM	2.520	2.200	1.210	1.480
	3.860	3.740	2.260	2.050
	3.470	3.610	2.380	2.040
	3.020	3.160	1.060	1.160
	3.260	3.100	1.125	1.360
PIZ8.1	5.150	4.110	1.750	1.070
	4.570	4.810	2.180	1.790
	5.290	5.000	2.010	1.500
PIZ8.2	5.360	5.420	2.390	2.120
	5.450	4.400	2.370	1.810
PIZ8.3	2.580	1.910	0.830	0.570
	2.980	2.480	1.500	1.000
	2.690	2.570	1.470	1.070
PIZ8.5	2.340	2.170	1.360	1.150
	2.570	2.410	1.300	1.080
	2.520	2.270	1.120	0.860
PIZ8.6	3.940	3.760	1.990	1.600

This table displays the raw data for the statistical analysis of the PIZ cell lines in section 5.2.7.

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