# ISOLATION OF NUCLEAR-CODED MITOCHONDRIAL GENES IN CHINESE HAMSTER CELLS.

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This thesis is dedicated to my mum and dad, my husband and my children.

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

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## ABBREVIATIONS

| | |
|---|---|
| 12S rRNA | - small subunit mitochondrial rRNA |
| 16S rRNA | - large subunit mitochondrial rRNA |
| Acetyl CoA | - acetyl coenzyme A |
| ADP | - adenosine 5'-diphosphate |
| ATP | - adenosine 5'-triphosphate |
| ATPase 6 and 8 | - ATP synthase subunits 6 and 8 |
| bis-acrylamide | - N, N' methyl-bis-acrylamide |
| cDNA | - complementary DNA |
| COI, II, III | - cytochrome c oxidase subunits I, II, III |
| CoQ | - coenzyme Q |
| $dH_2O$ | - distilled water |
| D-loop | - displacement loop |
| DNA | - deoxyribonucleic acid |
| DNase | - deoxyribonuclease |
| dNTP | - deoxyribonucleotide triphosphate |
| DTT | - dithiothreitol |
| EDTA | - ethylenediamine tetra-acetic acid |
| EtBr | - ethidium bromide |
| FAD | - flavin adenine dinucleotide (oxidised) |
| $FADH_2$ | - flavin adenine dinucleotide (reduced) |
| Fig. | - figure |
| HEPES | - N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid |
| HSP | - heavy strand promoter |
| IPTG | - Isopropyl-β-D-thiogalactopyranoside |
| LSP | - light strand promoter |
| MOPS | - 3-[N-morpholino]propane sulphonic acid |
| mRNA | - messenger RNA |
| mtTF1 | - mitochondrial transcription factor 1 |
| $NAD^+$ | - nicotinamide adenine dinucleotide (oxidised) |
| $NADH/H^+$ | - nicotinamide adenine dinucleotide (reduced) |
| ND1-ND6 | - NADH dehydrogenase subunits 1-6 |
| PEG | - polyethylene glycol |
| RNA | - ribonucleic acid |
| rRNA | - ribosomal RNA |
| RNase | - ribonuclease |

| | |
|---|---|
| SDS | - sodium dodecylsuphate |
| TCA cycle | - tricarboxylic acid cycle |
| TEMED | - N, N, N', N'-tetramethylenediamine |
| Tris | - tris(hydroxymethyl) amino ethane |
| tRNA | - transfer RNA |
| u | - units |
| URF | - unidentified reading frame |
| vol | - volume |
| X-gal | - 5-bromo-4-chloro-3-indolyl-B-galactopyranoside |

## UNITS

| | |
|---|---|
| bp | - base pairs |
| Ci | - Curies |
| cm | - centimetres |
| cpm | - counts per minute |
| $^{o}C$ | - degrees centigrade |
| g | - grammes |
| g | - centrifugal force equivalent to gravitational acceleration |
| hr | - hours |
| kb | - kilobases/kilobase pairs |
| l | - litres |
| mCi | - millicuries |
| M | - molar |
| mins | - minutes |
| ml | - millilitres |
| mm | - millimetre |
| mM | - millimolar |
| mMol | - milliMoles |
| mV | - millivolts |
| ng | - nanogrammes |
| nt | - nucleotides |
| nMol | - nanoMoles |
| uCi | - microcuries |
| ug | - microgrammes |
| ul | - microlitres |
| uFD | - microfarads |
| OD | - optical density |
| pH | - acidity [$-\log_{10}$(molar concentration of $H^{+}$ ions)] |
| rpm | - revolutions per minute |
| sec | - seconds |
| v | - volts |

**ACKNOWLEDGEMENTS**

I would like to thank Howy Jacobs for his unfailing support throughout the course of this study. I would also like to thank my family for their encouragement especially my mum for taking the kids off my hands while I've been writing this thesis. Thanks also to all my friends in the Genetics and Biotechnology Department for making it a pleasant place to work and a special thanks to Linda Kerr for helping me out on the word processor.

## SUMMARY

Both mitochondia and chloroplasts contain DNA which codes for a small subset of genes involved in organelle biogenesis. The mitochondrial gene content and organisation is now known for a number of species from higher plants to insects to mammals. The diversity in the gene content of different species is relatively small and all mitochondria need products encoded in the nucleus and imported into the organelle for their existence. The interaction between the mitochondrial and nuclear genomes is the subject of this thesis.

Most work performed on characterising nuclear-coded mitochondrial gene products has utilized yeast due to their ability to survive without aerobic respiration, and as a consequence many mutant phenotypes have been isolated which have a defect in mitochondrial function. This is in contrast to the situation in mammalian systems, where nuclear mutations in mitochondrial respiration, unless leaky, lead to a lethal phenotype. However, some respiration-deficient mutants have been isolated from Chinese hamster cells and three have been used in this study in an attempt to isolate and characterise the mutant nuclear genes in these cell-lines.

By labelling the mitochondrial proteins with $^{35}S$ methionine in the presence of emetine which blocks cytosolic translation, the effect of the nuclear mutation on mitochondrial protein synthesis can be assayed. I have observed by using this technique that in one of the mutant cell-lines, Gal 13, there is a reduction in the level of some of the mitochondrially encoded polypeptides. Those polypeptides which were reduced were tentatively assigned as subunits from Complex I of the electron transfer chain. This fits in with the results from a biochemical analysis on the level of activity of Complex I in Gal 13 cells which is 5% of the normal widtype level.

The same study performed with Gal 50 and Gal 73 cells shows no difference in the synthesis or accumulation of mitochondrially encoded polypeptides in these two cell-lines when compared to wildtype cells. This suggests that for Gal 50 and Gal 73

cells, unlike Gal 13 cells, the nuclear mutation is not involved in the translation of mitochondrial encoded mRNAs.

Gal 73 cells have a defect in Complex III of the electron transfer chain and an immunoprecipitation analysis of Complex III in Gal 73 cells suggests that the mutation in these cells is not present in a nuclear gene coding for a structural subunit of this complex.

In order to get a definitive answer as to the function of the mutant nuclear genes in Gal 73 and Gal 13 cells, a complementation and marker rescue scheme was adopted, to isolate for characterisation, the mutant genes.

To complement the cell-lines, a wildtype (V79) genomic cosmid library was constructed in the vector pWE15 which contains the $G418^R$ and $AMP^R$ genes and an SV40 promoter/enhancer. As a result of transfecting DNA from this library into Gal 73 cells, two cell-lines $Gal^+73A$ and $Gal^+73B$ exhibiting the required wildtype phenotype, were isolated. Southern blot analysis of DNA from $Gal^+73A$ and $Gal^+73B$ cells showed that two independent complementation events had taken place to restore phenotype. The analysis also showed that in $Gal^+73B$ cells especially, the recombinant cosmid clone had integrated tandemly. Integrated DNA sequences were isolated from $Gal^+73B$ cells by a marker rescue scheme. Another cosmid library was constructed, of $Gal^+73B$ DNA in the vector Lorist 6 which contains the $Kan^R$ gene. By plating this library on ampicillin, four overlapping Lorist 6 clones, L73B1-4, were isolated which spanned the region of integrated DNA in $Gal^+73B$ cells.

M13 subclones from L73B2 and 3 containing single-copy genomic DNA were generated, three of which, B1, E5 and C6, were characterised further.

Southern blot analysis of V79, Gal 73 and $Gal^+73B$ genomic DNA using B1, E5 and C6 as probes suggested that B1 and E5 contained genomic DNA that had been integrated into $Gal^+73B$ cells and could therefore be used as probes to screen for the original recombinant cosmid integrated into the $Gal^+73B$

genome.

C6, on the other hand, was shown to contain at least two M13 subclones, one of which C6-A, contained integrated DNA like B1 and E5. The other subclone C6-B, hybridised to a rearrangement in the DNA of the mutant cell-line Gal 73 which generated a novel EcoRI fragment. This fragment was not observed in V79 cell DNA or in the complemented $Gal^{+}73B$ DNA, suggesting that a gene conversion by homologous integration has occurred to restore wildtype phenotype in $Gal^{+}73B$ cells.

As a result of this work, One mammalian respiration-deficient cell-line has been complemented by wildtype DNA and three M13 probes generated to screen for the integrated wildtype sequences. Also one M13 subclone has been isolated which hybridises to a rearrangement in Gal 73 genomic DNA which could conceivably be the cause of the mutant phenotype.

# CHAPTER 1

# INTRODUCTION

## 1.1 MITOCHONDRIA

Mitochondria are present in most eukaryotic cells but are not the same structurally or physiologically in all tissue types: they undergo specialisation depending on the type of tissue in which they reside and the functions they have to perform in that tissue.

As early as 1850-1890 many cytologists observed granular particles in the cytoplasms of different cells (Lehninger, 1964). It was not until the development of oxidation-reduction stains such as Janus green, that the role in cellular respiration of these granular elements was revealed. With research into the biochemistry of respiration, the two areas of research converged with one another, and were helped enormously by the subsequent ability to isolate intact mitochondria by differential centrifugation.

The other major function of mitochondria is the citric acid or TCA cycle in which, acetyl CoA, the terminal product of fatty acid and pyruvate oxidation is metabolised, releasing high-energy electrons for passage along the respiratory chain resulting in oxidative phosphorylation and the release of free energy.

Structurally, the mitochondrion consists of a double membrane structure which defines two internal spaces, the intermembrane space and the matrix (reviewed in Alberts et al, 1989). From electron micrograph analysis there is a suggestion of pits and pores produced by the presence of many molecules of channel-forming proteins, located in the surface of the relatively smooth outer membrane. These allow small molecules to cross into the intermembrane space (Whittaker and Dank, 1979). The inner mitochondrial membrane is much more impermeable to exogenous molecules. This impermeability is believed to be produced in part by the high proportion of cardiolipid found in the inner mitochondrial membrane (Capaldi, 1982). However, the presence of a variety of specific transport proteins makes the inner membrane selectively permeable to those molecules, such as the fatty acyl CoA, which is metabolised by the

mitochondrial enzymes concentrated in the matrix space (Whittaker and Dank, 1979). In contrast to the relatively smooth outer membrane, the inner mitochondrial membrane is highly convoluted, forming a series of infoldings known as cristae, in the matrix space. Along with the high percentage of cardiolipid the inner mitochondrial membrane has a much higher percentage of protein, than is present in other membranes. This is accounted for by the protein complexes of the respiratory chain and oxidative phosphorylation (Capaldi, 1982). Thus, the mitochondrion can be split into four different compartments each with its own special functions.

The pores and channels in the outer membrane allow the passage of fatty acids, and the three-carbon molecule, pyruvate, which is produced by glycolysis in the cytosol. These pass on through the inner mitochondrial membrane and are broken down to acetyl-CoA by enzymes in the matrix. Acetyl-CoA can then be fed into the citric acid cycle and degraded further by matrix enzymes to release electrons for passage along the respiratory chain. As a result, the structure of the mitochondrial inner membrane is adapted to take into account the differences in functional demand of different tissues. Both in liver and cardiac muscle the cells are packed with large numbers of mitochondria which have different structures in the two tissues. In the liver, due to its detoxification function, the breakdown of metabolites and the citric acid cycle are more important than oxidative phosphorylation. In contrast, the requirement for respiratory energy in cardiac muscle is predominant, and this is reflected in the structure of the inner membrane. Large numbers of cristae are found in cardiac muscle mitochondria which increase the surface area of the inner membrane and therefore the number of redox complexes used in electron transfer and oxidative phosphorylation (Whittaker and Dank, 1979; Alberts et al, 1989).

## 1.2 THE CHEMICAL FUNCTION OF MITOCHONDRIA.

The citric acid cycle is so called because of the formation of the 6-carbon compound citric acid, when acetyl-CoA is condensed with oxaloacetate, a 4-carbon compound. During seven

subsequent enzymatic steps, each catalysed by a different enzyme, citric acid is reduced to regenerate oxaloacetate, 2 molecules of $CO_2$, 3 molecules of the high energy electron carrier NADH and 1 of $FADH_2$, along with 1 molecule of ATP via a GTP intermediate. The electrons carried by NADH and $FADH_2$ respectively, are passed on to the first and second of a series of electron carriers embedded in the inner mitochondrial membrane. The electron carriers include small molecules such as ubiquinone (co-enzyme Q), cytochromes, flavoproteins and iron-sulphur centres. As the electrons are passed along the chain they lose energy which is used ultimately to convert ADP to ATP. Each successive electron carrier has a greater redox potential than the last, as a result of which electrons move in a cascade fashion to lower energy levels until ultimately they are donated to oxygen, which has the greatest affinity of all for electrons, producing water (Whittaker and Dank, 1979; Lehninger, 1982; Alberts et al, 1989).

The energy generated by the passage of electrons to oxygen is not released as heat, but is stored as an electrochemical proton gradient across the inner mitochondrial membrane. The electrons from NADH and $FADH_2$ are produced from the splitting of their hydrogen atoms to electrons and protons. Whilst the electrons are passed along the respiratory chain, the protons are selectively pumped out of the matrix at three coupling sites along the respiratory chain, creating both a pH gradient and a voltage gradient across the inner mitochondrial membrane (Whittaker and Dank, 1979; Lehninger, 1982; Alberts et al, 1989).

The asymmetry in pH and voltage across the inner membrane causes protons to move back into the mitochondria through a large protein complex, ATP synthase. The movement of protons into the matrix drives the synthesis of ATP from ADP and inorganic phosphate, which are present in the matrix, by the ATP synthase complex. The ATP is then rapidly released by the mitochondria into the cytosol where it is hydrolysed back to ADP and inorganic phosphate during its use by the cell. For each acetyl-CoA molecule generated by the oxidation of fatty

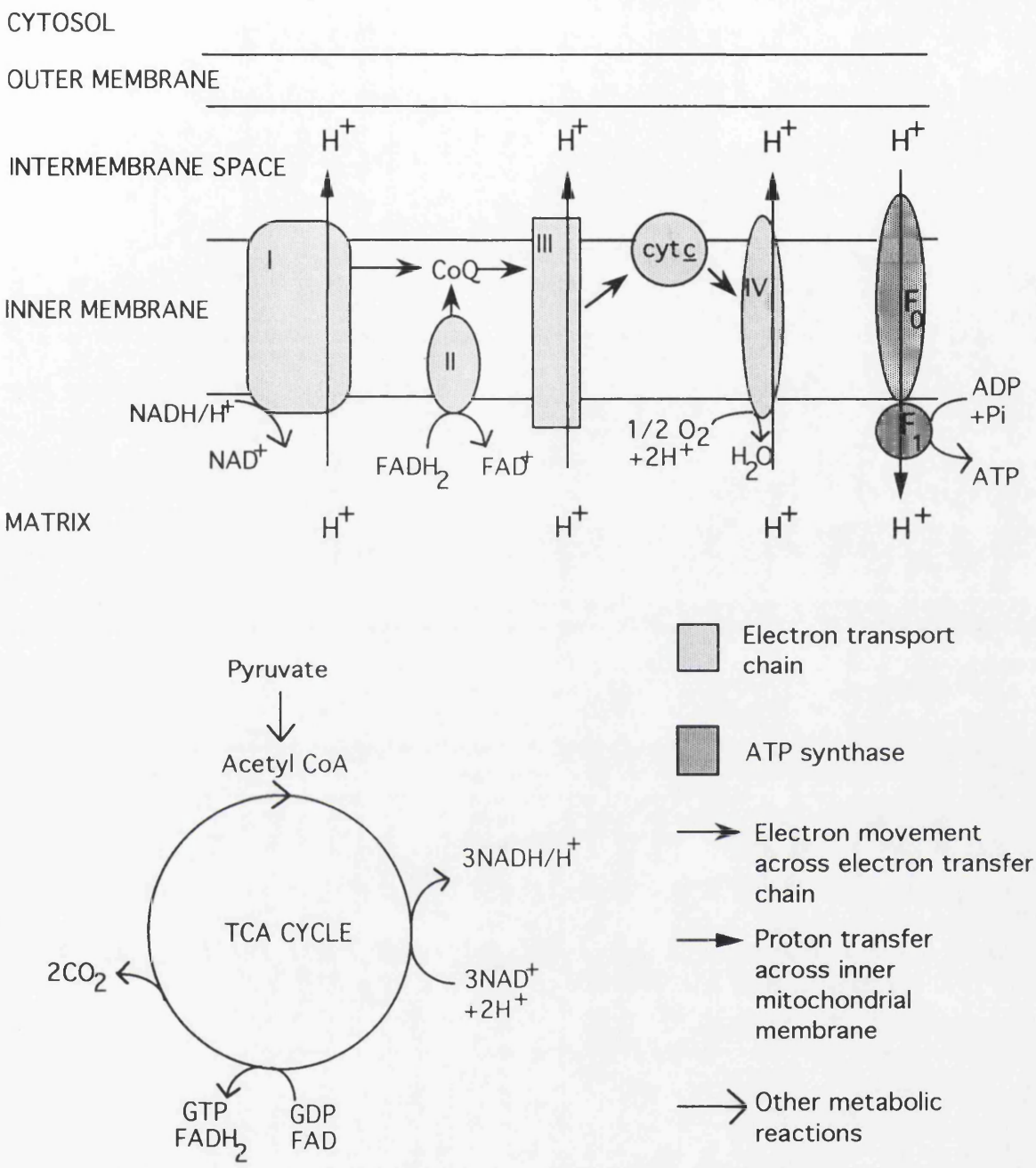



Fig. 1.1 Summary of the reactions involved in aerobic metabolism. Components of the electron transfer chain are shown as: I - NADH dehydrogenase (Complex I); II - succinate ubiquinone oxidoreductase (Complex II); III - ubiquinol cytochrome c oxidoreductase (Complex III); IV - cytochrome c oxidase (Complex IV). Not to scale.

acyl CoA or pyruvate, 12 ATP molecules are produced by the respiratory chain and oxidative phosphorylation, making the mitochondria the main free energy producers of the cell (Whittaker and Dank, 1979; Lehninger, 1982; Alberts *et al*, 1989).

## 1.3 PROTEIN COMPLEXES OF THE INNER MITOCHONDRIAL MEMBRANE.

The high protein content of the inner mitochondrial membrane is due to the presence of the protein complexes of the respiratory chain and oxidative phosphorylation (Capaldi, 1982). There are five main multi-subunit complexes, in which the number of subunits varies depending on the organism. These are: 1) NADH dehydrogenase (Complex I), which in mammals consists of about 41 subunits and is associated with ubiquinone, several iron-sulphur centres and flavoprotein as electron carriers (Walker, 1992); 2) succinate ubiquinone oxidoreductase (Complex II) which consists of 5 different protein subunits in mammals and, like Complex I, is also associated with ubiquinone, iron-sulphur complexes and flavoproteins (Capaldi, 1982); 3) ubiquinol cytochrome c oxidoreductase (Complex III) which is made up of 10 subunits in mammals, including 2 cytochromes *b* and $c_1$, and has a number of iron-sulphur centres and hemes as additional electron carriers (Capaldi, 1982); 4) cytochrome c oxidase (Complex IV), which is the last complex of the respiratory chain and contains 7-8 subunits in mammals and includes cytochromes *a* and $a_3$ and bound oxygen as electron carriers (Capladi, 1982). The fifth multi-subunit protein complex, ATP synthase, is not involved in the transfer of electrons but in the formation of ATP, by harnessing the energy produced by the flow of protons back into the matrix through this complex down the electrochemical gradient (Lippe *et al*, 1988; Nagley, 1988).

Complex I is a large complex with a monomeric molecular weight of 700,000 Da when isolated from bovine heart tissue (Galante and Hatefi, 1979; Walker, 1992). Of its 41 subunits, 7 are coded for by mammalian mitochondrial DNA, the rest by nuclear DNA (Chomyn *et al*, 1985; Chomyn *et al*, 1986). It is the most complex component of the respiratory chain which is reflected

in its relatively large number of subunits and its association with several redox centres including ubiquinone.

Complex II, succinate dehydrogenase, is a much smaller complex also associated with ubiquinone. The electrons from ubiquinone in Complexes I and II are passed straight to cytochromes b and $c_1$ in Complex III (Capaldi, 1982). All the subunits of Complex II in mammals are coded for by nuclear DNA, manufactured in the cytosol and transported into the mitochondria.

Complex III, ubiquinol cytochrome c oxidoreductase, consists of 10 subunits and its composition has been found to be similar in preparations from a number of different eukaryotes (Bell et al, 1979; Marres and Slater, 1977; Leonard et al, 1981 and Katan et al, 1976). X-ray crystallography and electron microscopy has greatly aided the investigation into the structure of Complex III. Indeed along with Complex IV (cytochrome c oxidase), it has been the most widely investigated of the redox complexes. In *Neurospora crassa*, low resolution electron microscopy has shown Complex III to span the inner mitochondrial lipid bilayer extending into both the matrix space and the intermembrane space (Leonard et al, 1981a and Leonard et al, 1981b). Of the 10 subunits in mammals, only one, cytochrome b, is encoded by mitochondrial DNA, all the others being transported from the cytosol (Bibb et al, 1981; Anderson et al, 1981; Anderson et al, 1982). The two hemes associated with cytochrome b pass on the electrons they receive from ubiquinone, to Complex IV.

Complex IV, cytochrome c oxidase, is the most widely studied component of the respiratory chain. Most eukaryotic forms of this complex consist of 8 subunits (Capaldi, 1990), but the mammalian complex contains a number of other subunits which when removed do not seem to affect the transfer of electrons or proton pumping (Capaldi, 1982; Capaldi, 1990) in vitro. The prosthetic groups appear to be associated with subunits I and II; they accept electrons from Complex III and donate them to bound oxygen (Winter et al, 1980). X-ray crystallography and electron microscopy have shown cytochrome c oxidase to have a

Y-shaped conformation spanning the lipid bilayer, with the stalk penetrating into the intermembrane space and the two arms into the matrix. Of all the subunits, only 3 are coded for by mammalian mitochondrial DNA (Bibb et al, 1981; Anderson et al, 1981; Anderson et al, 1982) .

ATP synthase is the fifth complex found in the inner mitochondrial membrane possessing as many as 18 protein subunits in mammals (Nagley, 1988). The complex is readily split into a water soluble $F_1$ fraction and a water insoluble lipid containing $F_0$ fraction (Lippe et al, 1988). The $F_1$ fraction made up of 5 subunits is involved in ATPase activity and is found on the matrix side of the lipid bilayer; the $F_0$ fraction is involved in proton translocation, and is found associated with the membrane. Of the 18 eukaryotic subunits, in mammals two subunits are encoded by mitochondrial DNA (Mariottini et al, 1983; Bibb et al, 1981; Anderson et al, 1981; Anderson et al, 1982).

## 1.4 MITOCHONDRIAL DNA.

Mitochondria along with plant chloroplasts are unique among cellular organelles in that they contain DNA. From the first when they were observed in the late 19th century, there was speculation that they contained a genetic function. In the mid 20th century mitochondrial DNA was discovered and with the advent of DNA sequencing and the development of other powerful molecular biological techniques the mitochondrial genomes of several organisms and plants were isolated, sequenced, and functionally analysed.

Mitochondrial DNA differs from nuclear DNA in several fundamental ways. It was found to consist of a single, circular double helix of 'naked' DNA whereas eukaryotic DNA of the nucleus is separated into a number of discrete linear chromosomes and is tightly associated with proteins. Mitochondrial DNA also uses a different genetic code from that of the nucleus (Barrell et al, 1980). The differences between mitochondrial DNA and nuclear DNA have led to speculations on the origin of mitochondria and their DNA, the most well known

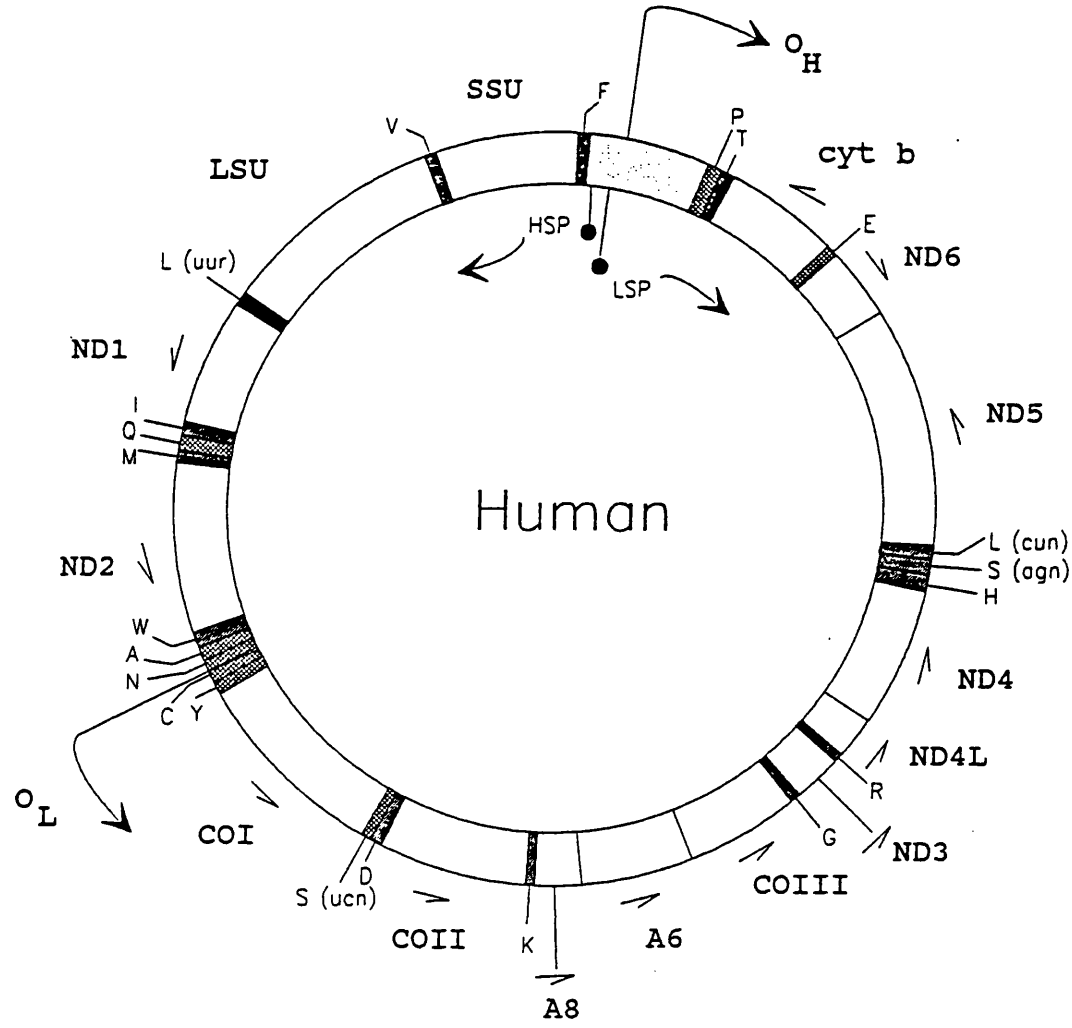



Fig. 1.2 Structure of the human mitochondrial genome. The origins of heavy strand replication and light strand replication are noted as $O_H$ and $O_L$ respectively. The D-loop is the shaded box. Genes for tRNAs are designated by the standard 1-letter code (P, T, E, $L_{cun}$, $S_{agn}$, H, R, G, K, D, $S_{ucn}$, Y, C, N, A, W, M, Q, I, $L_{uur}$, V, F). Transfer RNAs transcibed from the light strand are shown as checkered boxes and those transcibed from the heavy strand as striped boxes. The direction of transcription of the protein coding genes is shown as half arrows. The large ribosomal subunit and the small ribosomal subunit are designated LSU and SSU respectively. Other abbreviations used are described on page XX of this thesis.

being the endosymbiotic theory (Gray, 1989).

The endosymbiotic theory proposes that mitochondria and chloroplasts originated from an association between once free-living aerobic bacterial-like progenitors and a larger, non-respiring anaerobic organism, probably while the earth still had a low oxygen level. This resulted in an organism with the ability to adapt to aerobic living once the earth's oxygen level had increased ensuring the survival of the aerobic bacterial-like progenitor. Ultimately the association became so successful, the aerobic bacterium became a sub cellular-component which was unable to survive on its own as a distinct entity (Gray, 1989). This theory has been confirmed by the sequencing of mitochondrial DNA and investigations into its regulation.

Animal mitochondrial DNA, however, is a lot shorter than present-day bacterial DNA being more in the size range of typical viral DNAs.

### 1.4.1 ANIMAL MITOCHONDRIAL DNA.

#### 1.4.1.1 MAMMALIAN MITOCHONDRIAL DNA.

Mammalian mitochondrial DNAs are approximately 16.5 kb in length and contain the same basic set of genes in all species, that is 2 rRNAs, 22 tRNAs and the 13 protein-coding genes; NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5 and 6 (ND1-6), cytochrome oxidase subunits I, II and III (COI, COII, COIII), cytochrome b (cyt b), ATP synthase subunits 6 and 8 (A6 and A8), (Bibb et al, 1981; Anderson et al, 1981; Anderson et al, 1982). Two striking features of mammalian mitochondrial DNA are the general conservation of gene organisation and economy of sequence (fig. 1.2). Virtually all of the mitochondrial DNA is utilised as coding sequence, which has led to a lot of research into how the expression of the mitochondrial genes is regulated. Most of the mammalian mitochondrial genes are encoded on the heavy-strand of mitochondrial DNA, with the light strand encoding only one protein coding gene, ND6 and 8 tRNA genes. Only two extensive regions of mammalian

mitochondrial DNA were found to have no coding capacity, a very short region (32 bp) within a cluster of 5 tRNA genes and a relatively longer region (880 bp). These two were later shown to play a role in the regulation of replication and transcription of the mitochondrial genome (Clayton, 1982; Montoya et al, 1982; Clayton, 1984).

Variations in size of the mitochondrial genome between mammals are generally a result of differences in the size of the larger non-coding region. However, in all mammals so far studied, this non-coding region (commonly termed the displacement or D-loop) was found to contain the signal for heavy-strand replication (Clayton, 1982), along with two transcriptional promoters (Montoya et al, 1982; Clayton, 1984). The shorter non-coding region was found to be the origin for light-strand replication (Clayton, 1984). The complement of mitochondrial genes present and their organisation within the mammalian genome has been well conserved through evolutionary time with only the displacement-loop (D-loop) showing a significant level of sequence divergence between species (Clayton, 1982). No function other than in replication and transcriptional initiation has yet been assigned to it.

Mitochondrial transcription produces a single primary transcript of each strand, which requires processing to form the functional transcripts. This represents a problematic area for the economical mitochondrial genome (Montoya et al,1982; Clayton, 1984). Due to very few, if any, intervening nucleotides between coding sequences being present in mammalian mitochondrial DNA, 'conventional' methods for the regulation of processing can not function i.e. regulatory sequences to control the cleavage of the primary transcript. The hypothesis put forward to overcome the lack of cleavage regulatory sequences within primary transcripts revolves around the positions of the tRNA genes (Ojala et al, 1981). The tRNA genes are scattered around the genome and flank almost every longer gene (with a few exceptions). As transcription is proceeding, the tRNA regions, within the larger primary transcript, are presumed to fold into their

characteristic 'clover-leaf' secondary and tertiary structure, and are thought to act as recognition sites for the mitochondrial RNA processing enzymes, generating cleavages precisely at the beginning and end of the tRNA transcripts. To corroborate this, an RNase-P like enzyme has been isolated and purified from human mitochondria, which catalyses the cleavage of the precursor RNA on the 5' side of the tRNA sequences (Doerson et al, 1985). The lack of 5' untranslated leader sequences also poses a problem for the initiation and regulation of translation of mitochondrial mRNAs. In the cytosol, the signals for ribosome binding and initiation of translation are found in the 5' untranslated sequences of mRNAs. In contrast, mammalian mitochondrial mRNAs have little or no 5' untranslated sequences which could contain recognition signals for ribosome binding and the initiation of translation. How ribosome binding and translation initiation is controlled in mammalian mitochondrial DNA is as yet unknown.

The lack of non-coding sequence between mitochondrial genes poses a similar problem for the termination of mitochondrial translation as it does for initiation as many mitochondrial genes lack conventional stop codons. However, like nuclear messages, mamamalian mitochondrial transcipts are polyadenylated (Battey and Clayton, 1980; Anderson et al, 1981 and Ojala et al, 1981) and due to the presence of polyadenylation following (a) terminal U or UA residue(s), prior to the 5' end of the adjacent gene, can therefore generate the required UAA stop codons. In the human and bovine systems (but not apparently in the mouse), AGG and AGA codons also appear to function as stop codons, in addition to the above method of termination Barrell et al, 1980).

**1.4.1.2 NON-MAMMALIAN ANIMAL MITOCHONDRIAL DNA.**

Although the gene organisation of non-mammalian mitochondrial DNA is not as conserved as in mammals, the gene content is essentially the same and the genome size has been almost universally maintained at approximately 16 kb with most of the size variations being a product of differences within the

major non-coding region. However, through evolution and the differentiation of species, the non-mammalian mitochondrial genomes have undergone a series of translocations and inversions, which have changed the polarity of expression of some mitochondrial genes, but do not appear to have given cause for the development of alternative regulatory mechanisms for gene expression (Roberts et al, 1983; Clary and Wolstenholm, 1985; Roe et al, 1985).

### 1.4.2 YEAST MITOCHONDRIAL DNA.

In contrast to the compact genome of animal mitochondria, the yeast *Saccharomyces cerevisiae* mitochondrial genome is 70-75 kb in length and contains, between genes, relatively large areas of non-coding sequence that are highly A/T rich, interspersed with small clusters of G/C residues (Bernardi, 1980). There is one exception to this, the mitochondrial genomic organisation of the fission yeast *Schizosaccharomyces pombe* reflects that of mammalian mitochondrial DNA, with very small intergenic spacers and nearly all protein coding genes are separated by tRNA genes (Borst and Grivell, 1978). The budding yeast (*Saccharomyces cerevisiae*) mitochondrial genome shares a common set of genes with the mammalian mitochondrial genome, but genes exhibiting sequence similarity with the mammalian mitochondrially encoded NADH dehydrogenase subunits have not been found in yeast mitochondrial DNA (Borst and Grivell, 1978). The organisation of the genes common to yeast and mammalian mitochondrial genomes is completely different. In yeast mitochondrial DNA the two rRNA genes, (as in sea urchins (Roberts et al, 1983)), are not adjacent to one another but are separated by tRNA genes and by two genes for subunits of cytochrome c oxidase. Most of the tRNA genes are found in one main cluster again similar to the tRNA cluster found in the sea urchin mitochondrial genome (Christianson et al,1983; Zassenhaus et al, 1984), although seven are scattered around the genome and the excision of the tRNAs may be still important in rRNA and mRNA maturation by the action of specific endonucleolytic enzymes such as RNase-P (Martin and Underbrink-Lyon, 1981).

Another striking difference between the yeast and animal mitochondrial genomes and one which contributes to the large size of the yeast mitochondrial genome is the presence of long intron sequences found in the genes for cytochrome b (Nobrega and Tzagoloff, 1980; Lazowska et al, 1980), cytochrome oxidase I (Hensgens et al, 1983; Bonitz et al, 1980) and the large subunit (LSU) rRNA gene (Dujon, 1980). In some cases the number of introns is strain dependent (Tzagoloff and Myers, 1986). Not all of the introns in yeast mitochondrial DNA consist of entirely non-coding sequence. Whilst intron sequences do not, by definition contribute to the actual content of the protein itself, some introns have been found to contain open reading frames. Some intronic open reading frames have been shown to code for proteins which are required for the correct excision of the intron in which they reside. The proteins formed by these intronic open reading frames have been termed maturases, and mutations within these genes prevent the correct excision of the intron. Therefore, an aberrant respiratory chain subunit is formed, and as a consequence, yeast mitochondrial respiration is disrupted (Lazowska et al, 1980; Dhwahle et al, 1981; Weissbrummer et al, 1982; Anziano et al, 1982; Kruger et al, 1982 and Zaug et al,1983).

It may be argued that the presence in yeast mitochondrial DNA of non-coding sequences between and within genes could allow a more complex regulation of mtDNA replication and expression by the organelle itself than would be possible in animal mitochondrial DNA. The initiation of yeast mitochondrial transcription has been analysed (Christianson and Rabinowitz, 1983) using guanylyl transferase to cap the triphosphate 5' ends of the mitochondrial mRNAs, in order to assess the number and location of primary transcripts which are formed in yeast mitochondria. Unlike in mammals, where only one primary transcript of each strand is believed to be formed, some 20 primary transcripts have been identified in yeast, and further investigation of the 5' termini of yeast mRNAs has revealed a common 9 nucleotide sequence (5' ATATAAGTA 3') which characterises the initiation of transcription in yeast mitochondria (Christianson and Rabinowitz, 1983; Osinga et al,

1982). This, along with the semi-purification of the yeast mitochondrial RNA polymerase, and the development of a mitochondria-free transcription system, has confirmed the necessity of the nonanucleotide sequence for transcriptional initiation and has also revealed the requirement of other 5' sequence elements which have yet to be identified (Edwards et al, 1982; Edwards et al, 1983).

Unlike mammalian mitochondrial mRNAs, yeast mitochondrial mRNAs lack post-transcriptionally added poly A sequences (Groot et al, 1974) and have 5' untranslated leader sequences of various lengths.

The existence of mutations within yeast nuclear genes (PET mutants), which as a consequence, affect mitochondrial biogenesis has greatly enhanced our knowledge of yeast mitochondrial DNA expression and will be discussed in more detail in a later section.

### 1.4.3 PLANT MITOCHONDRIAL DNA.

Plant mitochondrial DNA is even larger and more complex than yeast mitochondrial DNA, with a genome size varying from 200-2500 kb depending on the species, although there can be great variations in size within species (Levings and Brown, 1989).

There are several unique features found in plant mitochondrial DNA. Plant mitochondria, like other eukaryotic mitochondria encode only a small number of proteins, but unlike other eukaryotes examined to date, plant mitochondrial DNA codes for three rRNAs (Leaver and Harmey, 1976; Dale et al, 1984 and Spencer et al, 1984). So far, 14 of the tRNAs have been located and it has been shown that four mitochondrial tRNAs of bean hybridise to nuclear and not mitochondrial DNA suggesting that some mitochondrial tRNAs in plants need to be imported from the cytosol (Green et al, 1987). 3 cytochrome oxidase genes (Fox and Leaver, 1981; Issac et al, 1985b and Heisel et al, 1987), and the cytochrome b gene (Dawson et al, 1984) as coded for by yeast and animal mitochondrial DNAs have also been found in plant mitochondrial DNA. Two open reading frames

with homology to animal ND1 and ND5 have been reported in some species (Bland et al, 1986; Stern et al, 1986 and Wissinger et al, 1988). The $F_1$-$F_0$ ATPase complex in plants contains four subunits that are mitochondrially coded (Levins and Brown, 1989) and there are several open reading frames which show sequence homology to small subunit ribosomal proteins (Bland et al, 1986; Schuster and Benicke, 1987 and Wahleithner and Wolstenholm, 1988). Group II introns have been found in the genes for cytochrome oxidase II, ND1 and ND5 (Fox and Leaver, 1981; Stern et al, 1986 and Wissinger et al, 1988).

In some species the presence of direct repeats in plant mitochondrial DNA allows intra recombinantion to occur between these repeat sequences producing a mitochondrial genome that consists of several smaller circular molecules (Synenki et al, 1978; Palmer and Shields, 1984).

## 1.5 NUCLEO-MITOCHONDRIAL INTERACTIONS.

Although mitochondrial genomes show extreme variations in size and genome organisation, the actual gene number and types of gene that the genome specifies show striking conservation, when the disparate eukaryotic taxa are compared. Mitochondrially encoded genes, all of which are involved in mitochondrial biogenesis, are only a small proportion of those required for the maintenance of full mitochondrial function. The remainder of the mitochondrial components are encoded by nuclear DNA, synthesised in the cytosol and then actively transported to the organelle. This requires the coordinate expression and regulation of genes in two distinct DNA-containing compartments to maintain mitochondria and their function. The coordinated expression and regulation of mitochondrial DNA is made even more complicated as different tissues, such as liver and muscle, make different functional demands on mitochondria through various developmental stages.

Most research to date, on the interaction of the nuclear and mitochondrial genomes, has used yeast as a model system. The ability of yeast to grow under anaerobic conditions, as well

as to respire aerobically, permits the isolation and study of conditional lethal mutants. Therefore, yeast has been a particulary fruitful organism in which a genetic approach has revealed a large number of mutations in nuclear genes involved in mitochondrial biogenesis. The reason why other organisms have not been fruitful sources of such mutants is the difficulty of maintaining them without respiratory metabolism. For this reason, most of the eukaryotic nuclear genes which have been characterised as being involved in mitochondrial biogenesis, whether structural or regulatory, were originally isolated from yeast. However, because of the similarity of mitochondrial function and genetic information across the whole spectrum of organisms, any knowledge gained from yeast is likely to be of relevance to the mammalian system. However, most mammalian nuclear mutations, unless leaky, which adversely affect respiration are probably lethal and therefore never observed.

### 1.5.1 DEPENDENCE OF YEAST MITOCHONDRIAL BIOGENESIS ON NUCLEAR GENES.

Like the classic yeast petite rho mutants (mutants with large regions of their mitochondrial genome deleted and are inactive in mitochondrial protein synthesis) and mit$^-$ mutants (mutants which have defects in their mitochondrial DNA usually deletions but which have retained active mitochondrial protein synthesis), the PET mutants (strains which carry mutations in nuclear genes required for mitochondrial function) are characterised phenotypically by their inability to grow on non-fermentable substrates e.g. glycerol or ethanol, even though they have normal or near-normal growth on glucose.

The range of components required by the mitochondria, which must be encoded by the nucleus, can be split into three groups: 1) nuclear-coded structural subunits of the respiratory chain; 2) the nuclear-coded components of the mitochondrial genetic system, including the proteins necessary for mitochondrial DNA replication, the regulatory factors involved in mitochondrial gene expression, and enzymes necessary for the synthesis, processing and modification of

both nuclear and mitochondrial encoded proteins, and 3) the nuclear-coded lipid and polypeptide components not already mentioned, which make up the inner and outer mitochondrial membranes.

All of the PET nuclear mutants which have been isolated so far, contain mutations in nuclear genes which fall into one or other of the above categories, with the largest group being those that code for essential components of mitochondrial protein synthesis.

### 1.5.2 NUCLEAR-CODED PROTEINS INVOLVED IN YEAST MITOCHONDRIAL DNA EXPRESSION.

#### 1.5.2.1 Replication.

Mitochondrial DNA replication in yeast is thought to rely on the initiation of transcription at a specific subset of mitochondrial DNA promoters, followed by site-specific cleavage of the RNA transcript giving rise to a functional replication primer (Osinger et al, 1981). Evidence supporting this hypothesis is the loss of mitochondrial DNA in yeast strains containing a disruption in the RPO41 gene, the nuclear gene coding for the catalytic subunit of the mitochondrial RNA polymerase (Schinkel et al, 1988; Ticho et al, 1988). However, other nuclear mutations that intefere with respiration also lead to the rearrangement and loss of mitochondrial DNA but yeast strains which lack replication control sequences (*rep* sequences, 300 bp regions of relatively G + C-rich DNA with 80% homology among the three or four locations) in the mitochondrial DNA have been shown to have stable mitochondrial genomes (Fangman et al, 1990). Therefore, in the former case it is not known whether a mutation in RPO41 is directly responsible for the loss of mitochondrial DNA or if the loss of DNA is due to a general disruption in respiration. There could be an alternative mechanism for replication initiation that is independent of RPO41 transcription. Fangman et al, (1990) showed that low levels of transcription occurred in two rho$^-$ strains, one containing the replication control sequence and one that did not, in both rho$^-$ strains the RPO41

gene had been disrupted. This suggests that there is a second minor RNA polymerizing system yet to be identified which could be used to form primers for mitochondrial DNA replication and that the loss of mitochondrial DNA in the RPO41$^-$ mutant is only an indirect consequence of the disruption.

### 1.5.2.2 Transcription.

Animal and yeast mitochondrial DNAs do not encode any component of the mitochondrial RNA polymerase which is mported into the mitochondria from the cytosol (Schinkel et al, 1988; Ticho et al, 1988). The mitochondrial RNA polymerase is a multisubunit complex, that could allow for the differential recognition of the 20 or so promoters contianing the consensus 5' ATATAAGTA 3' nonanucleotide sequence, and the specificity of recognition by the RNA polymerase is tuned by sequences outside the nonanucleotide motif (Christianson et al, 1983; Osinger et al, 1982; Edwards et al, 1983). The catalytic component of the yeast mitochondrial RNA polymerase is the product of the nuclear *RPO41* gene which contains regions with high sequence similarity to the genes coding for the RNA polymerases of bacteriophages T3 and T7 (Masters et al, 1987). This catalytic subunit combines with a factor which specifies the initiation of transcription at the correct nucleotide of the nonanucleotide motif i.e. the terminal A of the consensus (Wilcoxen et al, 1988; Schinkel et al,1987, 1988; Ticho and Getz, 1988). The nuclear gene (*MTF1*), coding for the yeast RNA polymerase specificity factor has since been cloned and encodes a 43 KDa protein, which when purified, restored transcriptional activity when added to the *mtf1* yeast mutant strain containing a disrupted *MTF1* gene (Lisowsky and Michaelis, 1989; Jang and Jaehning, 1991).

Another nuclear-coded protein ABF2, has been found to be very abundant in yeast mitochondria and binds most double-stranded DNA sequences with approximately equal affinity (Diffley and Stillman, 1992). ABF2 is thought to be involved in the packaging of yeast mitochondrial DNA due to its close relationship to the vertebrate HMG1 protein (Diffley and Stillman, 1992). This is supported by the ability of ABF2 to

introduced negative supercoils into a relaxed, double-stranded, circular DNA molecule. The binding of ABF2 to DNA however, is excluded from runs of A residues; as few as 9-11 A residues will prevent ABF2 from binding to the DNA in that location a highly significant finding given the high A+T content of yeast mitochondrial DNA. Footprints on the REP2 region, a putative replication origin in the yeast mitochondrial genome, have shown only two regions of significant ABF2 binding, one of which is immediately adjacent to its associated promoter. This binding near the promoter for mitochondrial RNA transcription and the similarity between ABF2 and a human mitochondrial transcription activator, mtTF1, (Fisher et al, 1992 see below section 1.6.2) suggest that ABF2 may also be an activator of transcription. This has been verified in vitro (Parisi et al, 1993). The other site of ABF2 binding is in the REP2 region is thought to be able to function as a minimal replication origin. No Nuclear genes controlling the differential transcription of mitochondrial genes have thus far been identified.

#### 1.5.2.3 RNA Processing and mRNA Stability.

Some mitochondrial genes do not have their own transcription initiation sites and are transcribed as part of longer multigenic precusors, such as the cytochrome b and $tRNA^{glu}$ precursor transcript. The correct cleavage of these precursors to generate messages with mature 5' and 3' ends requires the aid of nuclear-coded proteins. The 3' ends of yeast mitochondrial mRNAs lie within a conserved consensus sequence (Thalenfeld et al, 1983) identified as the dodecamer 5' AAUAAUAUUCUU 3'. This serves as a processing site (Osinga et al, 1984). Two mitochondrial mutations strongly confirm the importance of this sequence for gene expression. One is a double point mutation in the dodecamer sequence at the end of the open reading frame (termed FIT1) in the *omega* intron of the 21S rRNA (Zhu et al, 1987). The second mutation is a 195 base pair deletion downstream of the ribosomal VAR1 gene that removes its dodecamer and drastically reduces VAR1 expression.

Transcripts of the VAR1 gene with the deletion are reduced in abundance and have extended 3' ends (Zhu et al, 1989). A suppressor mutation SUV3-1, of the VAR1 dodecamer deletion mutant, was found to increase the translatability of the mRNA suggesting that a proper 3' end is required for efficient VAR1 translation in wild-type yeast mitochondria and that SUV3-1 relieves the requirement for a proper 3' end (Zhu et al, 1987). Surprisingly, however, the SUV3-1 mutation blocked normal processing at the wild-type dodecamer site of the intron-encoded FIT1 mRNA although it did not affect 3' processing of other mRNAs (Zhu et al, 1987). Therefore, it is difficult to interpret the specific role of the SUV3-1 wild-type gene product.

To complicate matters further, the presence of introns in some of the yeast mitochondrial genes necessitates additional processing of their mRNAs, before these messages are ready to be translated. Similar considerations apply to plant mitochondrial RNAs. Mitochondrial introns are different from nuclear pre-mRNA introns in that they do not conform to the GU...AG rule typical of pre-mRNAs are not spliced by a spliceosome complex (Cech, 1990). There are two main groups of mitochondrial introns, distinguished by the mechanism by which self-splicing occurs in vitro. Both group I and group II introns contain a distinct sets of conserved sequences for splicing and although both involve transesterification, these splicing mechanisms are different between the two (Grivell and Schweyen, 1989; Cech, 1990). The correct splicing of some mitochondrial introns in vivo also requires the aid of maturases encoded by the introns themselves, and of several nuclear-encoded proteins imported from the cytosol.

The analysis of mutations in a number of species revealed that some nuclear-coded mitochondrial splicing factors are mitochondrial amino acyl-tRNA synthetases. The *CYT18* nuclear gene in *Neurospora crassa* for example (Garriga and Lambowitz, 1986; Akins and Lambowitz, 1987), was found to encode a tyrosyl-tRNA synthetase which is also required for the splicing of group I introns. In yeast, the mitochondrial leucyl-tRNA synthetase, the product of the nuclear NAM2 gene,

| GENE | PROTEIN | INTRON(S) AFFECTED |
|---|---|---|
| **MITOCHONDRIAL DNA** | | |
| Intronic reading frames | RNA maturases | aI1,aI2,aI4,bI2,bI4 |
| **NUCLEAR DNA** | | |
| CBP2 | | bI5 |
| MSS18 | | aI5b |
| MSS51 | | aI1,aI2,aI4,aI5 |
| MSS116 | RNA helicase? | aI1,aI5a/b,bI1 bI2/3 |
| MRS1 | | bI3 |
| SUP-101 | | bI1 |
| NAM1 | | groups I and II |
| NAM2 | mt Leu-tRNA synthetase | aI4,bI4 |
| CYT18 | mt Tyr-tRNA synthetase | group I in *N. crassa* |

Table 1.1 Proteins required for splicing of mitochondrial introns (Grivell, 1989).

is necessary for the splicing of introns bI4 and aI4 in the genes for cytochrome oxidase subunit I and cytochrome b respectively (Herbert et al,1988). The other nuclear proteins involved in mitochondrial splicing known to date are listed in Table 1.1. Some of these proteins are necessary for the splicing of specific introns whilst others are involved in the splicing of 2 or more introns. However, it has been established that like the products of CYT18 and NAM2, which are required for translation of mitochondrial messages as well as splicing, many of the others could also have a dual function in mitochondrial protein synthesis. For example, respiratory competence is not restored in strains which contain mutations in the MSS116 and MSS18 genes even if they lack mitochondrial introns. In fact, the MSS116 gene product is a member of a protein family which includes the initiation factor eIf4A and several other nucleic acid helicases (Seraphin et al, 1989).

There is evidence that message stability is involved in regulating relative mRNA levels in yeast mitochondria. A mutation in the nuclear gene *CBP1*, for example, results in the absence of cytochrome b due to greatly reduced levels of its message (Dieckmann et al, 1984). The instability of the cytochrome b mRNA in the cbp1 mutant is due to the aberrant cleavage of the message from the adjacent $tRNA^{glu}$ transcript with which it is co-transcribed. Whether the CBP1 protein produces the cleavage via a specific endonuclease activity or whether it stabilises the message by binding to its 5' end is unknown.

#### 1.5.2.4 Translation

Many PET mutations so far characterised are in genes which regulate the translation of mitochondrial mRNAs. Translation can be considered under three headings: initiation, elongation and termination, of which mitochondrial initiation has been the most intensively studied. Most PET gene products known to be involved in translation are thought to regulate the initiation of mitochondrial translation by interacting with the 5' upstream sequences of the transcripts of specific

mitochondrial genes. An exception is PET123, which codes for a small subunit ribosomal protein (McMullin et al, 1990). More than one nuclear-encoded factor is generally required for the translation of each individual mitochondrial mRNA. Mutations in the nuclear genes PET494, PET111, PET54 and PET55, for example, all arrest the translation of cytochrome oxidase III mRNA, even though normal levels of the message exist (Muller and Fox, 1984; Costanzo et al, 1986; Costanzo and Fox, 1986; Fox et al, 1988; Fox, 1986; Poutre and Fox, 1987). The translation of all other mitochondrial mRNAs is unaffected. Cytochrome b mRNA translation is similarly regulated by the nuclear CBP6 and MK2 gene products (Fox, 1986; Fox et al, 1988). All of these PET mutants complement one another: therefore, cytochrome oxidase III and cytochrome b mRNAs in yeast, each require a number of exclusive factors for the correct initiation of translation. There is evidence for direct interaction between the proteins encoded by PET494, PET54, PET55, CBP6 and MK2, and the mRNAs for cytochrome oxidase III and cytochrome b. Suppressor strains of these mutants were isolated and their characterisation revealed mitochondrial DNA deletions within the 5' untranslated sequences of both cytochrome oxidase III and cytochrome b genes. The deletions of mitochondrial DNA resulted in the fusion of the amino-terminal coding sequences of cytochrome oxidase III and cytochrome b with various other sequences, mainly the 5' untranslated sequences of other mitochondrial genes (Fox et al, 1988). The area of the deletions in mitochondrial DNA which result in the suppression of the mutant phenotype and the amino acid comparison of these nuclear proteins could reveal how these protein factors might function. The deletions which suppress the PET494, PET54 and PET55 mutants all encompass the same region of the 5' untranslated leader of cytochrome oxidase III mRNA and the proteins encoded by PET494, PET54 and PET55 therefore probably bind to this region as a single multisubunit complex (Fox et al, 1988). Domains located within the PET proteins were revealed by amino acid sequence comparisons, to have clear homology to initiation factors eIf-2a from mammals and eIf-2B from yeast respectively (Costanzo and Fox, 1990).

Genes encoding homologues of *Escherichia coli* elongation factors $T_u$ (Nagata et al, 1983) and G (Tzagoloff and Dieckmann, 1990) have also been identified in yeast nuclear DNA, and mutations in these genes lead to a PET phenotype indicating that the functions of these factors is essential only in mitochondria.

### 1.5.2.5 Regulation of nuclear-coded mitochondrial gene expression.

As only a small number of mitochondrial proteins are encoded by the mitochondrial genome in all organisms, one would expect the nuclear-coded mitochondrial components to be regulated along with the mitochondrially encoded components.

Yeast can grow both aerobically and anaerobically and in response to a change in enviromental conditions the regulation of the nuclear-coded mitochondrial gene products could feedback both positively and negatively on the regulation of mitochondrial protein synthesis, to coordinate the expression of both systems. As of yet, how such a regulatory response might work is not known. What is known is that some nuclear-coded mitochondrial gene products are regulated in response to enviromental conditions. *CYC1*, the nuclear gene for mitochondrial iso-1 cytochrome c, is regulated transciptionally in response to oxygen and carbon source. Two upstream regions termed UAS1 and UAS2 of the CYC1 gene are responsible for transcriptional activation under different enviromental conditions and respond to a number of proteins. UAS1 was found to bind a nuclear protein, HAP1 which stimulates iso-1 cytochrome c transcription in the presence of haem under aerobic growth conditions. Similarly UAS2 was found to bind nuclear-coded proteins HAP2, HAP3 and HAP4 which probably bind as a single complex to activate transcription as a result of a shift to anaerobic conditions (Olesen and Guarente, 1990; Schider and Guarente, 1991).

There is an enormous input from the nucleus on the regulation of expression of both nuclear and mitochondrial genes involved in mitochondrial biogenesis, but does the mitochondrion

influence nuclear DNA expression? Studies of the steady-state levels of certain nuclear-coded mitochondrial RNAs show changes in response to the loss of mitochondrial DNA or mutations within the mitochondrial DNA (Parikh et al, 1987). How this occurs is as yet unknown, as is the extent to which the mitochondrion influences nuclear DNA expression.

### 1.5.3 IMPORT OF NUCLEAR-CODED PRECURSOR POLYPEPTIDES INTO MITOCHONDRIA.

Biochemical and genetic analysis have shown that the process of importing nuclear-coded respiratory polypeptides into mitochondria is itself mediated by nuclear-coded components. Most nuclear-coded mitochondrial proteins are imported into mitochondria as longer precursor proteins which are cleaved to their mature form once import into mitochondria has occurred. In *Neurospora crassa* two nuclear-coded proteins MOM19 (Sollner et al, 1989) and MOM72 (Sollner, 1990) were isolated from the outer mitochondrial membrane which were shown using antibodies to be the import receptors for most nuclear-coded mitochondrial precursor proteins and the import receptor for the ADP/ATP carrier precursor protein respectively. Antibodies raised against these membrane proteins block the import of most known nuclear-coded mitochondrial proteins, except apocytochrome c, the precursor for cytochrome c, suggesting that this polypeptide follows a different targeting pathway (Pfaller et al, 1988). In yeast, as in *Neurospora crassa*, a nuclear-coded protein, Mas70p (Hines et al, 1990), was isolated from the outer mitochondrial membrane and shown to be an import receptor for nuclear-coded mitochondrial proteins.

In contrast to most nuclear-coded mitochondrial polypeptides, apocytochrome c is thought to be able to insert into the mitochondrial membranes without the aid of proteins within the mitochondrial outer membrame (Stuart et al, 1990). Heme is added to apocytochrome c by the enzyme cytochrome c heme lyase in the intermembrane space (Nicolson and Hergersberg, 1988) and under conditions were heme cannot be added, import of the precursor is arrested (Henning and Neupert, 1981; Henning et al, 1983).

Yeast nuclear mutants have been isolated in which the import of nuclear-coded mitochondrial proteins is blocked. The temperature sensitive *Mas1* and *Mas2* strains plus the mif (mitochondrial import function) mutants block the import of several different nuclear-coded proteins to various degrees, in an unknown manner, but cloning and characterisation of these genes should help to characterise the import process further (Glick and Schatz, 1991).

The biochemical analysis of mutant strains in which precursors are only partially translocated across the mitochondrial membranes, and in which the membranes have been solubilised by urea or alkaline pH, suggest that the precursors are imported via a hydrophilic channel composed of protein subunits. One 42 KDa protein, termed import site protein 42 (ISP42) was identified by photo-crosslinking it to a partially translocated precursor (Rassow et al, 1989). Therefore, once the mitochondrial precursor proteins are identified by import receptors like MOM19 and MOM72, the precursor proteins are then thought to be passed on to generalised import site proteins at membrane contact points, where translocation occurs.

Polypeptides destined for mitochondria possess amino terminal presequences of various lengths, containing many positively charged amino acids. Amphipathic helices formed by such presequences, with the positively charged amino acids located on one side of the helix, are thought to be essential for mitochondrial targeting, and are believed to respond to the electrical membrane potential necessary for translocation of the precursor (Glick and Schatz, 1991). Presequences carry the targeting information required to direct proteins into mitochondria. This was found by constructing hybrid proteins between mitochondrial presequences and nonmitochondrial passenger proteins, that were imported into mitochondria (Van Loon et al, 1986; Hurt and Van Loon, 1986) as a result. As yet, motifs within presequences which influence its interaction with a specific mitochondrial outer membrane receptor have not been determined. In fact the presequences of

most imported mitochondrial proteins do not show extensive primary sequence homology, although most do show similar overall characteristcs i.e. rich in positively charged amino acids and hydroxylated amino acids, and a lack of acidic residues (Glick and Schatz, 1991). Mitochondrial presequences also function in intra-mitochondrial sorting into one or other of the 4 submitochondrial compartments. Proteins destined for the mitochondrial matrix are translocated directly into the matrix via contact sites . Those destined for the intermembrane space follow a more complex pathway. Most intermembrane space proteins are at first thought to be transported in their entirety into the matrix where the amino-terminal portion of the presequences are cleaved off and the protein translocated back through the inner membrane to the intermembrane space. Further processing occurs which releases the protein in its mature form (Hartl et al, 1987). Most of the intermembrane space precursors have bipartite presequences where two cleavages must occur before the mature protein is formed. However, there are exceptions.

For import into mitochondria, nuclear proteins must be in a partialy unfolded conformation so that the polypeptide chain can span both the outer and the inner mitochondrial membrane at sites where these two membranes are in contact with each other. This is achieved by the action of cytosolic proteins binding to the precursors. One so-called presequence binding factor (PBF) binds to the presequence of ornithine transcarbamylase and prevents folding prior to import (Murakami and Mori, 1990; Murakami et al, 1990). A group of cytosolic 70 KDa proteins (members of the heat-shock protein 70 family), are known to be associated with the precursor proteins and are thought to help keep the polypeptide chains in an unfolded state (Murakami et al, 1988). The hsp70 cytosolic proteins are then dissociated from the precusor as translocation across the mitochondrial membranes proceeds. All three processes, the association and dissociation of the hsp70 proteins with the precursor and the translocation of the precursor into mitochondria are ATP-dependent processes. However, it may be that not all mitochondrial precursors require cytosolic antifolding proteins for import. For

example, import *in vitro* of the artificial precursor pCOXIV-DHFR (consisting of the yeast cytochrome oxidase subunit IV presequence fused to mouse dihydrofolate reductase) is not affected by the absence of cytosolic proteins (Eliers and Schatz, 1986). Different precusors may thus exploit different strategies for attaining an import-competent conformation.

Once across the membranes, the presequences are processed by the nuclear-coded matrix peptidase, comprising the mitochondrial processing peptidase (MPP) and processing enhancing protein (PEP) (Pollock *et al*, 1988; Hawlitschek *et al*, 1988). The translocation of the major part of the precursor itself is thought to be driven by the association of the presequence upon entering the mitochondrion with the mitochondrial isoform of the cytosolic hsp70 proteins (Scherer *et al*, 1990; Kang *et al*, 1990). The mitochondrial hsp70 is encoded by the nuclear SSC1 gene, which has a high affinity for the unfolded state of the precursor and is thought to 'pull' the polypeptide chain further into the mitochondria. Once translocation is complete, the mitochondrial hsp70 is released like cytosolic hsp70 in an ATP dependent fashion leaving the precursor to become associated with another nuclear-coded heat-shock protein, hsp60 (Ostermann *et al*, 1989; Cheng *et al*, 1989). This appears to allow proper folding of the precursor and its eventual release as a mature protein into the enzyme complex to which it is destined.

### 1.5.4 ASSEMBLY OF MITOCHONDRIAL POLYPEPTIDES INTO FUNCTIONAL COMPLEXES.

Hsp60 not only plays a role in the transport of polypeptides across the mitochondrial membranes, but is also thought to act as a chaperone protein, involved in the assembly of the mitochondrial polypeptides into functional protein complexes, without being a structural part of the complex itself (Reading *et al*, 1989). The isolation of the yeast assembly mutant mif4 (*m*itochondrial *i*mport *f*unction) and its subsequent characterisation as a mutation within the gene coding for HSP60 has led to the proposal that hsp60 facilitates the correct folding of precursor polypeptides and then presents

them to the other components of the complex in a manner suitable for assembly. However the isolation of other assembly mutants showed that hsp60 does not act alone in facillitating the assembly of the complexes (Cheng et al, 1989; Reading et al, 1989).

Some subunits themselves confer stability on partially assembled subcomplexes. Subunits II, VII and VIII of Complex III of the respiratory chain, for example, are necessary for the correct assembly of the complex (Oudshoorn et al, 1987; Maarse et al, 1988; Schoppink et al, 1988; Schoppink et al, 1989a; Schoppink et al, 1989b). Mutations within subunits VII and VIII result in the proteolytic degradation of the Rieske FeS polypeptide, the apocytochrome b polypeptide and the holocytochrome b polypeptide. The mRNA levels of the three proteins, the Rieske FeS protein, the apocytochrome b protein and the holocytochrome b protein, are unchanged in these mutants (Schoppink et al, 1989a).

A mutation in the yeast nuclear gene COX10 specifically blocks assembly of cytochrome c oxidase. The COX10 product is homologous to a predicted protein (ORF1) of the cytochrome c oxidase operon of *Paracoccus denitrificans* (Raitio et al, 1987). A second nuclear gene required for oxidase assembly, COX11, is homologous to ORF3 of the same bacterial operon. Two other nuclear genes, CBP3 and CBP4, are specifically required for the assembly of Complex III cytochrome c oxidoreductase (Wu et al, 1989).

Other studies have suggested that correct assembly occurs in a stepwise fashion and requires the translation, of individual subunits in the correct order to stimulate correct assembly. This does not rule out the possibility that HSP60 or other nuclear factors or individual mitochondrial subunits confer stability on other subunits of the complex after their sequential translation and import.

## 1.6 NUCLEAR-CODED FUNCTIONS REQUIRED FOR MITOCHONDRIAL BIOGENESIS IN MAMMALIAN CELLS.

In the mammalian system, a mainly biochemical approach has been taken to analyse the various nuclear-coded functions required for mitochondrial biogenesis, in contrast to the mainly genetic approach taken with yeast. This is due firstly to the fact that whole organism mutants in oxidative phosphorylation would be lethal in aerobic organisms such as mammals and secondly to the diploid nature of mammalian cells and the absence of a true genetic system; in which most mutations would be recessive and the resultant phenotype never observed. Along with this was the difficulty, up until recently, of maintaining mammalian cells without respiratory metabolism, whereby any dominant mutations in nuclear-coded genes involved in respiration would probably be lethal to the cell. As a result the number of nuclear-coded genes identified, from mammalian cells, involved in mitochondrial biogenesis is limited. Although quite a number of the nuclear genes encoding structural subunits of the respiratory complexes have been isolated, nuclear genes encoding other components of mitochondrial biogenesis have not been readily identified.

### 1.6.1 Replication

As mentioned earlier in this chapter, mammalian mitochondrial DNA has two distinct origins of replication, the origin of heavy-strand synthesis and the origin of light strand synthesis. This is in contrast to yeast mitochondrial DNA which has several origins of replication. The origin of heavy-strand synthesis is located in the D-loop region which has evolved as the control site for both replication and transcription. Replication and transcription are closely connected: mitochondrial DNA replication arises due to the processing of an RNA precursor produced by the mitochondrial RNA polymerase to form a primer for mitochondrial DNA replication. The nuclear-coded mitochondrial RNA processing enzyme (RNase MRP) was isolated in 1987 from mouse and human mitochondrial extracts and was found to be a site-specific

endoribonuclease containing an essential nuclear-coded RNA component (Chang and Clayton, 1987). A mitochondrial DNA polymerase has been isolated from rat liver (Meyer and Simpson, 1970) and it was found that the major mammalian mitochondrial DNA polymerase is gamma. However, other factors are required mitochondrial DNA replication as under conditions in which both mitochondrial polymerases, but no other auxillary proteins are present, mitochondrial DNA replication does not occur. One of these factors could be the nuclear-coded mitochondrial transcription factor 1 (mtTF1), as mtTF1 was found to bind mitochondrial DNA near the site of cleavage of the RNase MRP enzyme (Fisher et al, 1992). Nuclear-coded topoisomerase(s) and helicases would also be required for mitochondrial DNA replication to occur. These have yet to be identified.

A methylation protection assay has been used to show a specific bovine nuclear-coded protein binds to mitochondrial DNA sequences located near the 3' end of the D-loop. The 3' end of the D-loop encompasses a relatively conserved region containing repeated A+T rich sequences elements designated the termination-associated sequences (TAS). This nuclear-coded protein could be involved, along with mtTF1, in the regulation of mitochondrial DNA synthesis. Footprinting experiments indicate that the 48 KDa nuclear-coded protein binds specifically to this region (5'-TACATTATGTCAAAT-3', 58 bp upstream of the D-loop 3' end) and not to other TAS-like elements scattered around the 3' end of the D-loop (Madsen et al, 1993).

Other trans-acting factors involved in mitochondrial DNA replication and encoded by mammalian nuclear DNA are as yet unknown.

### 1.6.2 Transcription

The mitochondrial RNA polymerase has been purified from human cells and was found to be very similar in protein composition and function to the yeast mitochondrial RNA polymerase (Chang and Clayton, 1984; Fisher and Clayton, 1985; Fisher et al,

1987). It is also assumed that like in yeast, the genes coding for the subunits of the mitochondrial RNA polymerase reside in the nucleus.

Recently, the first mammalian mitochondrial transcription factor designated mtTF1, was isolated from human cells (Fisher et al, 1992). In gel-retardation and footprinting experiments the 25 KDa mtTF1 polypeptide was found to bind directly to the mitochondrial DNA at sites upstream from both the heavy-strand and the light-strand promoters, and mutations in these areas led to a dysfunction in mitochondrial DNA transcription. The human nuclear gene for mtTF1 has now been cloned and sequenced; it is a member of the class of high-mobility-group proteins (Parisi et al, 1993). Previously identified as transcription factors in the nucleus. The homologue of human mtTF1 in yeast, is the transcription factor ABF2 (Diffley and Stillman, 1992), and mtTF1 can functionally replace ABF2 both in vivo and in vitro (Parisi et al, 1993).

### 1.6.3 RNA Processing

Unlike the yeast mitochondrial genome, the mammalian mitochondrial genome is trancribed as a single polycistronic transcript, which needs to be cleaved to release all the individual transcripts. Transfer RNAs are found flanking each of the mRNAs and this is thought to aid the processing of the large precursor transcript. An RNase P activity has been isolated from HeLa cell mitochondrial extracts (Doerson et al, 1985). Experimental evidence indicates that the mitochondrial RNase P is a homologue of the bacterial RNase P, as the mitochondrial RNase P cleaves the precursor to *E. coli* suppressor $tRNA^{tyr}$ at the same site as the *E. coli* enzyme. The mitochondrial RNase P, which, like its bacterial counterpart contains both a protein and an RNA moiety, is thought to be responsible for the endonucleolytic cleavage of the RNA transcript at the 5' side of the tRNA sequences (Doersen et al, 1985).

In contrast to yeast, mammalian mitochondrial DNA does not contain any introns and therefore genes equivalent to the

nuclear-coded mitochondrial splicing factors of yeast are not required and have not been identified.

### 1.6.4 Translation

Unlike yeast, from which many nuclear-coded genes have been identified that are involved in the translation of the mitochondrial transcripts, the homologues for these yeast genes have not yet been isolated from mammalian cells. However this is almost certainly due to the biochemical techniques which have had to be used to identify many of the mammalian nuclear-coded genes involved in mitochondrial biogenesis and the difficulty of mammalian cells in surviving without functional mitochondria.

These problems have also hindered the identification of nuclear-coded genes involved in the import of nuclear-coded mitochondrial proteins and their assembly into functional complexes.

## 1.7 PROTEINS INVOLVED IN THE EXPRESSION OF NUCLEAR-CODED MITOCHONDRIAL GENES.

Possibly the most important insight into the control of the coordinate expression of nuclear and mitochondrial respiratory genes in mammals comes from the discovery of a 68 KDa transcriptional activator known as NRF1 (nuclear respiratory factor 1) (Evans and Scarpulla, 1990). The function of NRF1 is somewhat similar to the function of HAP1 and HAP2 in yeast although whether NRF1 controls transcription constitutively or, like HAP1 and HAP2, in response to physiological conditions, is not yet known. NRF1 was initially characterised due to its ability to bind the promoter of the rat somatic cytochrome c gene. Subsequent analysis revealed the presence of functional NRF1 recognition sites in other nuclear-coded genes involved in mitochondrial respiration, namely the human cytochrome $c_1$ gene and the human ubiquinone-binding protein gene of Complex III, the rat cytochrome c oxidase subunit VIc gene of Complex IV, the ATP synthase gamma subunit gene of Complex V and the mouse and human genes encoding the RNA

moiety of the mitochondrial endonuclease MRP (Evans and Scarpulla, 1990; Chau et al, 1992). Transfectection experiments, using expression vectors containing NRF1 recognition sequences upstream of a reporter gene showed that NRF1 was able to stimulate transcription (Evans and Scarpulla, 1990; Chau et al, 1992). The presence of functional NRF1 sites in nuclear genes encoding structural mitochondrial proteins as well as in nuclear genes encoding components of mitochondrial replication suggests a method for the coordination of nuclear and mitochondrial DNA expression via the regulation of nuclear genes.

There have been other protein-binding sites exposed in the transcriptional control region of the gene for cytochrome $c_1$ and the gene for the ubiquinone-binding protein. The binding sites have been called Mt1 (5'-TATTCAGGT-3'), Mt3 (5'-ATCTGGCT-3') and Mt4 (5'-TGGTCA(T/G)AG-3') (Suzuki et al, 1990; Suzuki et al, 1991). Sequences highly homologous to these three mitochondrial sequence elements, have also been found in the 5' flanking regions for the beta subunit of human $F_0F_1$-Atpase and rat somatic cytochrome c. The Mt1 element is also similar to the GFII recognition sequence (5'-RTCACGTG-3') found in the yeast nuclear-coded genes for three subunits of Complex III (Suzuki et al, 1990; Dorsman et al, 1991). Sequences homologous to Mt3 and Mt4 have also been found in the D-loop and promoter regions of the mammalian mitochondrial DNAs (Suzuki et al, 1991). Gel retardation experiments disclosed that the same nuclear-coded factors from HeLa cell extracts bind to the mt sequence elements in both the nuclear-coded genes and the mitochondrial D-loop, suggesting that the Mt sequence elements, may play an important role in the coordinate expression of the two physically separated genomes during mitochondrial biogenesis (Suzuki et al, 1990; Suzuki et al, 1991).

A tissue-specific transcriptional element termed the OXBOX has been identified in the promoter of the human heart-skeletal muscle ADP/ATP translocator gene (ANT1) and the human ATP synthase beta subunit gene (ATPaseB) (Li et al, 1990). This element (5'-GGCTCTAAAGAGG-3') enhances transcription in muscle

cells but not in HeLa cells suggesting that it is muscle-specific, and initial gel-retardation experiments revealed a factor from C2C12 myogenic cells which specifically binds to the OXBOX element in the promoter of the ANT1 gene.

Subsequent gel-retardation experiments on the OXBOX element has revealed two unique but overlapping elements in this region, the 13bp OXBOX element and an 8bp REBOX element (Chung et al, 1992). The OXBOX element was found to bind factors only from myogenic cell-lines whereas the REBOX element bound factors from HeLa extracts as well as factor from myogenic extracts suggesting that the REBOX binding factors are ubiquitous. The binding of the REBOX factors was found to be sensitive to NADH and thyroxine, suggesting that the REBOX element may modulate oxidative phosphorylation gene expression in response to enviromental and hormonal changes (Li et al, 1990; Chung et al, 1992).

## 1.8 THE CHINESE HAMSTER GAL⁻ CELL-LINES.

The global aim of the project set out here was to try to devise a method for isolating and characterising mammalian nuclear genes which are involved in creating functional mitochondria. The closest equivalent in mammals to the yeast PET mutants are the respiration-deficient Chinese hamster cell-lines isolated by a number of laboratories. Due to the short generation time of mammalian cells in culture, the attainablilty of large population sizes and the develop ment of pure lines through single cell cloning, and the presence of a number of different cell-lines ease the process of gene manipulation and mutagenesis. However, with the Chinese hamster cells being diploid, it makes it difficult to perform a real genetic analysis on these cells in contrast to yeast which are haploid at the sporocyte stage in their development.

The Chinese hamster cell-lines from which most respiration-deficient mutants have been derived are from two different lung-tumour cell isolates, V79 and CCL16. The primary cultures of these cell-lines were diploid, but in culture some chromosomal rearrangments and loss of DNA has occurred, and

although not extensive is presumed to have made regions of the nuclear genome effectively haploid (Chu, 1985). Single cell cloning and the ability to store cells frozen have contributed to substantially alleviating the potential problem of DNA loss through prolonged growth. However, one must always be aware of the fact that spontaneous variation of somatic cells *in vitro* has been observed.

The two cell-lines, V79 and CCL16, and a number of mutagenesis methods were used by various laboratories to generate a number of different auxotrophic mutant strains. The method most employed being the 'Lethal growth method' (Puck and Kao, 1967; Kao and Puck, 1968; Chu *et al*, 1972; Chu, 1974; Sun *et al*, 1975). The wild-type strains V79 and CCL16 were exposed to a mutagen, and grown in nutritionally deficient medium containing 5' bromodeoxyuridine (BUdR). Cells which were not affected by the mutagen and grew normally incorporated the thymidine analogue and later were preferentially eliminated because of their increased sensitivity to UV light. The mutated cells which were unable to grow because of the nutritional deficiency remained static through this process and were then recovered by adding nutritionally sufficient medium. Many mutant cell-lines were isolated by this procedure including respiration-deficient mutants ($gal^-$ mutants) which were isolated due to their inability to grow on the fermentable substrate galactose substituted for a high concentration of glucose (Chu, 1974; Sun *et al*, 1975). Permeation of galactose into the cells was found to be unimpaired in the mutants, based on studies on their rate of uptake of radioactively labelled galactose (Sun *et al*, 1975).

The respiration-deficient mutations were shown, by the formation of hybrids between wildtype and mutants cells, to be recessive. The isolation of recessive mutations within an essentially diploid cell-line could be explained by the presence of effective hemizygosity in chromosome regions, for example the X-chromosome in male derived cell-lines, like V79 and CCL16, regions of irreversible heterochromatization, or regions of chromosomal rearrangements or loss. The Chinese

hamster cell-lines, as mentioned earlier, are known to exhibit spontaneous deletions and rearrangements causing hemizygosity in the autosomes, probably resulting in the ability to isolate the respiration-deficient recessive mutants. This would lead to the assumption that, predominantly, genetic markers located in deleted regions of the nuclear genome or in regions of functional hemizygosity, would be isolated.

More $gal^-$ mutants were isolated from V79 cells (63) than from CCL16 cells (3), which could indicate that V79 cells contain more regions of hemizygosity than CCL16 cells. It may also be significant that V79 cells have a lower rate of oxygen consumption and lower citric acid cycle activity than CCL16 cells (Ditta et al, 1976). Several gene loci may be involved during selection for a single altered phenotype, eg. the ability to utilise exogenous galactose. In the respiratory deficient $gal^-$ mutants there was a reduction in the activities of one or more of the following; $O_2$ uptake, citric acid cycle activity, succinate dehydrogenase, Complex I, Complex III, mitochondrial protein synthesis and a deficiency in the level of ubiquinone.

The phenotype of these $gal^-$ mutant cell-lines was found to be stable in cell progeny in the absence of selection. Intercistronic complementation was exhibited in cell-hybrid experiments, where cell fusion was induced between $gal^-$ mutant cells, leading to the conclusion that nearly all the mutations in the mutant strains so far examined behaved like recessive characters (Chu, 1974; Soderberg et al, 1979; Maiti et al, 1981) . Karyotypic analysis of $gal^+$ hybrid cells produced from fusion experiments between the Gal 2 $gal^-$ mutant strain (a temperature-sensitive strain deficient in $O_2$ uptake) and human lymphocytes identified the transfer of the human chromosome 2. Electrophoretic analysis identified the human galactose-1-phosphate uridyl transferase (gal-1-PUT) in the hybrid cells (Sun et al, 1975).

Cell fusion experiments, in which the $gal^-$ strains isolated by the groups of Chu (Chu, 1974), Scheffler (Soderberg et al, 1979) and Thirion (Maiti et al, 1981) were crossed with each

other enabled a complementation map to be drawn up. Seven complementation groups (groups I to VII) were identified by Chu's mutants, into which fell most of Scheffler's and Thirion's mutants, although two additional complementation groups were identified by Thirion (groups VIII and IX) (Table 1.2). The complementation map was found to be linear in which the complementing mutants were connected to one another by a series of non-complementing mutants, and Chu has suggested that the order of the complementing groups in the map is the same as the order of the corresponding mutational sites in the genetic map. Chu (1974) proposed that the overlapping complementation pattern may be due to interallelic complementation of missense mutations in single cistrons which code for multiple subunits of a protein. The formation of aggregates by the subunits are then thought to correct conformational defects in the partners' polypeptide chains during assembly. Another possible explanation is that complementing units may be linked and transcribed into a polycistronic mRNA (although unprecedented in eukaryotic RNA), and the mutation in the overlapping unit may involve the incorrect processing of the mRNA thereby affecting the activity of two or more mRNAs (Maiti et al, 1981). One gal$^-$ mutant Gal 20, did not complement mutants in either complementation groups I or II and was though to represent a double mutation or small deletion as this strain never reverted back to a wildtype phenotype even after being subjected to chemical mutagens (Soderberg et al, 1979). Hybrid formation for Gal 20 with mutants in complementation groups I and II was shown to occur at the same frequency as other cell-lines by introducing other selectable markers, like resistance to ouabain and thioguanine, which can be assayed, indicating that non-complementation was not due to a lack of cell fusion (Soderberg et al, 1979).

Biochemical experiments identified more specific defects in individual members of the complementation groups. Whitfield and co-workers (1981) examined 6 of the many such gal$^-$ mutants isolated by Chu from V79; Gal 3, Gal 13, Gal 17, Gal 49, Gal 50 and Gal 73, each being a member of a different complementation group. All these mutants were found to be

deficient in the respiratory chain. However, they were also reduced in one or more of a number of other capacities relating to respiration. Gal 13 and Gal 50 were both found to be defective in Complex I and did not complement one another when fused. Even though they did not complement one another, they behaved differently in fusions to Gal 49, which, Gal 50 complements, but Gal 13 does not. Gal 49 is deficient in Complex III and in fusion experiments does not complement Gal 73 which is also deficient in Complex III. However,

Gal 50 complement Gal 73 (Fig. 1.3). The bold lines which overlap indicate non-complementation. All the gal$^-$ mutants isolated to date are listed in Table 1.2 with details of their biochemical deficiency and notes on complementation.

The gal$^-$ mutants were isolated from V79 at a high frequency after mutagenesis; 1-15% of the surviving cells would not grow on exogenous galactose (Chu et al, 1972; Sun et al, 1975). Due to this high frequency of isolation and the observation that these mutants undergo spontaneous reversion at a frequency of 1 in $10^5$ to $10^7$, led to the belief that the pleiotropic mutant phenotypes exhibited by the gal$^-$ cell-lines were caused by a single base mutation of nuclear origin (Sun et al, 1975). The simplest explanation for the cause of a pleiotropic phenotype by a single base change is that it occurs in a nuclear gene which differentially regulates the expression of a number of other genes, like NRF1 that enhances transcription in a number of nuclear-coded subunits of different complexes of the respiratory chain (Evans and Scarpulla, 1990).

Although about 70 mammalian gal$^-$ mutants have been isolated these fall into a relatively small number (12) of complementation groups, indicating that many of the mutants isolated will be the same. In contrast, in yeast over 400 complementation groups of respiration-deficient mutants have been identified. This large number is almost certainly due mainly to the ability of yeast to grow anaerobically as well as aerobically. Most of the mammalian gal$^-$ cell-lines have a leaky phenotype, and this has contributed to the ease of their isolation. But the requirement for both a leaky phenotype and hemizygosity in the genome means that only a narrow range of mutants would be isolated by the methods available at the

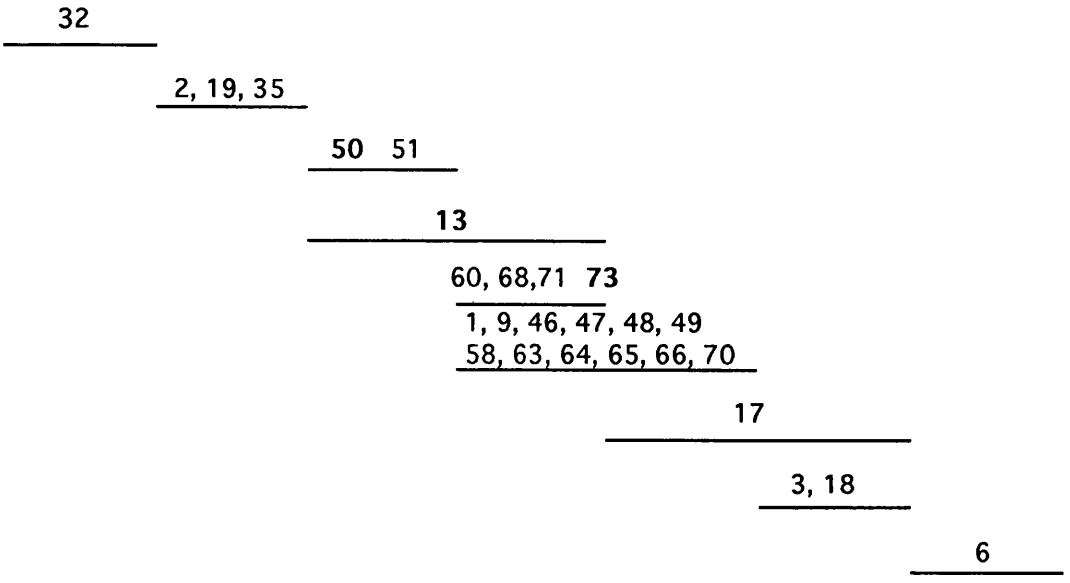



Fig. 1.3 Complementation map of 27 Chinese hamster gal$^-$ mutant V79 cell-lines based on cell fusion experiments (Chu, 1974). Overlapping lines depict non-complementing groups of mutants. Lines which do not overlap depict mutants which will complement one another. The numbers show the mutant cell-lines without the Gal prefix eg. 32 depicts Gal 32 etc.

Table 1.2 Assignment of Chinese hamster gal$^-$ mutants to complementation groups, with notes on complementation and biochemical deficiency (taken from Whitfield, 1985). All the mutants were derived from the V79 cell-line, except those designated CCL16. (P.T.O)

| Complementation group | mutants | Notes on complementation | Biochemical Deficiency |
|---|---|---|---|
| I | V79-G4,G5,G6,G8,G9, G10,G12,G13,G19,G21 G22,G23,G26,G28,G32, G37,G38,G43,G49,G50, G52 | | Complex I |
| I-II | V79-G20 | Does not complement groups I, II except complements G8 | Complex I |
| II | CCL16-B2<br>V79-G14 | | Complex I<br>Complex I |
| III | CCL16-B10<br>V79-G18,G35 | | Deficient in $O_2$ uptake, citrate cycle activity |
| IV | CCL16-B9 | | Succinate dehydrogenase |
| V | V79-G7 | | Mt protein synthesis |
| VI | V79-G11 | | Complex I |
| VII | V79-G29 | | Complex I |
| VIII | A13G9<br>34A13G32 | | Complex I |

Table 1.2 (cont.)

| Complementation group | Mutants | Notes on complementation | Biochemical Deficiency |
|---|---|---|---|
| XI | 2A13G14 | Does not complement Gal 14 Gal 11 and Gal 20 | Complex I |
| Gal 50 | Gal 50, 51 | Does not complement Gal 13 | Complex I |
| Gal 13 | Gal 13 | Does not complement Gal 50, 49 complements groups I-VII | Complex I |
| Gal 49 | Gal 1,9,46,47,48,49 58,63,64,65,66,70 | Does not complement Gal 13,17,73 | Complex III |
| Gal 73 | Gal 60,68,71,73 | Does not complement Gal 49 | Complex III |
| Gal 17 | Gal 17 | Does not complement Gal 3, 49 | Ubiquinone |
| Gal 3 | Gal 3, 18 | Does not complement Gal 17 | Ubiquinone |
| Gal 32 | Gal 32 | | Mt protein synthesis |
| Gal 5 | Gal 4,5,6 | | Similar to Gal 32, Mt protein synthesis |
| Gal 19 | Gal 2,19,35 | Temperature sensitive for growth on galactose | Temperature sensitive for $O_2$uptake |

time. However, Attardi et al (1988) have been able to emulate anaerobic growth conditions in HeLa cells, when mitochondrial DNA is eliminated using ethidium bromide, by modifying the growth media to maintain cell viability. Using the modified growth conditions, isolation of mutations which otherwise would be lethal, could occur and be analysed.

## 1.9 AIMS OF THE PROJECT.

1) It was decided to investigate in more detail several of the Chinese hamster gal$^-$ cell-lines described above in order to characterise the molecular defect in mitochondrial biogenesis, which manifests as the pleiotrophic phenotypes observed.

2) Three particular gal$^-$ cell-lines were selected for this study: Gal 13, Gal 50 and Gal 73. The initial study involved an analysis of mitochondrial protein synthesis in these cell-lines in order to determine whether the (nuclear-determined) biochemical defect was associated with abnormal mitochondrial gene expression, and if so, at what level. The three cell-lines above were selected because a) they all represent different complementation groups and b) although Gal 13 and Gal 50 belong to different complementation groups, biochemical studies have shown that both have a defect in Complex I.

3) The second aim of the project was to use complementation, via DNA-mediated gene transfer, to characterise and isolate wildtype genes which can restore gal$^-$ cell-lines to respiratory competence. Gal 73 and Gal 13 were selected for this task, because the protein synthesis analysis performed on these two cell-lines suggested that nuclear-coded mutant gene Gal 13 cells is involved in mitochondrial protein synthesis whereas the mutant gene in Gal 73 cells is not.

In undertaking this task a number of strategies were considered/attempted, including transfer of high molecular weight genomic DNA, Chinese hamster wildtype genomic DNA co-transfected with a selectable marker, or cloned Chinese hamster cDNA or genomic DNA from wildtype cells. One essential condition for such a strategy to succeed is that the

transfecting DNA should be marked in some way, so as to permit it to be easily reisolated from successful transformants by marker rescue or screening libraries for marker sequences. An alternative approach considered was a type of sib-selection: analysis of successively smaller pools of cloned DNAs for complementing activity.

## 1.10 ISOLATION OF MUTANT GENES BY COMPLEMENTATION.

DNA-mediated gene transfer is a powerful technique that can be used to restore wildtype phenotype to mutant cell-lines. Although many applications of this technique abound, complementation of mutations is perhaps the most widely used. Cloned or uncloned DNA can be transferred from cell to cell and genomic transfers are routinely achieved in several mammalian cell-lines such as HeLa, mouse L, mouse 3T3, african green monkey CV-1, CHL and CHO cells (reviewed by Gorman, 1985). Transformation frequencies vary however, depending on the cell-lines being used, and transformation efficiencies of 0.001% - 50% have been reported using different cell-lines under a variety of experimental conditions (Gorman, 1985; Chen and Okayama, 1987).

There are many methods which can be employed to mediate DNA transfer into cells including $CaPO_4$-DNA precipitation (Graham and Van der Eb, 1973; Graham et al, 1974), DEAE dextran (Milman and Herzberg, 1981; Sompayrac and Danna, 1981), protoplast fusion (Schaffner, 1980), microcell-mediated gene transfer (Fournier and Ruddle, 1977), microinjection (Cappechi, 1980; Anderson et al, 1980) and electroporation (Neumann et al, 1982), Before electroporation was developed the most widely used technique was the $CaPO_4$-DNA precipitation technique. This approach has been used for example, to transfer the purified Herpes simplex thymidine kinase gene to mouse L cells lacking thymidine kinase activity, using isolated viral DNA (Bacchetti and Graham, 1977; Maitland and McDougal, 1977) or the isolated HSV-tk gene fragment (Wigler et al, 1977). Many other genes have been isolated using this method including the alpha-amanatin resistance gene (Ingles and Shales, 1982), the adenine

phosphoribosyl transferase gene (Wigler et al, 1979), the hypoxyanthine phosphoribosyl transferase gene (Peterson and McBride, 1980; Lester et al, 1980; Jolly et al, 1982), the dihydrofolate reductase gene (Lewis et al, 1980; Wigler et al, 1980) and the chicken thymidine kinase gene (Perucho, 1980). Along with these, genes have been isolated whose exact physiological roles remain unclear, but have been isolated by virtue of their oncogenic activity (Goldfarb et al, 1982; Shih and Weinberg, 1982).

The $CaPO_4$-DNA precipitation method relies on the uptake by the cell of a precipitate formed between DNA, $CaCl_2$ and a solution containing phosphate ions, hence the name, $CaPO_4$-DNA precipitation. This technique can be used to introduce any DNA into mammalian cells for transient expression, or to achieve long-term transformation, provided that regulatory sequences appropriate for gene expression in mammalian cells are present in the donor DNA (Gorman, 1985).

The passage of the exogenous DNA from the cytoplasm to nucleus of the recipient cell has not been extensively examined. However, it is known that the DNA must be in the nucleus to be expressed (Capecchi 1980; Loyter et al, 1982). Once in the nucleus the DNA is initially expressed transiently at a high level, which decays, leaving a smaller number of stably expressing cells. There is a lag time of about 12 hours before transient expression of exogenous DNA is detected and reaches a peak in CV-1 cells after 48 hours and then declines (Gorman and Howard, 1983). After this point, the DNA appears to become stably integrated into the genome if the cells are kept under selective conditions. Although in some cases stability of integration is not maintained when selection is absent and the integrated DNA is lost (Gorman, 1985).

The most popular method of DNA-mediated gene transfer currently is electroporation, due to a higher and more reproducible frequency of integration and expression of exogenous DNA. Electroporation can be used to transform both eukaryotic and prokaryotic cells (Shigekawa and Dower, 1988). In addition to the introduction of DNA molecules it has been

used to load cells with dyes. Electoporation has been used to transform a wide range of cell-types which have not been accessible to other methods. It involves the exposure of the cells to a pulsed electric field, which is thought to create reversible pores in the plasma membrane (Chu et al, 1987). Because of this, the type of cell has a lesser effect on the frequency of transformation than for other methods of DNA mediated gene transfer. However, several parameters need to be optimised to achieve maximum transformation efficiencies for a given cell-line such as the electric field strength, pulse wave shape and duration, and temperature.

In general the transformation frequencies obtained using electroporation, approach 1% (Chu et al, 1987) and although under some conditions, with some cell-lines, the Ca-$PO_4$ precipitation method has been reported to yield frequencies of up to 50% (Chen and Okayama, 1987), electroporation tends to be more reliable and to give more consistent results.

Introduction of the DNA into cells by chemical methods, i.e. by $CaPO_4$-DNA precipitation, usually involves entry of the precipitated DNA by pinocytosis and passage through the cytoplasm before delivery to the nucleus. As the DNA is taken up as a precipitate, multiple copies of the DNA molecule usually integrate in the host chromosome as a linear concatamer (Robins et al, 1981). Since most of the integrated DNA inserts as a single large DNA concatamer at a single site, it is difficult to achieve integration of one or a few copies of the exogenous DNA by chemical methods. Electroporation is more likely to involve the direct movement of individual DNA molecules from the extracellular space into the cytoplasm through micropores, giving a lower probability of concatamer formation and a higher probability of individual DNA molecules integrating at separate sites in the host chromosome. In a number of studies, cells transformed by electroporation incorporated single or few copies of the exogenous DNA and these copies tended to be in distinct, random sites (Neumann et al,1982; Potter et al, 1984; Riggs and Bates, 1986) This would seem to be a disadvantage for experiments requiring co-transformation of two unlinked types

of DNA. (Co-transformation is a strategy which can be used when transforming cell-lines with DNA which can not be easily selected for. It involves co-transforming cells with a cloned selectable marker as well as uncloned genomic DNA, and sequentially selecting for the cloned marker and then the genomic gene of interest). However, co-transformation by electroporation with two unlinked markers has been successfully achieved with efficiencies in the range of 23-100% of succeessfully transfected cells (Tongeguzzo et al, 1986).

In this piece of research both $CaPO_4$-DNA precipitation and electroporation have been applied with varying degrees of success using both cloned and uncloned wildtype DNA to complement the $gal^-$ mutant cell-lines in an attempt to isolate nuclear-coded mitochondrial genes.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 BACTERIAL STRAINS

**DS941** thr-1, leuB6, hisG4, thi-1, ara14, λ(gpt-proA)62, argE3, galK2, supE44, xyl-5, mtl-1, txs-33, lacY1, rpsL31, recF143, laczΔM15, $lacI^q$.
Sheratt (pers. comm.).

**HB101** supE44, hsdS20($r_B^-m_B^-$), recA13, ara14, proA2, lacY1, galK2, rspL20, xyl-5, mtl-1.
Bolivar and Blackman, 1979.

**JM101** rec A1, end A1, gyr A96, thi, hsd R17, Sup E44, rel A1, $\lambda^-$Δ(lac-pro AB)[F'Tra D36 pro AB Lac IqZΔM15].
Yanisch-Perron et al, 1985.

**JM109** recA1, supE44, endA1, hsdR17, gyrA96, relA1, thiλ(lac-proAB) F'[traD36proAB$^+$ $lacI^q$ lacZΔM15].
Yanisch-Perron et al, 1988.

## 2.2 CLONING VECTORS.

**pWE15** A pBR322-related cosmid vector, containing the SV40 promoter sequences in cis with the $neo^R$ gene from the transposon Tn 5. (Berg, 1986)

**Lorist6** A phage lambda-replication origin containing cosmid vector containing the bacterial kanamycin resistance gene. (Gibson et al, 1987)

**M13mp18** Bacteriophage vector derived from M13 and used for subcloning and to propagate ssDNA. (Norrander et al, 1983).

**pBluescript** Cloning vectors designed to simplify commonly used cloning and sequencing procedures. Contains the alpha-peptide of *LacZ* for blue-white screening for insertion. pBluescript (+) and (-) are available with two polylinker orientations. (Stratagene, 1989).

## 2.3. MAMMALIAN CELL-LINES.

### 2.3.1 CELL-LINES USED IN THE PROJECT

| | |
|---|---|
| **V79** | Chinese hamster lung cell-line. |
| **Gal 73** | $Gal^-$ mutant cell-line derived from V79. Chu *et al*, 1972. |
| **Gal 13** | $Gal^-$ mutant cell-line derived from V79. Chu *et al*, 1972. |
| **Gal 50** | $Gal^-$ mutant cell-line derived from V79. Chu *et al*, 1972. |
| **HeLa** | Human cervical carcinoma cell-line. |

### 2.3.2 CELL-LINES GENERATED IN THE COURSE OF THE PROJECT.

| | |
|---|---|
| **Gal 73 $neo^R$ cl.1** | Gal 73 stably transformed with the plasmid $pSV_2neo$, (clone 1). |
| **Gal 73 $neo^R$ cl.2** | Gal 73 stably transformed with the plasmid $pSV_2neo$, (clone 2). |
| **$Gal^+$73A** | Clone of Gal 73 rescued to wildtype phenotype by complementation (see results, section 4.2.5). |
| **$Gal^+$73B** | Clone of Gal 73 rescued to wildtype phenotype by complementation (see results, section 4.2.5). |

## 2.4. CHEMICALS

| | **SOURCE** |
|---|---|
| **GENERAL LAB CHEMICALS** | Sigma, BDH, BRL, Formachem, May & Baker, Rose Chemicals Ltd. |

| | |
|---|---|
| **RADIOCHEMICALS** | NEN. |
| **ANTIBIOTICS** | Sigma, BRL, Gibco. |
| **RESTRICTION ENZYMES** | BRL, Biolabs, Boehringer Mannheim. |
| **DNA MODIFYING ENZYMES** | BRL. |
| **TISSUE CULTURE MEDIUM** | Gibco. |
| **MICROBIOLOGICAL MEDIA** | Difco, Oxford. |
| **AGAROSES** | BRL. |

## 2.5. MICROBIOLOGICAL CULTURE MEDIUM

### 2.5.1 MEDIA

**L-BROTH** 10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose and 20mg thymine, made up to 1 litre with distilled $H_2O$ and adjusted to pH7 with NaOH.

**L-AGAR** As for L-broth without glucose but with 15g per litre of agar.

**SOFT AGAR** 2 parts L-broth to 1 part L-agar.

**2xYT** 10g tryptone, 10g yeast extract & 5g NaCl made up to 1 litre with distilled $H_2O$.

### 2.5.2 ANTIBIOTICS

All were stored as solutions at -20$^{o}$C.

| | STOCK SOLUTION | SOLVENT | WORKING SOLUTION |
|---|---|---|---|
| **Ampicillin** | 100 $mg.ml^{-1}$ | $H_2O$ | 100 $ug.ml^{-1}$ |
| **Kanamycin** | 50 $mg.ml^{-1}$ | $H_2O$ | 25 $ug.ml^{-1}$ |
| **Geneticin G418 sulphate** | 80 $mg.ml^{-1}$ | $H_2O$ | 800 $ug.ml^{-1}$ |

| | | | |
|---|---|---|---|
| **Chloramphenicol** | 100 mM | 100% ethanol | 1 mM |
| **Emetine** | 100 mM | $H_2O$ | 1mM,100 uM,10 uM |
| **Tetracycline** | 12.5 $mg.ml^{-1}$ | 50% $H_2O$/ 50% ethanol | 12.5$ug.ml^{-1}$ |

### 2.5.3 INDICATORS

X-gal (5-bromo-4-chloro-3-indolyl-B-galactoside; Sigma) was added to molten agar when required in conjunction with IPTG (Isopropyl-B-D-thiogalactopyranoside; Sigma). X-gal dissolved in dimethylformamide and IPTG in distilled water were both stored at -20°C as 20 $mg.ml^{-1}$ stock solutions and were used at a final concentration of 20 $ug.ml^{-1}$.

## 2.6 GENERAL SOLUTIONS AND REAGENTS

### 2.6.1 SOLUTIONS

**TE** 10 mM Tris/HCl, 1 mM EDTA pH8.0

**20XSSC** 3 M NaCl, 300 mM sodium citrate

**20XSET** 400 mM Tris/HCl pH7.8, 3 M NaCl, 20 mM EDTA

**Ethidium Bromide** (EtBr) 20 $mg.ml^{-1}$ stock solution

**PBSA** 5 mM $NaHPO_4$, 7.5 mM $KH_2PO_4$, 2.75 mM KCl, 170 mM NaCl pH7.2

### 2.6.2. ORGANIC SOLVENTS

**PHENOL** Phenol was equilibrated with 1 M Tris/HCl pH8.0 and 0.1 M Tris/HCl pH8.0. 0.1% 8-hydroxyquinoline was added and stock aliquots were stored under 0.1 M Tris/HCl pH8.0 protected from light at -20°C.

**CHLOROFORM** Analytical grade chloroform was mixed with isoamyl alcohol (24:1 v/v) and stored protected from light.

**ETHANOL** Analytical grade ethanol and 70% ethanol (v/v) used for DNA precipitation were stored at room temperature.

**FORMAMIDE** Analytical grade was stored protected from light at 4°C.

**OTHER ORGANIC SOLVENTS** Methanol, isopropanol, acetic acid, formaldehyde were stored protected from light at room temperature and used directly.

## 2.7 STERILIZATION

All growth media were sterilized by autoclaving at 120°C for 15 mins., supplements and buffer solutions at 108°C for 10 mins. and $CaCl_2$ at 114°C for 10 mins. Heat sensitive reagents were sterilized by filtration using disposable 0.45um membrane filters. Plasticware was sterilized by autoclaving at 120°C for 90 mins.

## 2.8 BUFFER SOLUTIONS

### 2.8.1 ELECTROPHORESIS BUFFERS

**50 x TAE** 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH8.0, made up to 1 litre with distilled water.

**10 x TBE** 109 g Tris base, 55 g boric acid, 9.3 g $Na_2EDTA.2H_2O$ made up to 1 litre with distilled water. The final pH was 8.3.

**10 x MOPS** 21 g NaMOPS (3-[Morpholino]propanesulphonic acid), 5.44 g sodium acetate trihydrate and 1.85 g EDTA, made up to 1 litre with distilled water and adjusted to pH7.0 with sodium hydroxide. The solution was stored at 4°C in the dark.

### 2.8.2 GEL LOADING BUFFERS

**SEQUENCING** 90% deionized formamide, 10mM EDTA, 10mM NaOH, 0.1-0.3% each of xylene cyanol and bromophenol blue.

**AGAROSE** 0.25% bromophenol blue, 0.25% xylene cyanol, 0.25% orange G and 25% ficoll 400.

**FORMALDEHYDE/ AGAROSE** 50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol.

### 2.8.3 ENZYME BUFFERS

BRL REact restriction buffers were generally used.

**10 x REact 1** 500 mM Tris/HCl pH8.0, 100 mM $MgCl_2$
**10 x REact 2** 500 mM Tris/HCl pH8.0, 100 mM $MgCl_2$, 500 mM NaCl
**10 x REact 3** 500 mM Tris/HCl pH8.0, 100 mM $MgCl_2$, 1 M NaCl
**10 x REact 4** 200 mM Tris/HCl pH8.0, 100 mM $MgCl_2$, 500 mM KCl

**10 x Ligation buffer** 660 mM Tris/HCl pH7.6, 60 mM $MgCl_2$, 100 mM DTT. Stored at -20°C.

**10 x Nick translation buffer** 500 mM Tris/HCl pH7.2, 100 mM $MgSO_4$, 1 mM DTT, 500 $ug.ml^{-1}$ BSA. Stored at -20°C.

**10 x Polymerase reaction buffer** 70 mM Tris/HCl pH7.5, 70 mM $MgCl_2$, 500 mM NaCl Stored at -20°C.

**5 x Reverse transcriptase buffer** 250 mM Tris/HCl pH8.3, 375 mM KCl, 50 mM DTT, 15 mM $MgCl_2$. Stored at -20°C.

### 2.8.4 PLASMID PREPARATION BUFFERS

Alkaline lysate buffers for small or large scale plasmid preparations.

**Birboim-Doly I** 50 mM glucose, 25 mM Tris/HCl pH8.0, 10 mM EDTA

**Birboim-Doly II** 0.2 M NaOH, 0.2% SDS (freshly prepared)

**Birboim-Doly III** 5 M KAc made by mixing equal volumes of 3 M potassium acetate and 2 M glacial acetic acid to a final pH of 4.8.

### 2.8.5 NUCLEIC ACID HYBRIDIZATION BUFFERS

#### 2.8.5.1 SOUTHERN BLOT HYBRIDIZATION BUFFERS

**0.5 x Denaturing solution** 0.5 M NaOH, 1.5 M NaCl

**1 x Neutralizing solution** 0.5 M Tris/HCl pH 7.5, 1.5 M NaCl

**HYBRIDIZATION BUFFER**

4 x SET
5 x Denhardt's solution
100 ug.ml$^{-1}$ denatured salmon sperm DNA
0.02 M Phosphate buffer pH6.8 (as defined below)
5% Dextran sulphate
20 ug.ml$^{-1}$ Poly A
20 ug.ml$^{-1}$ Poly C
0.1% SDS

**50 x Denhardt's solution**

1% ficoll
1% polyvinylypyrrolidone
1% BSA
1% SDS

**Phosphate Buffer pH6.8**

1 M $Na_2HPO_4$
1 M $NaH_2PO_4$
mixed together to pH6.8

**20 x SET**

3 M NaCl
400 mM Tris/HCl pH7.8
20 mM EDTA

#### 2.8.5.2 NORTHERN BLOT HYBRIDIZATION BUFFER

5% Dextran sulphate

50% formamide
5 x SET
1 x Denhardts solution
50 $ug.ml^{-1}$ Poly rA
50 $ug.ml^{-1}$ Poly rC
0.02 M Phosphate buffer pH6.8
0.1% SDS
100 $ug.ml^{-1}$ denatured salmon sperm DNA

## 2.9 TRANSFORMATION OF *E.coli*

### 2.9.1 Preparation of competent *E.coli* cells for transformation.

100 ml of L-broth was inoculated with a single colony of DS941 or JM109 and grown overnight at 37°C with shaking. 400 ul of this was added to 20 ml of fresh L-broth and grown as before for 90 mins. or until the $OD_{600}$ was 0.3. The cells were pelleted by centrifugation at 5K r.p.m for 5mins. at 4°C, and resuspended in 10 ml ice cold 50 mM $CaCl_2$. This was left on ice for 20 mins. and then respun as before. This time the cell pellet was resuspended in 2 ml of ice cold 50 mM $CaCl_2$ and used directly.

### 2.9.2 Preparation of competent HB101 cells for phage-lambda infection.

100 mls of L-broth supplemented with 10 mM $MgSO_4$, 0.2% maltose was inoculated with a single colony of HB101 cells and grown overnight at 37°C with shaking. 400 ul of this was added to 20 mls of L broth again supplemented as above and grown for 4hrs. or until the $OD_{600}$ was between 0.6 - 0.65. The cells were pelleted by centrifugation at 5K r.p.m., 4°C for 5 mins. and resuspended in 2 ml of ice cold 10 mM $MgSO_4$ and used directly.

### 2.9.3 TRANSFORMATION OF COMPETENT *E.coli* CELLS WITH PLASMID DNA.

10 ng, 1 ng, 100 pg dilutions of plasmid DNA was taken up in 20 ul of TE and added to 100 ul of competent *E.coli* cells.

After an incubation on ice of 15 mins. the cells were heat shocked at 42°C for 2 mins. and then returned to ice for a further 15 mins. The cells were subsequently plated out on L-agar plates with the appropriate antibiotic and incubated overnight at 37°C.

### 2.9.4 TRANSFORMATION OF COMPETENT JM109 CELLS WITH SINGLE-STRANDED M13 SUBCLONES.

100ng and 200ng of single-stranded M13 DNA was made up to 20ul with TE and added to 100ul of competent JM109 cells. The transformation proceeded as for the transformation with plasmid DNA but was mixed into soft agar with 20ul IPTG (28.5 $mgml^{-1}$), 20ul X-gal (40 $mgml^{-1}$) and 100ul of JM109 from a fresh overnight culture before being plated on L-agar plates and incubated at 37°C overnight.

### 2.10 PLASMID PREPARATIONS.

Both large and small scale preparations of double-stranded covalently closed circular plasmid DNA were conducted following the alkaline lysis protocol of Birboim and Doly (1979).

### 2.10.1 LARGE SCALE PREP.

100 mls of L-broth with the appropriate antibiotic was inoculated with a single colony and grown overnight with shaking at 37°C. The cells were pelleted by centrifugation at 5K r.p.m., 4°C for 10 mins. The pellet was resuspended in 4 mls Birboim-Doly I solution and incubated on ice for 5 mins. 8 mls of freshly prepared Birbiom-Doly II solution was added, mixed and left on ice for 4 mins. 6 mls precooled Birbiom-Doly III was mixed gently into the cell lysate until the viscosity had dropped and a white flocular precipitate appeared. This was separated from the lysate by centrifugation at 15K r.p.m., 4°C for 20 mins. with no brake. The supernatant was poured though a funnel plugged with siliconized glass wool into 12mls of isopropanol. This was mixed well and the DNA allowed to

precipitate at room temperature for 15 mins. The DNA was pelleted by centrifugation in a prewarmed rotor at 15K r.p.m., 20°C for 10 mins. and then washed with 70% ethanol, dried and resuspended in 7 mls TE ready for purification by CsCl density gradient centrifugation.

**2.10.2 SMALL SCALE PREPS.**

1.5 mls of fresh overnight culture of cells transformed with plasmid was centrifued in a benchtop microfuge for 1 min. The cell pellet was resuspended in 100 ul ice cold Birbiom-Doly I solution and lysed with 200 ul of freshly prepared Birbiom-Doly II solution. The lysate was incubated for 5 mins. on ice before 150 ul of ice cold Birbiom-Doly III solution was added and incubated for a further 5 mins. on ice. The white flocular precipitate was removed by centrifugation for 5 mins. at 4°C in a microfuge. The supernatant was extracted with phenol and then with sevag (24:1 chloroform:isoamyl alcohol) before the DNA was precipitated with ethanol. Depending on the size of the DNA pellet it was generally resuspended in 20 - 50 ul of TE containing 20 $ug.ml^{-1}$ RNase A.

**2.10.3 DNA PURIFICATION BY CsCl DENSITY GRADIENT CENTRIFUGATION.**

To the resuspended plasmid DNA 1 g of CsCl and 50 ul (10 $mg.ml^{-1}$) EtBr was added per ml of plasmid solution. The density was assessed and more CsCl or sterile TE was added to bring the density to approximately 1.58=0.01 g. This was then loaded into quick seal tubes (Beckman), balanced, sealed and set up in the rotor (VTi 50 or VTi65). Centrifugation at 49K r.p.m., 20°C lasted for 16 hours. The plasmid band was isolated, extracted twice with isopropanol and dialysed for 16 hrs. in 5 l of TE with 2 changes of TE. The DNA was then precipitated in ethanol and taken up in an appropriate volume.

**2.10.4 PREPARATION OF SINGLE-STRAND M13 DNA**

To 100 mls of L-broth, 1 ml of fresh overnight JM109 was added along with a single plaque. This was incubated at 37°C with

shaking for 6 hours. 30 mls was centrifuged at 10K r.p.m. for 5 mins. to remove the cell debris and then the supernatant respun for a further 5 mins. 5 ml of 20% PEG, 2.5 M NaCl solution was added to the supernatant and left to stand at room temperature for 15 mins. This was centrifuged again for 5 mins. at 10K r.p.m. and the supernatant discarded. The phage pellet was resuspended in 100 ul of sterile TE and extracted with 50 ul of phenol and 50 ul of sevag. The phage DNA was ethanol precipitated overnight before being pelleted, dried and resuspended in 50 ul TE buffer. The DNA was stored at -20$^{o}$C until needed.

## 2.11 GEL ELECTROPHORESIS.

### 2.11.1 AGAROSE GELS FOR THE SEPARATION OF DNA MOLECULES.

1%, 0.7% and 0.3% horizontal agarose gels were used to separate DNA molecules according to their size. 2ul of agarose gel loading buffer was added to the DNA samples prior to loading and the gel was run overnight at 30V or for 4 hours at 150V.

### 2.11.2 FORMALDEHYDE AGAROSE GELS FOR THE SEPARATION OF RNA MOLECULES.

3 g of ultra-pure agarose was melted in 200 ml $dH_2O$ and allowed to cool to 60$^{o}$C. 15 ml of 20 x MOPS (running buffer) and 50 ml of 37% formaldehyde solution was added and the volume made up to 300 ml with $dH_2O$, before casting the gel. 1 x MOPS was used as the running buffer. Prior to loading the RNA sample (in 4.5 ul of DEPC treated $dH_2O$), 2 ul of formaldehyde/agarose gel loading buffer, 3.5 ul formaldehyde and 10 ul formamide was added, and the mixture heated to 55$^{o}$C for 15 mins. and then cooled on ice. The gel was run overnight in a fumehood at 30V.

### 2.11.3 SDS-POLYACRYLAMIDE GELS FOR THE SEPARATION OF PROTEINS.

Cell (protein-containing) lysates were separated using a Tris/HCl/glycine buffer system in combination with a 12% SDS-

polyacrylamide gel, following the protocol of Laemmli (1970).

### 2.11.3.1 STOCK SOLUTIONS

**30% acrylamide solution** 30% acrylamide , 0.8% bis-acrylamide.

**Tris buffers** 1 M Tris/HCl pH8.8 for the resolving gel, 0.5 M Tris/HCl pH6.9 for the stacking gel.

The resolving gel was prepared as follows.

12% acrylamide solution, 0.375 M Tris/HCl pH8.8, 12.5% glycerol and 0.15% SDS. This was degassed in a vacuum system for 30 mins. prior to the addition of the polymerization catalysts.

The stacking gel was prepared as follows.

5.8% acrylamide solution, 0.06 M Tris/HCl pH6.9, 0.15% SDS

The polymerization catalysts used were ammonium persulphate (AMPS) and N'N'NN-tetramethylethylene diamine (TEMED). The amounts used for the polymerization of the stacking and resolving gels were as follows.

**Stacking gel** 0.03% AMPS, 2% TEMED
**Resolving gel** 0.012% AMPS, 0.4% TEMED

Electrophoresis was carried out using a constant voltage of 30V for 16-20 hours. The electrophoresis buffer used was 0.025 M Tris base, 0.192 M glycine and 0.1% SDS. 50 ul of 1% bromophenol blue was added to the top tank buffer before the current was turned on.

### 2.11.3.2 Preparation of samples for polyacrylamide gel electrophoresis

Tissue culture cells (approx. 2 x $10^5$ cells) were lysed in 500 ul of SDS sample buffer. 20 ul of this was heated to 100°C for 10 mins. prior to loading on the polyacrylamide gel.

**SDS SAMPLE BUFFER** 2.3% SDS, 3.5 mM Tris, 10% glycerol
5% 2-mercaptoethanol

### 2.11.4 PULSED-FIELD GEL ELECTROPHORESIS

A 1% agarose gel was prepared in 0.5 x TBE buffer. The gel was run in 5 litres of cold 0.5 x TBE buffer for 12 hours at 135V. The current was switched at a ratio of 3:1 i.e. 3 seconds forward and 1 second back.

### 2.11.5 VISUALIZATION OF DNA OR RNA IN AGAROSE AND FORMALDEHYDE/AGAROSE GELS.

30 ul of 20 $mg.ml^{-1}$ EtBr was added to approximately 200 ml of $dH_2O$ and the gels soaked in this for 20 mins. They were either destained in 200 ml of $dH_2O$ for a further 20 mins. (agarose gels) or overnight (formaldehyde/agarose gels) and then photographed using a polaroid camera with red safety filter. Alternatively, for non formaldehyde/agarose gels the EtBr was added straight to the molten agarose (5 ul of 20 $mg.ml^{-1}$ per 150 ml agarose).

### 2.11.6 VISUALIZATION OF PROTEINS ON POLYACRYLAMIDE GELS.

Gels were stained by soaking in a Coomassie Brilliant Blue solution (0.1% Coomassie Blue R250, 50% methanol, 10% acetic acid) for 2 hours or until sufficiently stained, and then destained in 10% methanol, 10% acetic acid overnight. If setting the gel up for fluorography, marker tracks were cut off and stained as above and the rest of the gel soaked for an hour in $En^3Hance$ fluorography enhancer (Dupont-NEN). The gel was then washed in $dH_2O$ for 30 mins., dried with heat under vacuum, fluorographied against Kodak film for an appropriate

length of exposure.

## 2.12 GENERAL NUCLEIC ACID PURIFICATION TECHNIQUES.

Nucleic acids were deproteinised using tris-saturated phenol-sevag (50:50) followed by sevag. In both cases extractions were carried out by adding an equal volume of the solvent, mixing by inversion and centrifugation for 3 mins. in a microfuge for small samples or for 20 mins. at 5K r.p.m in a benchtop centrifuge for larger samples. This separates the organic phase where the proteins are retained and the aqueous phase. The residual solvent from the aqueous phase was removed by dialysis in TE buffer or by ethanol precipitation.

## 2.13 ETHANOL PRECIPITATION OF NUCEIC ACIDS.

To concentrate or to remove organic solvents from nucleic acid samples ethanol precipitations were performed. A 1/10th volume of 3 M sodium acetate pH6.0 and 2.5 volumes of absolute ethanol were added and the samples left to precipitate for at least an hour at -20$^{o}$C. The DNA or RNA was then recovered by centrifugation at 5K r.p.m. for 20 mins. in a benchtop centrifuge for large samples or 15 mins. in a microfuge for smaller samples. The nucleic acid pellet was then washed in 70% ethanol, dried under vacuum and resuspended in an appropriate volume of TE or $dH_2O$.

## 2.14 DNA SEQUENCING.

Single-stranded M13 DNA sequencing was performed using the Sequenase version 2.0 sequencing kit (United States Biochemicals, USB) and following the stated protocol. All the solutions in the kit were ready to use unless stated otherwise in the protocol, and stored at -20$^{o}$C until required. No modifications to the protocol were made.

### 2.14.1 PRIMER ANNEALING

1 ul of single-strand M13 DNA in 7 ul of $dH_2O$ was mixed with 1 ul of 17 bp sequencing primer (0.5 pmol/ul) and 2 ul of 5 x

sequenase reaction buffer (Sequenase protocol, Stratagene). The mixture was heated to 65°C for 2 mins. and then allowed to cool slowly to room temperature over a period of 30-45 mins.

### 2.14.2 LABELLING REACTION

Once the temperature of the sample was below 30°C 1 ul 0.1 M DTT, 2 ul of diluted dGTP labelling mix (diluted 5 fold with $dH_2O$: Sequenase protocol, Stratagene), 0.5 ul *-$^{35}$S dATP (10 uci/ul) and 2 ul of diluted sequenase enzyme (diluted 8 fold with enzyme dilution buffer: Sequenase protocol, Stratagene) was added. This was mixed thoroughly and incubated for 2-5 mins. at room temperature.

### 2.14.3 TERMINATION REACTIONS

For each reaction tube, 4 other tubes marked A,T,G and C were prepared containing 2.5 ul of the appropriate dideoxy termination mix (Sequenase protocol, Stratagene). These were prewarmed to 37°C for 1 min. and then 3.5 ul from the labelling reaction was added to each tube and incubated for 3-5 mins. at 37°C. 4 ul of stop solution (Sequenase protocol, Stratagene) was added to each tube and stored on ice until ready to load onto the gel.

### 2.14.4 SEQUENCING GELS

5% polyacrylamide sequencing gels were prepared as follows: 60 g of ultra-pure urea was dissolved in a solution containing 37 ml $dH_2O$, 12 ml 10 x TBE and 20 ml of 30% acrylamide, 0.8% bis-acrylamide solution. After cooling and degassing, polymerization was catalysed by the addition of 1 ml 10% ammonium persulphate and 35 ul TEMED. Before polymerization the acrylamide solution was poured between glass plates separated by conventional spacers to give a flat gel. A shark's tooth comb was used to form the wells. The gels were prerun in 1 x TBE for 1 hour. Immediately prior to loading, the samples were denatured by heating to 90°C for 2 mins., quenched on ice and then 2 ul was loaded onto the gel. Gels were run at 60W until the bromophenol blue had reached the

bottom of the gel for short runs, or 2-3 times this for long runs. The gels were fixed for 30 mins. in 10% methanol, 10% acetic acid and dried dowm before being visualized by autoradiography.

## 2.15 RESTRICTION ENDONUCLEASE DIGESTIONS.

Restriction digests were usually carried out in a final volume of 20 ul, containing between 0.25-1 ug of DNA, 2 ul of the appropriate 10 x concentration restriction digest buffer, 1 ul (8-10 units) of restriction enzyme and $dH_2O$ to take the final volume to 20 ul. Reactions were allowed to proceed for 1-2 hours at 37$^{o}$C and then stopped either by the addition of gel loading buffer or by phenol extraction and precipitation with ethanol.

## 2.16 DEPHOSPHORYLATION OF VECTOR ENDS.

Calf intestinal alkaline phosphatase (CIP: BRL) was used to dephosphorylate the 5' phosphate ends of the vector DNA. The 5' hydroxyl product cannot be ligated to a 3' hydroxyl end of DNA thus preventing the recircularization of the linearized cloning vector and improving the efficiency of cloning experiments by reducing the proportion of non-recombinant clones. An excess amount of vector DNA (20 ug) was digested by an appropriate restriction enzyme for 1 hour, prior to the addition of 5 ul (5 units) of CIP and incubation for 30 mins. at 37$^{o}$C. The dephosphorylated DNA was subsequently phenol extracted, treated with sevag and ethanol precipitated.

## 2.17 LIGATION OF VECTOR TO INSERT DNA.

$T_4$ DNA ligase catalyses the formation of a covalent phosphodiester bond between adjacent 5' phosphate and 3' hydroxyl groups in duplex DNA, thereby joining DNA fragments together. The concentration of the restriction fragments, taking into account the susequent packaging reaction, was such that the vector ends were in 2 fold excess over the insert ends. 1 ul 10 x ligation buffer, 1 ul 10 mM ATP were added to the DNA fragments and made up to 9 ul with $dH_2O$. As the

ligations performed were sticky end ligations, 1 ul of $T_4$ DNA ligase at a concentration of 1 unit per ul was then added, and the solution incubated overnight at 16°C.

## 2.18 HYBRIDISATION ANALYSIS OF NUCLEIC ACIDS IMMOBILISED ON NYLON FILTERS

### 2.18.1 SOUTHERN BLOTS

Southern blotting was carried out by a method adapted from Southern (1975) and described in Maniatis et al (1982).

After electrophoresis and photography, agarose gels were soaked in denaturing solution (0.5 x denaturing solution; see section 2.8.5.1) for 1 hour, in 1 x neutralizing solution for 1 hour and then in 20 x SSC (section 2.6.1) for 30 mins. Agarose gels which contained high molecular weight genomic DNA were soaked in 0.25 M HCl prior to soaking in 0.5 x denaturing solution, in order to promote strand scission and thereby aid transfer. DNA was transferred to Pall Biodyne nylon filters by capillary action due to 20 x SSC draining from a reservoir through the gel and the filter into subsequent layers of tissue paper. Transfer was carried out for 16 hours after which the filter was air dried and baked for 2 hours at 80°C.

### 2.18.2 NORTHERN BLOTS

After electrophoresis, formaldehyde agarose gels were immediately set up for capillary blotting as for southern blots, but without the denaturing/renaturing soaking steps.

### 2.18.3 AQUEOUS HYBRIDIZATIONS

After baking, the nylon filter was prehybridized in aqueous hybridization buffer (sections 2.8.5.1/2; approx. 1ml buffer per 1 $cm^2$ filter) for 1 - 4 hours prior to the addition of the radioactive probe. The DNA to DNA hybridization was carried out at 68°C overnight in the same aqueous hybridization solution. Hybridisations were carried out in a shaking water

bath.

### 2.18.4 50% FORMAMIDE HYBRIDIZATIONS.

After transfer and baking the filters were soaked for 20 mins. in 4 x SET, 0.1% SDS before being prehybridized for at least 1 hour in 50% formamide hybridization buffer (see section 2.8.5.2) at 42$^{o}$C. The denatured radioactive probe was then added to the filter and hybridization was carried out overnight at the same temperature.

### 2.18.5 PROBING M13 SUBCLONES.

2 ul of each of the phage supernatants from the M13 subclone stocks were spotted onto sterile $dH_2O$ saturated biodyne membranes. The filter was then denatured in 0.5 x denaturing solution (section 2.8.5.1) for 30 secs., neutralised in 1 x neutralising solution (section 2.8.5.1) for 1 min. and then soaked twice in 4 x SSC (section 2.6.1) for 5 mins, prior to baking at 80$^{o}$C for 2 hours.

The filters were prehybridized, hybridized with a radioactive probe and washed as for southern blots.

### 2.18.6 WASHING OF FILTERS.

The filters from both types of blotting were washed twice in 5 x W (5 x SSC, 0.1% SDS) for 20 mins. at 65$^{o}$C, twice in 1 x W (1 x SSC, 0.1% SDS) for 20 mins. at 65$^{o}$C and twice in 0.1 x W (1 x SSC, 0.1% SDS) for 20 mins. at 65$^{o}$C before being sealed moist, in plastic and set up for autoradiography.

## 2.19 PREPARATION OF $^{32}$P LABELLED RADIOACTIVE PROBES.

### 2.19.1 NICK TRANSLATION.

Nick translation was carried out by the proceedure of Rigby et

al (1977). The labelling mixture was set up as follows:

5 ul 10 x NT buffer
24 ul $dH_2O$
1 ul 500 $ug.ml^{-1}$ DNA
1 ul 20 mM 2-mercaptoethanol
10 ul 5 x NT-N (500 uM deoxynucleotide mix without dATP i.e. the labelled nucleotide.)
50 uCi $^{32}P$ dATP (Specific activity 600 Ci/mmol)
3 ul 100 $ng.ml^{-1}$ DNase 1
1 ul DNA polymerase 1

This reaction mix was incubated at 14°C for 2-3 hours before 1 ul 5 $mg.ml^{-1}$ proteinase K and 1 ul 500 mM EDTA were added. 1 ul 10 $mg.ml^{-1}$ yeast tRNA was added prior to loading on a G50 Sephadex column saturated with 2 ul 10 $mg.ml^{-1}$ yeast tRNA to separate the unincorporated radioactive dATP. The probe was then denatured by the addition of an equal volume of formamide, heating for 10mins. at 70°C and quenching on ice for 5 mins. The probe was then added to the hybridization reaction.

### 2.19.2 PREPARATION OF $^{32}P$ LABELLED SINGLE-STRAND DNA PROBE

1 ug of single-strand DNA was annealed to (200 ng) of the 17bp M13 forward sequencing primer as follows. 2 ul of single-strand DNA was mixed with 2 ul of primer, 1 ul 10 x polymerization buffer (section 2.8.3) and 5.5 ul $dH_2O$. They were incubated at 90°C for 1 min., 55°C for 5 mins. and then allowed to cool slowly to room temperature. 1 ul of $A^o$, $T^o$, $C^o$ and $G^o$ were added along with 1 ul 0.1 M DTT, 1 ul $^{32}P$ dATP (specific activity 600 Ci/mmol) and 1 ul Klenow and incubated at 50°C for 1 min prior to the addition of 1 ul 500 mM EDTA. The probe was denatured as for the nick translated probe before being added to the hybridization reaction.

### 2.19.3 PREPARATION OF $^{32}p$ LABELLED TOTAL cDNA PROBES.

50 uCi of alpha-$^{32}P$ dCTP and 50 uCi of alpha-$^{32}P$ dATP (specific activity 600 Ci/mmol) was lyopilized to a final

volume of less than 5 ul. To this 5 ul 5 x reverse transcriptase buffer (Section 2.8.3), 2.5 ul 5 mM dGTP and dTTP, 2.5 ul oligo dT primer (100 ug.ml$^{-1}$), 20 ug total RNA, 2 ul mouse MLV reverse transcriptase (400 units) was added and then the final volume made to 25 ul by $dH_2O$. This was incubated for 90 mins. at 37$^{o}$C prior to the addition of 1 ul of 10 mM dCTP and 1 ul of 10 mM dATP and a further incubation period of 60 mins. at 37$^{o}$C. 27 ul of 0.6 M NaOH, 20 mM EDTA solution was added and incubated at 65$^{o}$C for 30 mins. to hydrolyse the RNA. The cDNA probes were separated on a Sephadex G100 column equilibrated in NETS (0.1 M NaCl, 10 mM Tris/HCl pH7.5, 0.1 mM EDTA, 0.1% SDS) and used directly.

## 2.20 CELL CULTURE

### 2.20.1 CELL CULTURE MEDIUM

The mammalian cell-lines were maintained in Dulbecco-Modified Eagles Medium (DMEM) containing 10% foetal calf serum and supplemented as follows:

**DMEM-superglu**

| | |
|---|---|
| 1 x DMEM | 500ml |
| 20% glucose | 9ml |
| 4.5mg.ml$^{-1}$ asparagine | 5ml |
| 5mg.ml$^{-1}$ uridine | 5ml |
| 100mM sodium pyruvate | 5ml |
| 200mM L-glutamine | 5ml |
| foetal calf serum | 50ml |

### 2.20.2 CELL CULTURE CONDITIONS.

Cells in routine culture were maintained in 25cm$^2$ plastic tissue culture flasks (Bibby) under 10 ml of the appropriate medium at 37$^{o}$C in an atmosphere containing 5% $CO_2$, and were passed when confluent, approximately every 5-6 days. In order to pass cells the medium was aspirated off and the cells were washed in 10 ml PBSA (see section 2.6.1). The PBSA was also

aspirated off and the cells detached by treatment with 1 ml trypsin-versene (0.025% Trypsin, 500uM EDTA, 0.0015% phenol red in PBSA) for 1 min. THe cells were dislodged by tapping the flask and were dispersed in 9 ml of fresh medium. Fresh cultures were initiated with 1/10th to 1/200th dilutions made up to 10 ml with fresh medium.

### 2.20.3 SELECTIVE MEDIUM CONDITIONS.

Cell-lines under selection were maintained in DMEM-galactose medium, 10% foetal calf serum and supplemented as follows:

#### 2.20.3.1 DMEM-GALACTOSE MEDIUM.

| | |
|---|---|
| DMEM minus glucose and glutamine | 500 ml |
| foetal calf serum | 50 ml |
| 1 M galactose | 12.5 ml |
| 4.5 $mg.ml^{-1}$ asparagine | 5 ml |
| 200 mM glutamine | 10 ml |

## 2.21 PREPARATION OF HIGH MOLECULAR WEIGHT GENOMIC DNA

2 large flasks of tissue-culture cells were grown to confluence, washed in 20 ml PBSA, trypsinized using 3 ml of TV and resuspended in 7 ml of the appropriate culture medium. The cells were separated from the medium by centrifugation in sterile universals at 3K r.p.m. for 5 mins. The medium was removed by aspiration and the cell pellet resuspended in 5 mls of 1 x SSC (section 2.6.1). The cells were lysed by the addition of 5 ml of cell lysis buffer (100 mM Tris/HCl pH7.5, 100 mM NaCl, 10 mM EDTA, 1% sarkosyl) and treated with 100 $ug.ml^{-1}$ proteinase K for either 2 hours at $55^{o}C$ or overnight at $37^{o}C$. Phenol and sevag extractions were carried out and the extracted genomic DNA dialysed in 5 litres of TE for 16 hrs with two changes of TE.

## 2.22 RNA EXTRACTION

### 2.22.1 ISOLATION OF RNA FROM TISSUE CULTURE CELLS.

3 large confluent flasks of tissue-culture cells were washed, trypsinized, dispersed in culture medium and pelleted by centrifugation as for genomic DNA preparations. The cell pellet was resuspended in 8mls of guanidinium isothiocyanate solution (4.5 mM guanidium isothiocyanate, 50 mM EDTA, 50 mM HEPES pH7.0 and 5% v/v b-mercaptoethanol) and to this 1 g of CsCl was added per 2.5mls of cell homogenate. The solution was vortexed until the CsCl was dissolved. To SW 50 tubes 1 ml of a CsCl cushion solution (5.7 M CsCl, 0.1 M EDTA pH7.5, 50 mM HEPES) was added and the cell/guanidinium/CsCl homogenate layered onto it. The tubes were spun at 35K r.p.m in a SW50 rotor for 16 hours at 20$^{o}$C. The supernatant was carefully removed, the RNA pellet dried and resuspended in 200ul RNase free $dH_2O$. The RNA was stored as an ethanol precipitate until required.

### 2.22.2 POLY $(A)^+$ mRNA ISOLATION.

100 mg of oligo dT cellulose (BRL) was cleared of fines by adding 1 ml of 1 x BB and allowing the oligo dT cellulose to settle before removing the supernatant and repeating this proceedure three to four times, until the supernatant was no longer cloudy. This was then layered onto a 1 ml RNase free G100 sephadex column prepared in a 5 ml plastic syringe. 1 mg of total RNA was diluted to approximately 100 $ug.ml^{-1}$ in 1 x BB, heated to 65$^{o}$C for 5 mins., cooled rapidly on ice for 2 mins. and run through the column. 5 ml aliquots were collected, heated and cooled as before and then reloaded onto the column. This was repeated 3 times. The column was then washed with approximately 20 x the column volume of 1 x BB. The mRNA was eluted by passing RNase free $dH_2O$ in 1 ml aliquots through the column. 12 fractions were collected and the $OD_{260}$ taken of each. The fractions containing the majority of the mRNA were pooled and stored as an ethanol precipitate until required.

## 2.23 TRANSFECTION OF MAMMALIAN CELLS WITH PLASMID DNA

### 2.23.1 TRANSFECTION BUFFERS

**2.23.1.1 1 x HeBS**

5g Hepes
8g NaCl
0.37g KCl
0.125g $Na_2HPO_4$
1g glucose
$dH_2O$ to 1 litre
Adjusted to pH7.0 with NaOH
Filter sterilized

**2.23.1.2 1 x TBS**

8g NaCl
0.37g KCl
0.125g $NaH_2PO_4$
1g glucose
3g Tris HCl pH 7.4
$dH_2O$ to 1 litre
Filter sterilized

### 2.23.2 CALCIUM PHOSPHATE TRANSFECTIONS

The appropriate concentration of plasmid/genomic DNA was ethanol precipitated overnight, vacuum dessicated and resuspended in HeBS to a final concentration of 10 $ug.ml^{-1}$. To form a DNA-calcium phosphate co-precipitate a 1/15th volume of sterile 2 M $CaCl_2$ was added and the mixture was vortexed. The samples were incubated at room temperature for 20 min. until a fine precipitate had formed. The medium was removed from tissue culture cells seeded 24 hours and grown to 60% confluency. The DNA-calcium phosphate precipitate was layered over the cells and allowed to stand for 20 min. at room temperature. 10 ml fresh DMEM-superglu medium was added gently and the cells incubated at 37°C in 5% $CO_2$ for 5 hours. The medium was aspirated off and the cells were "glycerol shocked" by exposure to 20% glycerol (v/v) in TBS for 2 min. The glycerol was aspirated off and the cells were washed in 10 ml TBS. 10 ml fresh DMEM-superglu medium was added and the cells were incubated at 37°C in 5% $CO_2$ for 48 hours. The cells were

detached with trypsin-versene, dispersed in fresh DMEM-superglu medium and were counted using a haemocytometer. Several dilutions of cells were used to initiate cultures under the appropriate selection conditions. Further dilutions were also plated in non-selective medium to allow the viable cell count to be determined. The cells were incubated at 37$^{o}$C in 5% $CO_2$.

The medium was replaced after 24 hours and every 3-4 days thereafter. When colonies were visible, 3-4 days in non-selective medium, 10-14 days or longer in selective medium, the cells were either stained with Leishman's stain (1.5 g per litre in methanol) for 5 min, rinsed with water and allowed to drain, or cloned to initiate a transfected cell-line.

### 2.23.3 TRANSFECTIONS USING ELECTROPORATION.

Growing cells were washed with PBSA, detached from the flask with Trypsin-versene and dispersed in DMEM-superglu medium. The cells were counted and approximately 2 x $10^8$ cells were pelleted and resuspended in 10 ml of 1 x HeBS. The cell suspension was incubated on ice for 10 min. with the appropriate concentration of plasmid DNA prior to loading into 12 electroporation cuvettes.
The cells were exposed to a single voltage pulse at room temperature (300V, 960uFD), and allowed to remain in the buffer for 10 min. Then they were plated into 6 large tissue culture flasks (175cm$^2$) in 50 ml DMEM-superglu and incubated for 48 hrs at 37$^{o}$C in 5% $CO_2$.

Relative cell viability was measured by plating cells exposed or not exposed to the electric field in parallel flasks, allowing them to grow for 72 hrs, and then measuring relative cell number directly by counting with a haemocytometer.

After the 48 hr incubation the cells were washed, trypsinized and placed in various dilutions under the appropriate selection conditions. The transformed colonies were stained or cloned as explained previously.

### 2.23.4 CLONING OF TRANSFORMED COLONIES.

The medium was removed from the flasks and the cells washed with PBSA as before for the general maintenance of stocks. A hole was cut in the top of the flask and a chosen colony isolated using a sterile metal cloning ring coated with vaseline which had a diameter slightly larger than the colony itself. The cloning ring was filled with Trypsin-versene and the cells detached by pipetting gently up and down after 1 min. The trypsin-versene plus the cells was added to a $25cm^2$ flask containing 10 ml of the appropriate culture medium. The cells were then incubated until confluent at $37^oC$ in an atmosphere of 5% $CO_2$.

## 2.24 PREPARATION OF SIZED GENOMIC DNA FOR COSMID LIBRARY CONSTRUCTION.

### 2.24.1 Partial digestion of high molecular weight genomic DNA.

High molecular weight genomic DNA was extracted from tissue culture cells as explained previously. After dialysis, 300 ug in approximately 1.8 ml of TE was aliquoted and 200 ul 10 x REact 4 buffer was added. Dilutions of Sau3AI enzyme were made in REact 4 and added to the DNA. The samples were incubated at $37^oC$. Because of the failure of small scale serial dilutions to be scaled-up, the viscosity of the DNA was used as an indication of the extent of digestion. Therefore, the viscosity of the DNA was tested at 5 min. intervals by pipetting. Once the viscosity had started to drop the reaction was stopped by the addition of EDTA to a final concentration of 20 mM. 20 ul of each sample was run on a 0.3% agarose gel to visualize the extent of the digestion.

### 2.24.2 Sucrose-density gradient centrifugation.

38 ml 10-40% sucrose gradients were prepared in a dual chamber gradient maker 1 hr before centrifugation. 2 mls of the Sau3AI-digested DNA was layered carefully on top of the

gradient, balanced and loaded into the SW 28 rotor (Beckman). Centrifugation was carried out for 18 hrs. at 26 000 r.p.m. at 10$^{o}$C. The gradients were fractionated into 1 ml aliquots by piercing the bottom of the tube with a hot syringe needle. 20 ul of every other aliquot was checked by electrophoresis on a 0.3% agarose gel. Due to the effect of the high salt concentration in the sucrose solution on DNA mobility, high salt sucrose solution to about 30% was added to the marker tracks.

**2.24.3 Separation of DNA from sucrose.**

To each chosen aliquot 2 volumes of ethanol was added and then a further 2 ml of 70% ethanol to avoid precipitation of the sucrose. This was left at -20$^{o}$C for at least 18 hrs. The DNA was pelleted by centrifugation for 15 min. in a microfuge, washed twice with 70% ethanol, dried and resuspended in 30 ul TE. 5 ul was checked on a 0.3% agarose gel and the appropriate fractions pooled and reprecipitated with ethanol. The DNA was pelleted by centrifugation as above, dried and resuspended to give a final concentration of 1-2 ug.ml$^{-1}$.

## 2.25 $^{35}$S METHIONINE PULSE-LABELLING OF TISSUE CULTURE CELLS.

The medium was removed from cells at 60% confluency in 25cm$^{2}$ flasks and replaced with special DMEM-superglu medium which did not contain methionine, but contained $10^{-3}$M, $10^{-4}$M, $10^{-5}$M or no emetine or $10^{-5}$M emetine plus 100 mM chloramphenicol. The cells were incubated in this medium for 30 mins. prior to the addition of 250 uCi per flask of $^{35}$S methionine. The cells were incubated from 1 hr upto 24 hrs. at 37$^{o}$C in 5% $CO_2$ before the removal of the medium, washing in 10 ml PBSA and the addition of 500 ul of SDS-sample buffer. (2.3% SDS, 3.5 mM Tris, 10% glycerol, 5% 2-mercaptoethanol).

## 2.26 IMMUNOPRECIPITATION OF COMPLEX III FROM TISSUE CULTURE CELLS.

The cells were labelled with $^{35}$S methionine as above but lysed

with 500 ul of RIPA solution (150 mM NaCl, 25 mM EDTA, 10 mM Tris/HCl pH7.2, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) instead of SDS sample buffer. 110 ul of each sample was preincubated twice with 25 ul of protein-A agarose on ice for 2 hrs. The protein-A agarose beads were washed 2-3 times in RIPA solution before use. After preincubation the beads were pelleted and 100 ul of the supernatant was added to 5 ul of rabbit anti-beef heart Complex III antiserum (kindly donated by Ian Ragan, Southampton University) overnight at $4^{o}C$. 25 ul of prewashed protein-A agarose were added for 2 hrs and incubated on ice and the beads were then pelleted and the supernatant discarded. The beads were washed 5 times in 1 ml of RIPA solution and the bound proteins eluted by the addition of 25 ul SDS sample buffer and boiling for 10 mins. 20 ul of the supernatant was loaded directly onto a 12% polyacrylamide SDS gel (see section 2.11.3).

# CHAPTER 3

## ANALYSIS OF MITOCHONDRIAL PROTEIN SYNTHESIS IN RESPIRATORY MUTANT CELL-LINES OF THE CHINESE HAMSTER.

## 3.1 INTRODUCTION.

The existence of Chinese hamster cell-lines containing nuclear mutations which affect mitochondrial biogenesis and/or respiratory functions, allows us to use current biochemical and genetical technical knowledge to analyse the effects that these mutations have on the function of the mitochondrion. These mutations could occur within nuclear genes which code for one of the following; (i) a protein subunit of one of the redox complexes, (ii) a regulatory product which interacts with mitochondrial RNA or DNA, (iii) a non regulatory component of the mitochondrial DNA replication, transcription, translation or RNA processing machinery, or (iv) a component of the machinery of mitochondrial protein import or processing.

As mentioned previously in Chapter 1, studies in yeast have resulted in the isolation of respiration-deficient nuclear mutants falling into over 400 complementation groups (Michaelis et al, 1982). Of these, some 60-70 correspond to proteins which fall into the first category above. Another dozen or so code for biosynthetic enzymes for various co-factors such as ubiquinone and heme etc. The vast majority however, appear to encode funtions required for the correctly regulated expression of the very modest number of genes specified by mitochondrial DNA itself.

Identification of such a large number of complementation groups in yeast, compared with mammalian cells is mainly due to the ability of yeast to grow anaerobically as well as aerobically whereas, up until recently, no way was known whereby mammalian cells could be compensated for loss of mitochondrial respiratory function. Also yeast cells go through a haploid stage in their development whereas mammalian cells are diploid making it hard to isolate recessive mutations. Therefore the isolation of respiration-deficient cell-lines relied upon mutations being leaky, resulting in the identification of only about a dozen complementation groups (Chu, 1974; Sun et al, 1976; Soderberg et al, 1979 and Maiti et al, 1981) in contrast to the large number in yeast. Many of

the complementation groups in yeast contain mutants which have been shown to affect the regulation of mitochondrial protein synthesis.

To extend the analysis of the effects of the mutations previously identified on mitochondrial biogenesis in respiration-deficient cell-lines, a study was carried out of mtDNA-encoded protein synthesis in three mutant cell-lines Gal 73, Gal 50 and Gal 13, by treating the cells with the antibiotics emetine and chloramphenicol whose mode of action is described below. If the mitochondrial translation products of the mutant cell-lines in any way differ from those produced by the wildtype parental cell-line, V79, the affected subunit or subunits could be identified, and Northern blot analysis of the messages coding for the affected proteins could give an indication at which level, i.e. transcription or translation, the mutation is acting. Although previous biochemical studies have pin-pointed the position in the electron transfer chain at which the defects occur in the various mutant cell-lines (Sun et al, 1975; Breen and Scheffler, 1979; Maiti et al, 1981 and Whitfield et al, 1981), analysis of mitochondrial protein synthesis should, in principle enable the mutations to be placed in on of the categories mentioned above. However, the possibility cannot be ruled out that the mutation affects a nuclear-coded subunit of the respiratory chain which indirectly affects the levels of the mitochondrially encoded subunits of the complex to which it belongs, e.g. by acting posttranslationally through degradation due to non-assembly of the complex. In this instance the pattern of mitochondrial protein synthesis might be hard to distinguish from category (ii) above i.e. a regulatory component of mitochondrial DNA expression.

### 3.2 MITOCHONDRIAL TRANSLATION PRODUCTS IN GAL MUTANTS.

Cells from the parental cell-line V79, as well as from the mutants Gal 13 and Gal 50 (both of which are primarily deficient in Complex I - NADH ubiquinone reductase) and Gal 73 (primarily deficient in Complex III - ubiquinone cytochrome c reductase) were pulse-labelled with $^{35}S$ methionine for upto

24hrs in the presence of increasing concentrations of emetine from 0 to $10^{-3}$ M, and 1 mM chloramphenicol in conjunction with $10^{-5}$ M emetine. Emetine blocks the initiation of cytosolic translation, but should have little or no effect on mitochondrial protein synthesis when present at appropriate concentrations (Ching and Attardi, 1975 and Oliver and Wallace, 1982). Chloramphenicol, on the other hand, inhibits mitochondrial ribosomes, thereby blocking mitochondrial protein synthesis (Elliot, 1973; Neirhaus and Neirhaus, 1973). By taking advantage of the differential mode of action of these two antibiotics, mitochondrial translation products i.e. those which are emetine-resistant but chloramphenicol-sensitive can be studied by SDS-polyacrylamide gel electrophoresis.

Figure 3.1 shows the results of an experiment to determine the concentrations of emetine required, respectively, to block nuclear and mitochondrial protein synthesis in Gal 73 cells. Emetine at a concentration of $10^{-3}$M resulted in a blockage of all protein synthesis both nuclear and mitochondrial (Fig. 3.1, track 2). When the emetine concentration was dropped 10 fold or 100 fold, emetine-resistant polypeptide synthesis was observed with increasing abundance. Therefore, $10^{-4}$ and $10^{-5}$M emetine was used in subsequent experiments. The presumptive mitochondrial protein synthesis products synthesized in the presence of $10^{-5}$M emetine were more heavily labelled and therefore were clearer on the autoradiograph. However, in some experiments $10^{-5}$M emetine did not give total suppression of nuclear protein synthesis, but the comparison of these tracks with the $10^{-4}$M emetine-treated samples allowed a distinction to be drawn between the mitochondrial (emetine-resistant) polypeptides and the background (emetine-sensitive) polypeptides.

The emetine-resistant polypeptides observed when Gal 73 and V79 cells were labelled in this way (figs. 3.1 and 3.2) ranged, in apparent molecular weight, from approximately 67kD to 4kD. The smallest polypeptides in Fig. 3.1 i.e. those which appear to have a high mobility through polyacrylamide, have not been separated from the marker dye at the bottom of the

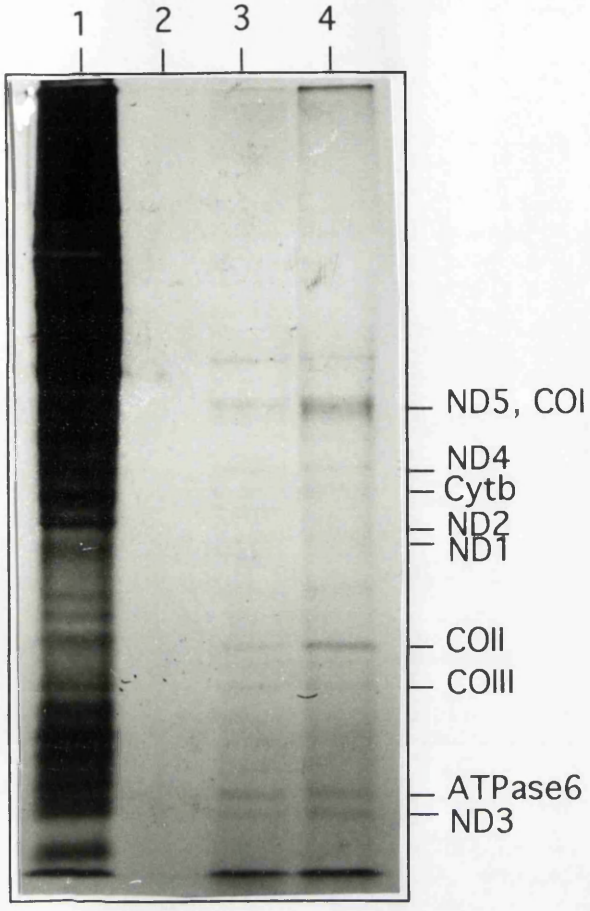



**Fig. 3.1 ELECTROPHORETIC PATTERN OF MITOCHONDRIAL TRANSLATION PRODUCTS FROM GAL 73 CELLS LABELLED *IN VIVO* WITH $^{35}S$ METHIONINE IN THE PRESENCE OF INCREASING CONCENTRATIONS OF EMETINE.**

4 flasks each containing 2 x $10^5$ cells were grown overnight in glucose medium at 37°C in 5% $CO_2$. The cells were washed in PBS solution and grown in 10 ml methionine-free glucose medium for 1 hour prior to the addition of 250 uCi $^{35}S$ methionine and no emetine (track 1), $10^{-3}$ M emetine (track 2), $10^{-4}$ M emetine (track 3) and $10^{-4}$ M emetine (track 4). The cells in each flask were then lysed with 500 ul of SDS sample buffer of which 50 ul from each sample was loaded onto a 12% SDS polyacrylamide gel. The smaller mitochondrial translation products, such as ATPase8, ND4L and ND6, are not visualised because they contain less $^{35}S$ methionine. The mitochondrially synthesised polypeptides are tentatively assigned according to their molecular weight in this gel system and by comparison to the work of Whitfield and Jefferson (1990).

gel. To get resolution of the smaller polypeptides, a higher percentage polyacylamide gel would have to be used. For Gal 73, the resolution of the smaller polypeptides was not thought necessary at the time as Gal 73 has its major loss of activity in Complex III, ubiquinone cytochrome c reductase (Whitfield et al, 1981). Of the 10 subunits of this complex only 1, cytochrome b, is mitochondrially encoded. Bovine cytochrome b has a predicted molecular weight of approximately 42 kD (Capaldi, 1982) and Chinese hamster cytochrome b an apparent molecular weight of approximately 33kD (Whitfield et al, 1990), and therefore if the mutation affects the synthesis of this subunit it would be within the molecular weight range of the polypeptides observed in Fig. 3.1. The pattern of putative mitochondrial polypeptides in this study showed a slight variation to that observed by Whitfield et al, (1990) for another mutant Chinese hamster cell-line, Gal 32, which could possibly be accounted for by differences in the gel electrophoresis conditions. Due to this similarity it was possible tentatively to assign the individual subunits observed on the autoradiographs as particular products of the mitochondrial genes. However, even if some are misassigned, the initial comparison of mitochondrial protein synthesis between wildtype and mutant cell-lines would still be informative and if differences were observed they could then be studied in greater detail and the identity of individual subunits discovered using antibodies.

Although around 13 polypeptides appeared to be synthesised in these experiments, the comparison of emetine-resistant protein synthesis in V79 and Gal 73 cells, showed no reproducible difference in those polypeptides which were synthesized, nor in their relative amounts.

Another cell-line, Gal 50 was analysed in the same way. Gal 50 was chosen because it contains a mutation which affects the activity of Complex I, NADH-ubiquinone reductase (Whitfield et al, 1981). Of the 34 subunits of Complex I so far identified, 7 are encoded for by the mitochondrial genome in mammals (Chomyn et al, 1985) unlike Complex III where only 1 subunit is mitochondrially encoded (Bibb et al, 1981; Anderson et al,

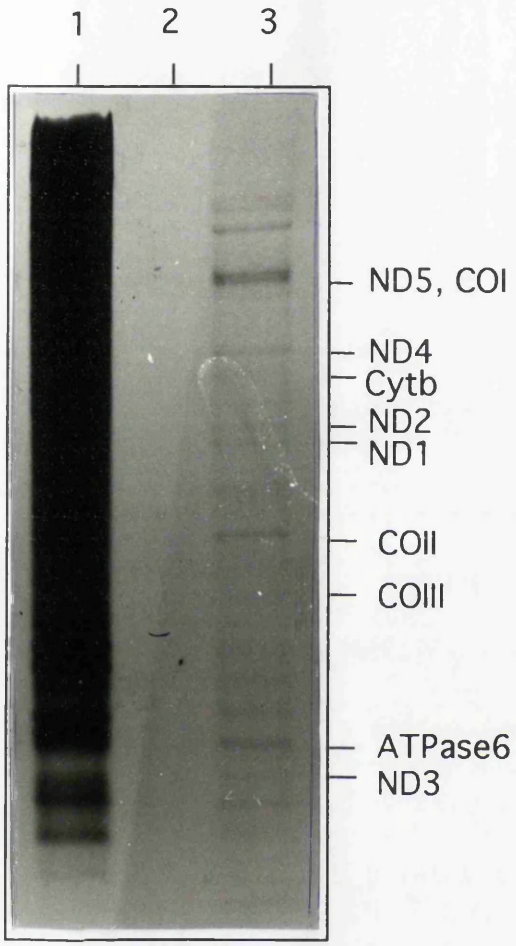



**Fig. 3.2 ELECTROPHORETIC PATTERN OF MITOCHONDRIAL TRANSLATION PRODUCTS FROM V79 CELLS LABELLED *IN VIVO* WITH $^{35}S$ METHIONINE IN THE PRESENCE OF EMETINE.**

The V79 cells were grown and labelled as for Fig. 3.1 in various concentrations of emetine; no emetine (track 1), $10^{-4}$M emetine (track 2) and $10^{-5}$M emetine (track 3). The putative mitochondrial polypeptides were identified as for Fig. 3.1.

1981 and Anderson et al, 1982).

Fig. 3.3, tracks 1 and 4 show the total protein synthesis of Gal 50 and V79 respectively. V79 cells repeatedly incorporated a higher concentration of $^{35}S$ methionine into their translation products than the mutant cell-lines Gal 73 and Gal 50. The reason for this is unknown, and is demonstrated in both figs. 3.3 and 3.4, and applies both to the polypeptides inferred as mitochondrially synthesized and to the 'background' polypeptides, presumably synthesized in the cytosol. This was seen in almost all the experiments performed and had to be taken into account when comparisons of the relative abundance of translation products were made. Although all the protein bands were compared, only those polypeptides observed in fig. 3.1 (under increased concentrations of emetine) were assumed to be mitochondrially encoded in fig. 3.3, and special attention was paid to these.

As in the case of Gal 73, the products analysed on an SDS polyacrylamide gel showed no quantitative differences between V79 and Gal 50 in the pattern and relative abundance of the mitochondrially encoded polypeptides, although the increased level in background protein synthesis present in this experiment hinders the ability to identify and quantify definitively the emetine-resistant polypeptides.

Thus in two gal$^-$ cell-lines, Gal 73 and Gal 50, which carry nuclear mutations affecting a different complex of the electron transport chain (Whitfield et al, 1981), the mutation itself does not appear to affect the synthesis or accumulation of the mitochondrially encoded components of these complexes. This implies that these two mutations do not occur within nuclear genes coding for a structural component of the mitochondrial transcription, processing or translation machinery e.g. a ribosomal protein, or a regulatory product which interacts with mitochondrial RNA or DNA to facillitate the correct expression of mitochondrial DNA.

Similar experiments carried out on another respiratory mutant cell-line, Gal 13, showed a mitochondrial protein synthesis

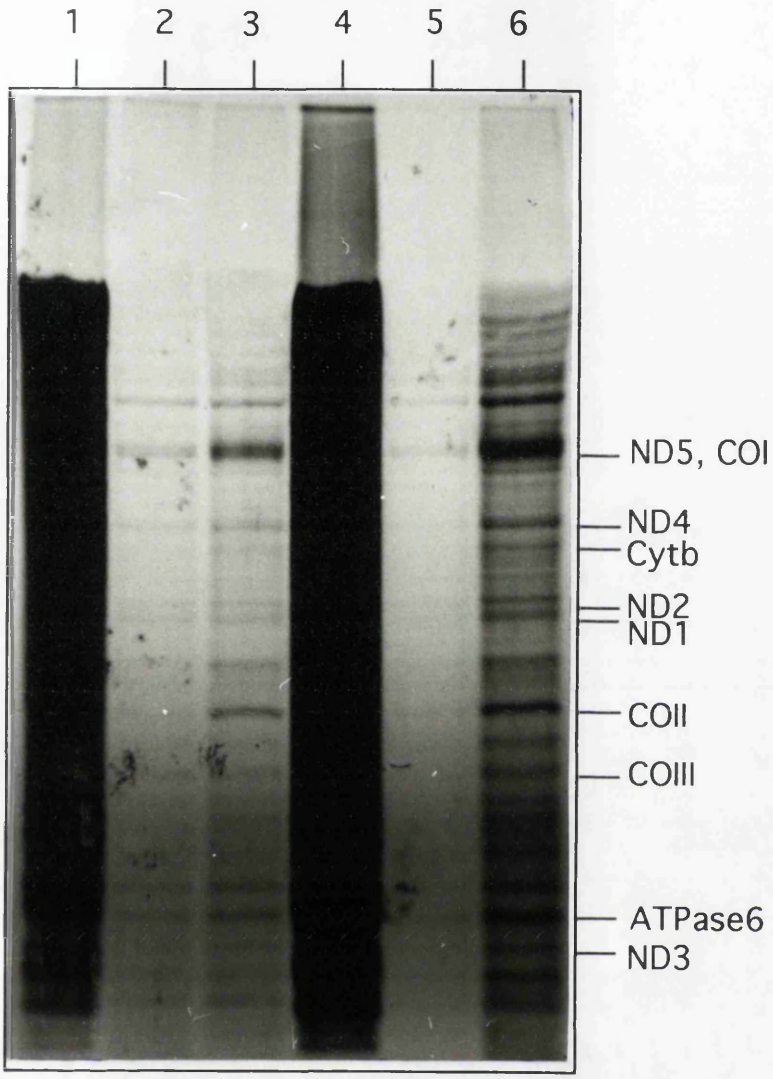



**Fig. 3.3 ELECTROPHORETIC PATTERN OF MITOCHONDRIAL PROTEIN SYNTHESIS PRODUCTS FROM V79 AND GAL 50 CELLS LABELLED *IN VIVO* WITH $^{35}S$ METHIONINE IN THE PRESENCE OF EMETINE.**

The growth and radiolabelling of the cells was performed as detailed in fig. 3.1. The putative mitochondrial protein synthesis products were assigned as for Fig. 3.1. The order of the samples on the SDS polyacrylamide gel are as follows: Gal 50 cells, without emetine (track 1), Gal 50 cells in $10^{-4}$ M emetine (track 2), Gal 50 cells in $10^{-5}$ M emetine (track 3), V79 cells without emetine (track 4), V79 cells in $10^{-4}$M emetine (track 5) and V79 cells in $10^{-5}$M emetine (track 6).

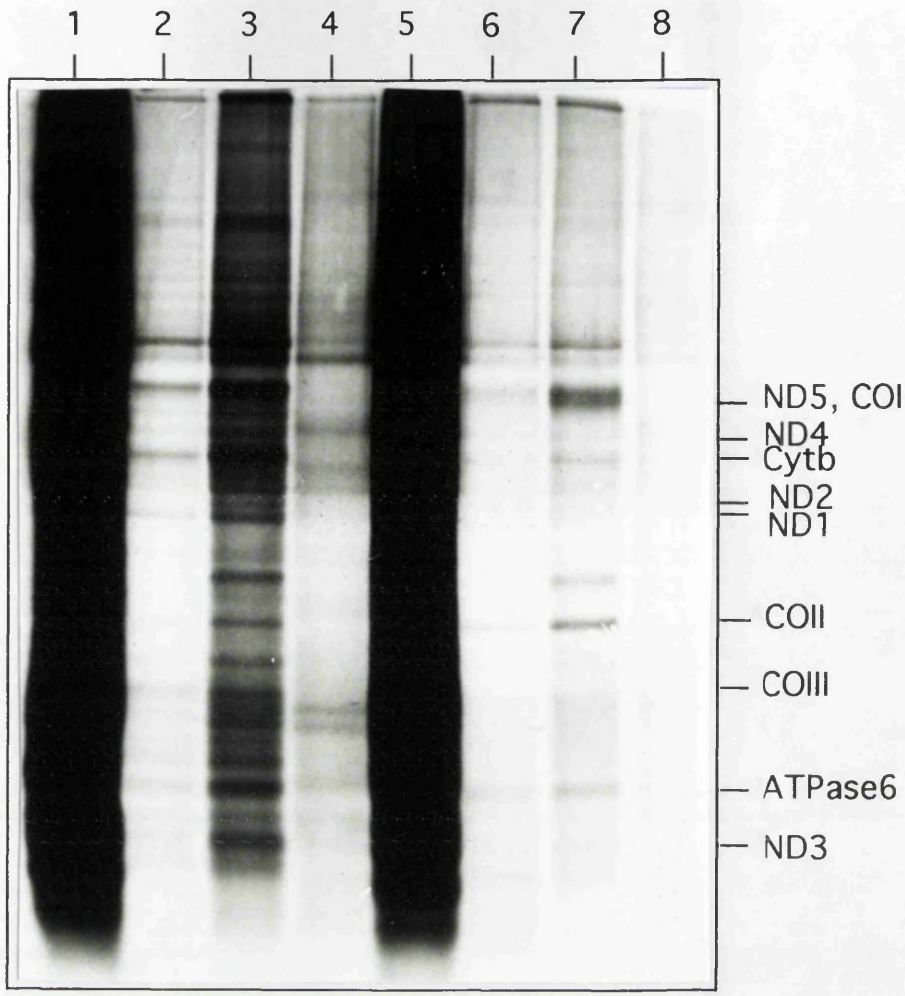



**Fig. 3.4 ELECTROPHORETIC PATTERN OF MITOCHONDRIAL TRANSLATION PRODUCTS FROM V79 AND GAL 13 CELLS *IN VIVO* LABELLED WITH $^{35}S$ METHIONINE IN THE PRESENCE OF EMETINE AND CHLORAMPHENICOL.**

The growth and radiolabelling of the cells were performed as detailed for Fig. 3.1. Chloramphenicol at a concentration of 1 mM along with $10^{-5}$ M emetine was added to two additional samples of both cell lines and the cells radiolabelled as those with emetine alone. The putative mitochondrial protein synthesis products were assigned as for Fig. 3.1. The order of the samples on the SDS polyacrylamide gel are as follows: V79 cells, no emetine (track 1); V79 cells, $10^{-4}$ M emetine (track 2); V79 cells, $10^{-5}$ M emetine (track 3); V79 cells, $10^{-5}$ M emetine/1 mM chloramphenicol (track 4); Gal 13 cells, no emetine (track 5); Gal 13 cells, $10^{-4}$ M emetine (track 6); Gal 13 cells, $10^{-5}$ M emetine (track 7); Gal 13 cells, $10^{-5}$ M emetine/1 mM chloramphenicol (track 8).

pattern different from that observed for wildtype (V79) cells.

Gal 13 cells were labelled with $^{35}S$ methionine in the presence of emetine as for V79, Gal 73 and Gal 50 cells. Due to a higher level of background of non-mitochondrial protein synthesis products observed in the earlier experiment shown in Fig. 3.3, an additional control was introduced in parallel to the other labelling regimes. V79 and Gal 13 cells were pulse labelled with $^{35}S$ methionine in the presence of $10^{-5}$ M emetine with the addition of chloramphenicol to a final concentration of 1 mM. Chloramphenicol blocks mitochondrial protein synthesis (Elliot, 1973; Nierhaus and Nierhaus, 1973) but not nuclear protein synthesis, and it was hoped that those polypeptides which were emetine-resistant at this concentration but chloramphenicol-sensitive, i.e. indicative of mitochondrial protein synthesis, could be clearly distinguished from all others. Taking this into account, any proteins in the samples treated with emetine corresponding to proteins which appearred in the chloramphenicol treated samples could be discarded from the analysis (Fig. 3.4).

About twelve clear polypeptide bands were found to fit the criteria for production via mitochondrial DNA expression. As with Gal 73 and Gal 50 some of the presumptive mitochondrial polypeptides were tentatively assigned according to size and comparison to those identified by Whitfield *et al* (1990), as indicated in Fig. 3.3. In Gal 13 cells however, there was a marked difference in the abundance pattern of the emetine-resistant, chloramphenicol-sensitive polypeptides, suggesting that the nuclear mutation in Gal 13 cells affects a gene whose product is involved in mitochondrial protein synthesis. Several of the presumptive mitochondrial polypeptides showed an alteration in their relative abundance when compared to their abundance in wildtype V79 cells. At first it was thought that the polypeptides which might be altered in abundance were the mitochondrially encoded NADH dehydrogenase subunits, as the mutation in Gal 13 cells primarily affects the NADH dehydrogenase complex of the electron transport chain (Whitfield *et al*, 1981). When some of the mitochondrial translation products were assigned as specific subunits of the

electron transfer chain, according to Whitfield et al, (1990), this seemed to be the case. Those polypeptides which were most altered in relative abundance were tentatively assigned as, ND1, ND3 ,ND4, ND5, and one unassigned polypeptide all of which were lowered in abundance and COIII which increased in abundance (Fig. 3.4), with the remaining polypeptides being unaffected at the level of abundance from wildtype. There was also no appearance of new protein bands in Fig. 3.4, track 7 which could correspond to a change in size of one of the polypeptides with lowered abundance, either due to incomplete processing or the formation of a truncated protein.

The results do seem to indicate that the mutation in Gal 13 cells interferes with the synthesis and/or accumulation of the mitochondrially coded NADH dehydrogenase subunits, but to confirm this, immunoprecipitations of translation products and western blots of steady state proteins using antibodies raised against the mitochondrially encoded subunits of Complex I would have to be performed on both V79 and Gal 13 cells, and the relative abundance of the various subunits compared.

### 3.3 COMPLEX III BIOSYNTHESIS IN GAL 73.

Although in Gal 73 the nuclear mutation does not appear to affect mitochondrial gene expression this does not rule out the possibility that the affected gene codes for a nuclear-coded subunit of complex III of the electron transport chain. Complex III consists of 10 subunits (Capaldi, 1982) including cytochrome b (mitochondrially coded) and the mutation could affect the expression or function of any one of these, except cytochrome b. So far, only the effect which the mutation could possibly have on the synthesis, accumulation, stability and electrophoretic mobility of cytochrome b has been investigated. To study the expression of the nuclear-coded subunits of Complex III, immunoprecipitations of total protein synthesis products from V79 and Gal 73 cells were carried out using a rabbit antibody raised against beef heart complex III (kindly donated by Dr. Ian Ragan, University of Southhampton). The immunoprecipitations were carried out as described in Chapter 2, section 2.26, and the precipitated polypeptides

Tracks 1 and 5 correspond to total protein synthesis in V79 and Gal 73 cells respectively.
Tracks 2 and 3 correspond to the immunoprecipitation of Complex III from V79 cells which were lysed with RIPA solution containing 0.1% SDS.
Track 4 is as for tracks 2 and 3 but the V79 cells were lysed with RIPA solution containing 0.01% SDS.
Tracks 6, 7 and 8 are the same as tracks 2,3 and 4 respectively but repeated with Gal 73 cells.

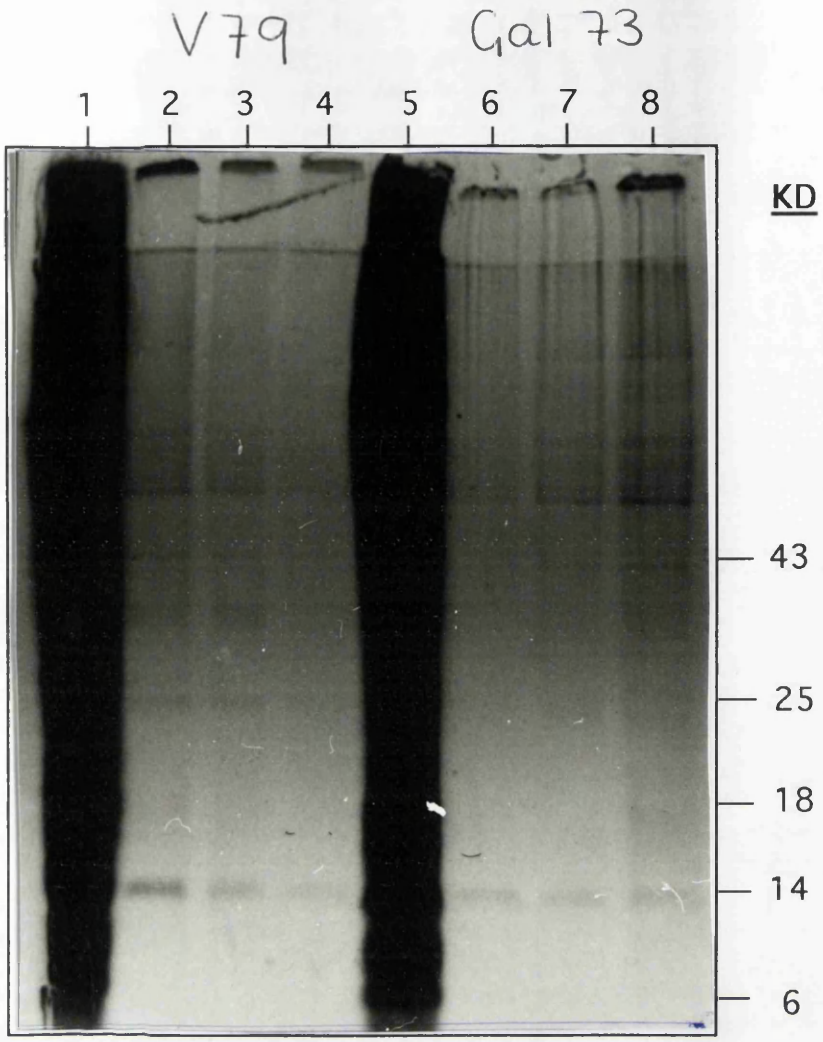



**Fig. 3.5 IMMUNOPRECIPITATION OF NASCENT COMPLEX III SUBUNITS FROM V79 AND GAL 73 CELLS.**

Flasks containing 2 x $10^5$ V79 and Gal 73 cells were labelled for up to 24 hrs with 250 uCi $^{35}S$ methionine per flask in methionine-free medium and then lysed in 500 ul RIPA solution containing either 0.1% SDS or 0.01% SDS. 110 ul of cell lysate was preincubated with 25ul of RIPA washed Protein-A agarose beads for 2hrs on ice. The beads where removed by centrifugation and 5ul of the beef heart Complex III antibody added to the supernatant and incubated at 4$^{o}$C overnight. 25ul of prewashed Protein-A agarose was added and again incubated on ice for 2hrs. The supernatant was discarded and the beads washed 5x in 1ml RIPA solution. Complex III subunits were eluted from the Protein-A agarose beads by boiling for 10 mins. in SDS sample buffer and the immunoprecipitated subunits analysed by SDS gel electrophoresis. Low molecular weight protein markers (BRL) were used as size standards and visualized by Coomassie blue staining .

analysed by SDS-polyacrylamide gel electrophoresis.

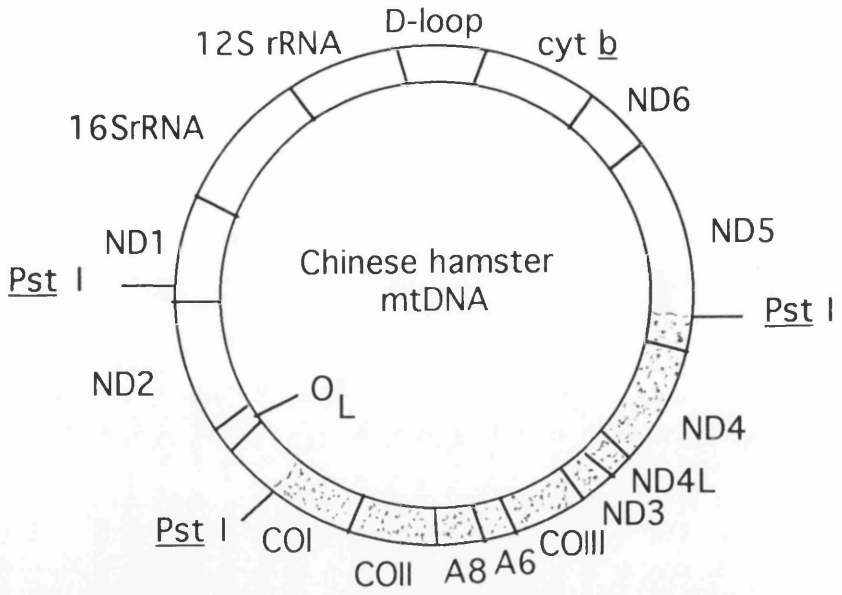
Under the wash conditions employed, i.e. 5 washes with 1 ml RIPA solution, the antibody precipitated more than 10 polypeptides ranging from the largest, which migrated with an apparent molecular weight of approximately 190 kD, to the smallest, which migrated at an approximate molecular weight of 10 kD (Fig. 3.5). The larger polypeptides detected, those with an apparent molecular weight of above 95kD, were probably not subunits of Complex III but may have resulted from non-specific binding to the antibody,or aggregation artifacts, as the human core protein subunit I has the highest molecular weight of the subunits of the complex, at around 45kD. The smaller proteins precipitated were present in Gal 73 cells (fig. 3.5, tracks 4-6) at the same relative abundance as in V79 cells (fig. 3.5, tracks 1-3). This includes a polypeptide of around 34kD which could represent cytochrome b. Although probably not all Complex III subunits were detected by the antibody and some polypeptides which were precipitated may not have been Complex III subunits, the synthesis, accumulation, stability and apparent electrophoretic mobility of all polypeptides detected in this assay appeared unaffected by the nuclear mutation in Gal 73 cells.

### 3.4 MITOCHONDRIAL TRANSCRIPTS IN GAL 13 CELLS.

There are a number of stages from DNA to protein at which a mutation could disrupt protein synthesis, most obviously at the levels of transcription or translation. In Gal 13 cells, the stable accumulation of some mitochondrially encoded translation products appears to be affected by the nuclear mutation. To investigate whether the defective accumulation of these putative mitochondrially encoded polypeptides in Gal 13 cells was due to a deficiency at the RNA level, the sizes and relative abundance of some of the mitochondrial RNAs were studied.

A 6.5Kb PstI fragment of Chinese hamster mitochondrial DNA, cloned into pUC9 (kindly donated by Dr. Richard Wilson, University of Glasgow) was used in this experiment. The

A


D-loop
12S rRNA
cyt b
ND6
16SrRNA
ND5
ND1
Chinese hamster
mtDNA
Pst I
Pst I
ND2
O L
ND4
ND4L
ND3
Pst I
COI
COII
A8
A6
COIII

B

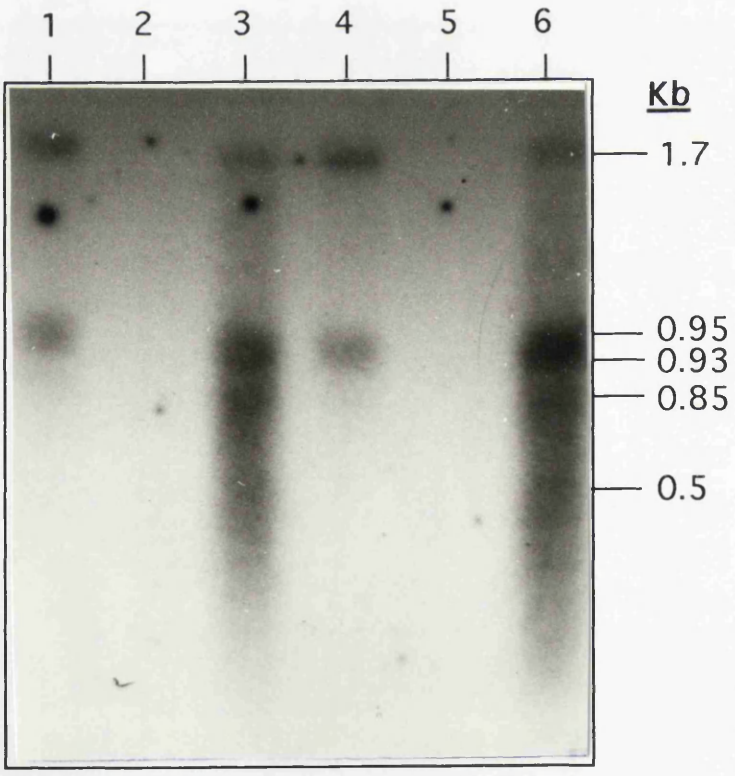

1
2
3
4
5
6
Kb
1.7
0.95
0.93
0.85
0.5

**Fig. 3.6** (A) Genomic map of the Chinese hamster mitochondrial DNA. The positions of the PstI sites are shown. The probe fragment used in the northern blot below is the PstI fragment from ND5 to COI (shown as the dotted segment).
(B) Northern blot analysis of mitochondrial mRNA transcripts in V79 and Gal 13 cells. Poly $A^+$ RNA was isolated from both V79 and Gal 13 cells as described in Chapter 2. 1 ug each of total V79 RNA (track 1), V79 poly $A^-$ RNA (track 2), V79 poly $A^+$ RNA (track 3), Gal 13 total RNA (track 4), Gal 13 poly $A^-$ RNA (track 5) and Gal 13 poly $A^+$ RNA (track 6) was loaded onto a 1% agarose gel containing formaldehyde and capillary blotted onto biodyne nylon membrane. Hybridisation to a 6.5Kb PstI mitochondrial DNA fragment was analysed at 42°C and the filters washed in 0.1 x W at 42°C.
The sizes of the transcripts are shown in kilobases.

fragment contains DNA sequences from the genes ND5, ND4, ND4L, COI, COII, COIII, ATPase6 and ATPase8. Northern blots were carried out using this as a probe. Total RNA was extracted from V79 and Gal 13 cells from which poly $A^+$ RNA was purified.

Fig. 3.6 tracks 1,3,4 and 6 show transcripts of 1.7 kb, 0.95 kb, 0.93 kb, 0.85 kb, 0.72 kb and 0.5 kb. These transcripts correspond to COI and ND4/4L (1.7 kb), ATPase6/8 (0.95 kb), COIII (0.93 kb), COII (0.72 kb) and ND3 (0.5 kb) based on the mitochondrial mRNA sizes published by Whitfield and Jefferson (1990) and Van Etten et al (1982). The only mRNA not detected by this mitochondrial DNA probe fragment is the ND5 mRNA. ND5 is processed from the primary precursor transcript to form a 2.4 kb mRNA coding for both ND5 and ND6. The failure to detect a transcipt of this size in both the wildtype and mutant RNA samples could be due to the small regionof ND5 contained in the cloned PstI fragment and the fact that the ND mRNAs are notoriously difficult to detect on northerns. However, the relative abundance of at least three ND mRNAs is unaltered by the nuclear-coded mutation in Gal 13 cells when compared to wildtype, two of which (ND3 and ND4) have been shown to be lowered in relative abundance at the protein level. Also they appear to be processed correctly to their mature mRNAs when compared to wildtype (Fig. 3.6, track 3), although fine structure analysis using mitochondrial gene-specific probes was not carried out.

## 3.5 DISCUSSION.

The respiratory mutant cell-lines descibed here each carry a nuclear DNA mutation which disrupts the normal respiratory function of the mitochondrion, and disables the cells from growth on non-fermentable substrates such as galactose. The mitochondria rely heavily on nuclear products for the expression of their small subset of mitochondrially encoded genes and therefore, there is the potential for disruption by mutations in the nucleus, at every level of mitochondrial DNA expression.

### 3.5.1 RELIABILITY OF THE PROTEIN SYNTHESIS ANALYSIS ON THE GAL MUTANTS.

In order to investigate at which level the disruption to respiratory activity might occur an analysis of mitochondrial protein synthesis was carried out.

In the process of doing these experiments it was noticed that the level of incorporation of $^{35}S$ methionine into the emetine-resistant polypeptides was not very consistent. In some experiments the concentration of emetine used, did not completely suppress all cytosolic protein synthesis, which made identification (according to Whitfield et al, 1990, for Chinese hamster cells) of the putative mitochondrially encoded polypeptides difficult. However, the pattern and relative abundance of those polypeptides tentatively assigned as mitochondrially encoded in each experiment was similar. It also became clear that the pattern of potential mitochondrial translation products produced in this study for Chinese hamster cells was reproducibly different to that published by Chomyn et al in 1985 for the human cell-line HeLa (compared to that for Chinese hamster cells observed both in this study and that seen by Whitfield et al, 1990). This could be due to a number of reasons. Firstly, the gel system used here was not the same as that used by Chomyn et al, 1985 and this could change the relative migration characteristics of some or all of the mitochondrial polypeptides. All of the mitochondrially encoded polypeptides are components of complexes of the inner mitochondrial membrane and are quite hydrophobic in their amino acid sequences. Therefore the discrepancies in the sizes of some of the Chinese hamster mitochondrial proteins compared to their human counterparts could be due to the well documented aberrant migration of hydrophobic proteins on SDS polyacrylamide gels. This, coupled with the different gel system used, could have greatly increased the difference in pattern between the two species.

Although in all published mammalian mitochondrial genomes the amino acid sequence homology is high, only a few amino acid changes would be necessary to change the overall

hydrophobicity of a polypeptide or to change the charge of a region of the polypeptide and in consequence change its migration through SDS polyacrylamide gels. As a result, the pattern of the putative mitochondrial polypeptides observed for the respiratory mutant cell-lines was not compared to the pattern observed by Chomyn et al, 1985 for HeLa cells but to the pattern observed for Chinese hamster cells by Whitfield et al, 1990. Special attention was paid to the presence and abundance of these polypeptides and compared to wildtype levels.

It was also observed that the incorporation of $^{35}S$ methionine into newly synthesized polypeptides was more efficient in V79 cells than in the mutant gal$^-$ cell-lines. This was also observed by Whitfield et al, (1990) when they performed the same protein synthesis analysis for Gal 32 cells, and they concluded it was due to the difference in the growth rates of the two cell-lines during the labelling period which, although relatively short, still produced a detectable variation in the level of the incorporation of the radioactively labelled amino acid.

### 3.5.2 RESULTS OF THE ANALYSIS OF MITOCHONDRIAL PROTEIN SYNTHESIS IN MUTANT CHINESE HAMSTER CELL-LINES.

The mitochondrial polypeptides observed in V79 (fig. 3.2) were all synthesized and accumulated in the two mutant cell-lines Gal 73 (fig. 3.1) and Gal 50 (fig. 3.3) to the same level once the effects of the increase in the incorporation of $^{35}S$ methionine in V79 cells had been taken into account. These results seem to indicate that the mutation does not occur in a gene whose function is involved in the expression or regulation of genes encoded in mitochondrial DNA. Gal 73 and Gal 50 have major defects in Complex III and Complex I of the electron transport chain respectively, but there is the possibility that the mutant genes code for a structural subunit of either of these two complexes. Antibodies raised against beef heart Complex III were obtained to test this hypothesis with regard to Gal 73. Even though some non-specific binding was observed when Complex III was

immunoprecipitated from V79 and Gal 73 cells (fig. 3.5), the pattern and abundance of those polypeptides precipitated, was unchanged in Gal 73 when compared to V79. Although probably not all of the 10 subunits of Complex III were precipitated by the antibody directly, i.e. only those subunits which have epitopes which appear on the outside of the complex would bind the antibody, it was hoped that the subunits which could not directly bind would still be precipitated by virtue of their association with the other subunits of the complex. However, this might not occur if some subunits are only loosely bound in which case subunit-specific antibodies would have to be used. If not all the subunits of Complex III are precipitated, the mutation could conceivably affect one of the unprecipitated subunits or alternatively, the mutation could be a point mutation in one of the subunits precipitated which does not alter its mobility on SDS polyacrylamide gels but is detrimental to the activity of the complex once formed.

#### 3.5.2.1 Possible effects on mitochondrial protein synthesis of a mutation in the transport of nuclear-coded mitochondrial subunits.

All of the nuclear-coded mitochondrial subunits of the complexes of the electron transport chain need to be transported into the mitochondrion from the cytoplasm and be targeted to their correct location within the organelle (reviewed by Pfanner and Neupert, 1990; Neupert et al, 1990). For this to happen, most nuclear-coded mitochondrial polypeptides have amino-terminal presequences (Van Loon et al, 1986) which are cleaved off by a mitochondrial protease once the polypeptides have been transported through the mitochondrial membranes and targeted to their correct location (Hawlitschek et al, 1988; Pollock et al, 1988). If the mutation in Gal 73 occurs within the presequence of a nuclear-coded subunit of Complex III, then the polypeptide might not get as efficiently transported into the mitochondria and the level of activity of Complex III in the cell-line would be decreased. A decrease in the level of Complex III activity in Gal 73 cells was observed in a biochemical analysis of the electron transport chain within this cell-line performed by

Whitfield, 1981. Exactly how the inefficient transport of a subunit of Complex III would affect the accumulation of the other subunits of the complex is not known. However, there are a number of possibilities i.e. (i) that there is no affect and all the other subunits are accumulated normally, or (ii) because of the absence of one, the other subunits can not be assembled properly and therefore undergo some degradation. From the results observed for the immunoprecipitation of Complex III from V79 and Gal 73 cells, the accumulation of Complex III subunits in Gal 73 cells shows little difference to that occurring in wildtype indicating that the inefficient transport of one (hypothetical) subunit does not appear to affect the accumulation of the other subunits. However, in the absence of direct evidence for a transport defect, it cannot be regarded as more likely than, for example, a point mutation in a protein coding subunit of Complex III.

For Gal 50 cells, the mutation could be in a nuclear coded subunit of Complex I of which there are at least 27, as the synthesis and accumulation of the nuclear-coded subunits were not studied. However, the mutation could also occur in the presequence of one of the nuclear-coded subunits, in which case, it would seem that the decrease in the availability of one subunit does not seem to affect the accumulation of, at least, the seven mitochondrially coded subunits. No difference was observed in the synthesis and accumulation of these subunits compared to wildtype.

In contrast, in Gal 13 cells the synthesis and accumulation of at least five mitochodrially encoded polypeptide subunits, which may all be of Complex I, was distinctly altered compared to wildtype. In this case, the transport or structure of a nuclear-coded subunit of Complex I may be defective e.g. due to a mutation in its presequence, resulting in the complex not assembling efficiently. The mitochondrially encoded subunits for this complex might then be supposed to be degraded (to varying degrees) in this cell-line. It might also be the case that the remainder of the nuclear-coded subunits fail to accumulate, and undergo rapid turnover. To test whether this is the case, the accumulation and steady state

levels of all the subunits of the complex in Gal 13 cells would have to be quantified. This could be achieved by the immunoprecipitation of the subunits of Complex I from Gal 13 cells and comparison with wildtype.

### 3.5.2.2 Possible implications on mitochondrial protein synthesis of a mutation in the assembly of the redox complexes.

Another process which can be affected by a nuclear mutation is the assembly of the subunits into functional complexes of the electron transport chain. In yeast the assembly of the redox complexes has been found to require the services of a nuclear coded mitochondrial heat shock protein, hsp60 (Cheng et al, 1989; Ostermann et al, 1989; Reading et al, 1989). Hsp60 is a member of the chaperonin family of protein factors, which assemble subunits into complexes but are not a part of the complex themselves (reviewed by Ellis, 1991), and include the E.coli groEL protein (Georgopoulos et al, 1973; Sternberg, 1973) and the Rubisco large subunit binding protein of chloroplasts (Hemmingson et al, 1988). Analysis of conditional lethal mutants of the HSP60 gene in yeast has revealed defects in the assembly of all of the mitochondrial complexes (Cheng et al, 1989). The nuclear mutations present in the Chinese hamster respiratory deficient cell-lines, mainly affect one aspect of the electron transport chain, but they also simultaneously affect other parts to a lesser extent. The mutations in Gal 73 and Gal 50 could concievably be mutations in different domains of an hsp60 homologue or in distinct additional components which might be involved in the assembly of Complex III or Complex I respectively. Mitochondria could contain an equivalent to the groES protein, which in E.coli seems to cooperate with groEL in various assembly functions by an unknown mechanism (Chandrasekhar et al, 1986; Tilly et al,1981) and which in mitochondria could conceivably act as a specificity factor associated with hsp60.

If there is more than one chaperone involved in the assembly of the redox complexes, and they assemble specific complexes, then a mutation in one chaperone would only affect certain

complexes. If assembly fails completely one might expect the subunits of that complex to be less stable and undergo some degradation. However, in yeast, mutations in HSP60 result in the accumulation of intermediate-sized mitochondrial precursor polypeptides and some mature-sized mitochondrial polypeptides (Cheng et al, 1989). Although such an accumulation of mitochondrial polypeptides was not observed in Gal 73 cells (at least for those subunits of Complex III which were immunoprecipitated) a mutation in an assembly factor cannot be ruled out.

As no degradation of mitochondrially encoded polypeptides was observed in yeast with a mutation in HSP60, this would suggest in an indirect manner that inefficient assembly due to inefficient transport of a nuclear-coded subunit with a mutation within its presequence would not result in the degradation of the other subunits and so for both Gal 73 and Gal 50 cells, a mutation in a transport presequence or assembly protein could give the mitochondrial protein synthesis pattern observed.

For Gal 13 cells, the nuclear mutation leads to a decrease in abundance of mitochondrially encoded polypeptides tentatively identified as Complex I subunits. Following the argument above that a mutation in transport or assembly might not necessarily lead to the degradation of the mitochondrially encoded polypeptides, then for Gal 13 cells, a mutation in either of these is not necessarily implied, and might be construed as unlikely. Due to the decrease in the synthesis or accumulation of the putative ND subunits a decrease in the activity of Complex I of the electron transport chain might be suggested, which is in fact the case in Gal 13 cells. NADH dehydrogenase activity in Gal 13 cells is only approximately 5% of the wildtype level (Whitfield et al, 1981). This would imply that the mutation must be in a gene whose major function is in the regulation of expression of the mitochondrial subunits of this complex and not in an assembly factor or the presequence of a nuclear-coded subunit of the complex.

### 3.5.2.3 Possible effects of a mutation on the translation of mitochondrial mRNAs.

The results from the northern blot hybridization experiment using a 6.5Kb fragment of mitochondrial DNA as a probe to assess the levels of mitochondrial mRNA in Gal 13 compared to V79 show that for at least three of the 7 mitochondrial-coded subunits of Complex I, the regulation exerted by the mutant nuclear gene is beyond the level of transcription. The abundance of all the mRNAs hybridizing to this mitochondrial DNA fragment appears to be the same as wildtype. Therefore for the mRNAs detected here the mutant gene must regulate either the translation of these messages or the stability of their translation products.

In yeast strains carrying mutations affecting the translation of mitochondrial mRNAs, two types of mutations have been identified , the second type being of most importance here. In the first type, mutations occur in the 5' untranslated leaders of mitochondrial mRNAs and lead to the accumulation of stable, but untranslatable mRNAs (Hibbs et al, 1987; Ooi et al, 1987). In the second type, untranslatable mitochondrial mRNA also accumulates, but this time as a result of mutations in nuclear regulatory genes (Decoster et al, 1990; Fox et al, 1988; Poutre and Fox, 1987; Costanzo and Fox, 1986; Costanzo et al, 1986a; Costanzo et al, 1986b; Fox, 1986; Muller and Fox, 1984; Dieckmann et al, 1984). A number of these mutations have been isolated along with suppressor mutations (usually mitochondrial DNA rearrangements) which bypass the translational block caused by the nuclear mutation (Muller et al, 1984; Rodel et al, 1985; Rodel, 1986; Rodel and Fox, 1987). The most likely function for these mutant yeast proteins encoded by the nucleus, is that of mitochondrial mRNA specific initiation factors. Support for this idea is given by the results of sequence comparisons between the genes encoding these mutant proteins in yeast and previously characterised translation initiation factors, which reveal evidence of homology to both prokaryotic and eukaryotic initiation factors (Grivell, 1989). Although in Gal 13 there is no accumulation in the mRNAs analysed, the possibility of a defect in an

initiation factor for the translation of the mitochondrially encoded ND subunits cannot be ruled out and maybe sequence comparison of the mutant gene in the three respiratory deficient cell-lines will reveal relationships to initiation or assembly factors.

For all three of the cell-lines analysed above, it is still not clear as to what functions the mutant gene products have during mitochondrial biogenesis. To answer this question definitively, the mutant genes in Gal 13, Gal 50 and Gal 73 cells need to be cloned and characterised. Then their expression and the interaction of their gene products with the other components of the appropriate redox complexes and the machinery for their biogenesis can be studied.

# CHAPTER 4

## DNA-MEDIATED RESTORATION OF WILDTYPE PHENOTYPE IN GAL$^-$ MUTATNT CELL-LINES BY COMPLEMENTATION.

## 4.1 INTRODUCTION.

Although useful, analysing the effect of the nuclear mutations on mitochondrial protein synthesis in the Chinese hamster cell-lines yielded only suggestive information on the function of the mutated gene. To find a more definitive explanation as to the function of these genes, they need to be cloned and characterized. A general strategy for cloning genes whose genomic locations and functions are unknown but have been highlighted due to the presence of mutations within those genes, is by complementation. This approach has been used extensively and has been successful in isolating other mammalian genes like the chicken thymidine kinase gene (Perucho *et al*, 1980) and two human bladder oncogenes (Goldfarb *et al*, 1982; Shih and Weinberg, 1982), and subsequently in many other instances.

In an initial series of experiments (see section 4.3, below), extensive attempts to restore Gal 73 cells to respiratory competence, by co-transfection of randomly sheared high molecular weight genomic DNA prepared from the parental V79 cell-line, or from HeLa cells, with the plasmid pSV2neo (Berg *et al*, 1984) using the calcium phosphate precipitation method, gave no success. As, this technique measures only the integration of the wildtype gene which could complemented the mutation within Gal 73 cells, by growth in galactose medium, it was impossible to know if the genomic DNA was being taken up by the nucleus along with the plasmid DNA and integrated into the genome. Therefore a wildtype genomic cosmid library using the vector pWE15 which contains the dominant eukaryotic selectable marker of resistance to the antibiotic G418 (neo) and the strong SV40 enhancer in *cis*, was constructed for transfection into Gal 73 cells, thereby allowing for the detection of integrated genomic DNA via its linked plasmid sequences.

In an attempt to complement the Chinese hamster mutant cell-line Gal 73, DNA was prepared from pools of 20 000 cosmid recombinant clones and transfected by electroporation into Gal 73 cells, which were then scored for growth on galactose

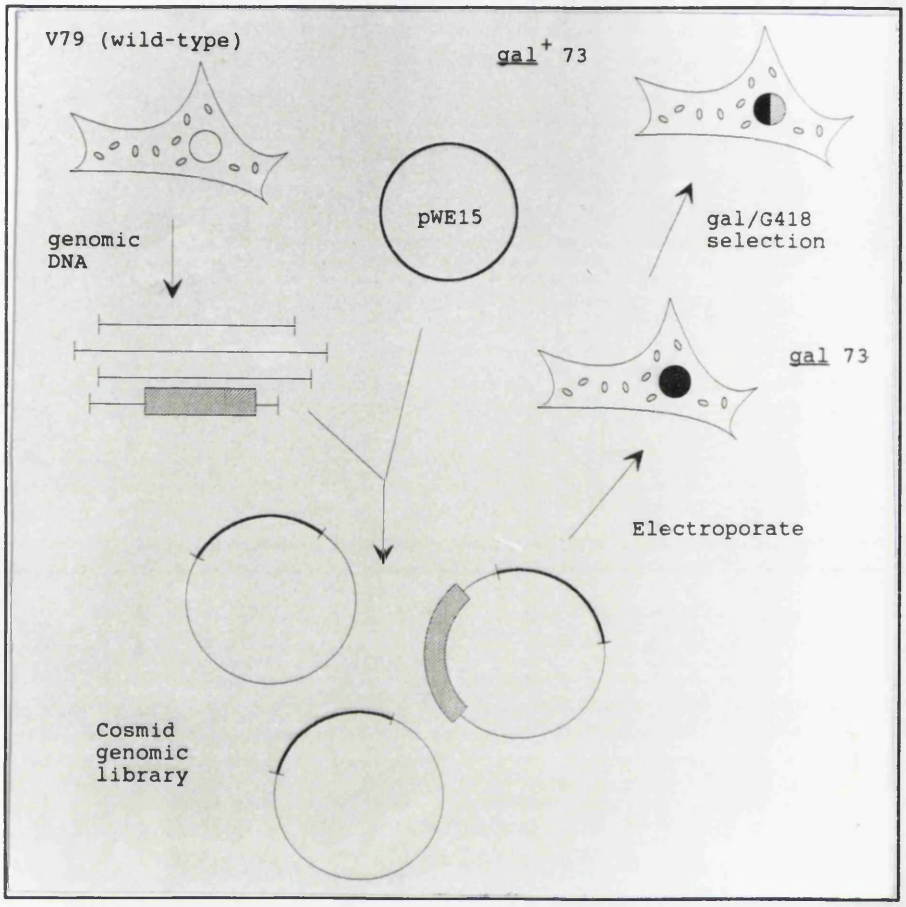



**Fig. 4.1 Scheme for screening wildtype (V79 cell) genomic DNA for sequences capable of restoring gal$^+$ phenotype on Gal 73 cells.**

The genomic sequences of interest are shown as a shaded box, and the pWE15 vector sequences are depicted by bold lines. Gal 73 nuclei containing the gal$^-$ mutant gene are shown as filled circles, those containing the gal$^+$ wildtype gene are diagonally shaded. The presence of both alleles is shown by both kinds of shading together.

medium in the presence of G418 (Fig. 4.1).

## 4.2 RESULTS

### 4.2.1 ANALYSIS OF THE SPONTANEOUS REVERSION RATES OF GAL 73 AND GAL 13 CELLS.

The $gal^-$ Chinese hamster mutant cell-lines were originally isolated after treatment of the cells with E.M.S, a chemical mutagen. E.M.S. mutagenesis normally generates point mutations or small deletions and experimenters found that under selection for respiratory competence, a portion of the $gal^-$ mutant cells survived (Puck and Kao, 1967; Kao and Puck, 1968; Chu et al, 1972; Smith and Chu, 1973; Rosenstraus and Chasen, 1975; Robins, 1979). The published frequency for reversion back to wildtype phenotype was reported to be between 1 in $10^5$ to 1 in $10^7$ cells (Whitfield et al, 1981). At this frequency, spontaneous reversion of the $gal^-$ cell-lines would result in the isolation of false positives in complementation experiments, unless the frequency of complementation of the $gal^-$ cell-lines was markedly higher than the spontaneous reversion frequency.

To check the spontaneous reversion frequency of Gal 73 and Gal 13 cells, $10^9$ cells for each cell-line were plated out in galactose selective medium. This number of cells with a spontaneous reversion frequency of 1 in $10^5$ to $10^7$ should generate between 100 to 10 000 $gal^+$ colonies. However, no such colonies were observed. This experiment was repeated for each cell-line with the same results. This would suggest that the spontaneous reversion frequency for both Gal 73 and Gal 13 cells was less than 1 in 2 x $10^9$ cells. The large discrepancy between the published frequency and the frequency observed here could be due to differences in the selection conditions imposed on the cells in the two experiments. However, the lower spontaneous reversion frequency observed here, along with screening for double selection (i.e. $Gal^+$, $G418^R$), suggests that isolating spontaneous revertants would be extremely unlikely, though cannot be discounted absolutely.

### 4.2.2 CO-TRANSFORMATION OF GAL 73 CELLS WITH pSV2NEO AND NAKED GENOMIC DNA.

It is already known that the respiratory deficient cell-line Gal 73 has a mutation within a nuclear gene whose product is involved in the respiratory function of mitochondria. Although previous biochemical studies have shown that the mutation affects Complex III activity of the electron transport chain in particular (Whitfield et al, 1981), nothing is yet known about the mutated nuclear gene itself and how its product interacts with the other components of the organelle. However, the study of the effect of the mutation in Gal 73 on mitochondrial protein synthesis, detailed in the previous chapter, gives an indication that the product of the mutated gene is not involved in the direct regulation of mitochondrial protein synthesis. Only by the cloning of the mutated gene is it likely that the precise mitochondrial function of its gene product can be clarified. This may also help us to understand aspects of the co-ordination of nuclear and mitochondrial gene expression in mitochondrial biogenesis.

In an attempt to do this high molecular weight V79 and HeLa cell genomic DNAs were isolated and co-transfected with plasmid pSV2neo DNA (Fig. 4.2), which carries a neomycin resistance gene (neo) conferring resistance to the antibiotic G418 when expressed in eukaryotic cells. Complementation of deficient cell-lines to wildtype phenotype has previously been successful using a co-transfection strategy. The approach involved co-transfecting mouse L cells with cloned selectable marker DNA, as well as uncloned wildtype genomic DNA, and sequentially selecting for the integrated marker and then the genomic DNA of interest (Kavathas and Herzenberg, 1983a; Kavathas and Herzenberg, 1983b; Kuhn et al, 1984). The first step enriches for cells that take up DNA. Co-transfection is a common event, and cells that do take up DNA can take up a large amount, up to 2000 Kb of DNA (Perucho et al, 1980).

Published data reporting the frequency of transformation of various mammalian cell-lines using different transfection

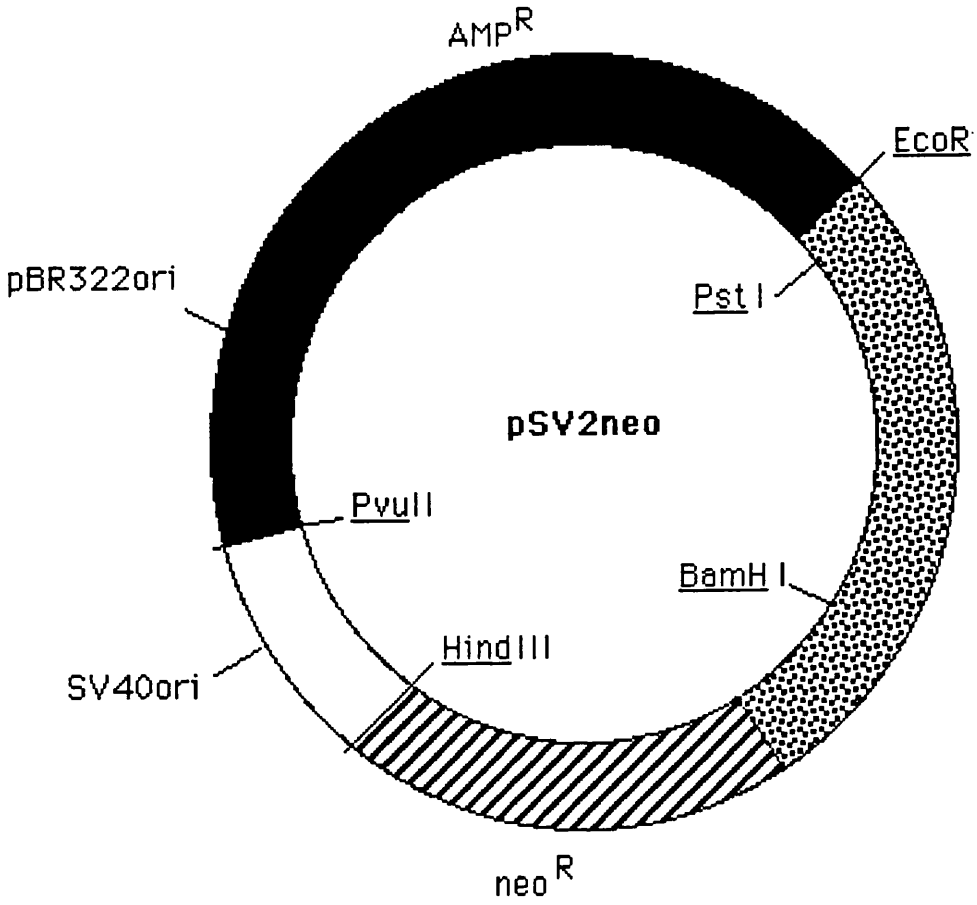



**Fig. 4.2 Structure of pSV2neo.**

The plasmid pSV2neo is composed of DNA segments from various sources: pBR322, represented by the solid black arc, contains the pBR322 (ColE1) origin of replication and the ampicillin resistance marker gene (Amp$^R$); the hatched segment represents the G418$^R$ marker gene; SV40 sequences are indicated as the dotted segments. The SV40 origin of replication (SV40 ori) and the SV40 early promoter are present on a small fragment immediately 5' to the neomycin gene. The restriction sites are indicated as follows: HindIII - H; PstI - P; EcoRI - R.

methods varied greatly. Transfection using the calcium phosphate precipitation approach was reported to give high frequencies of transfection and was the most widely used transfection approach at the time when these experiments were performed (Graham and van der Eb, 1873; Graham et al, 1974; Gorman et al, 1983; Chen and Okayama, 1987). Basically, exogenous DNA is mixed with calcium chloride and is then added slowly to a solution containing phosphate ions. A calcium phosphate/DNA co-precipitate is formed which is readily taken up by mammalian cells in culture, resulting in high levels of exogenous gene expression.

Using the calcium phosphate precipitation method, the frequency of transformation of Gal 73 cells, to $G418^R$ was tested, using the plasmid pSV2neo. Initially, the frequency varied from between 0.0035% to 0.1% (table 4.1), but with practice the frequency stabilised around 0.05%.

Attempts were made to complement the deficiency in Gal 73 cells by co-transfection with either V79 or HeLa cell genomic DNAs and pSV2neo DNA. In a number of experiments, over $10^9$ Gal 73 cells were transfected, with a transformation frequency to $G418^R$ of 0.05%. This gave a total of $5 \times 10^5$ $G418^R$ Gal 73 cells which were screened for complementation of the mutation by growth in galactose medium ($gal^+$). From these, two unhealthy $gal^+$, $G418^R$ colonies were obtained. Both were isolated from experiments in which HeLa cell genomic DNA had been used, but both colonies died before cloning. Due to the unexpected death of the $gal^+$, $G418^R$ colonies, stable integration of exogenous HeLa cell genomic DNA into Gal 73 cells could not be proven.

After extensive attempts to obtain other $gal^+$, $G418^R$ clones using this strategy were unsuccessful, it was thought that the original $gal^+$, $G418^R$ colonies obtained were unstable transformants or that secondary site insertional mutations had been isolated.

Transformation to respiratory competence of several $gal^-$ lines has been reported by Garnett et al (1985), using a DNA-

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| 5ug | 0 | 120secs | 0.02% |
| 0.5ug | 0 | 120secs | 0.05% |
| 0.05ug | 0 | 120secs | 0.18% |
| 0.5ug | 4.5ug | 120secs | 0.04% |
| 0.05ug | 4.95ug | 120secs | 0.44% |
| 5ug | 0 | 0 | 0.0007% |
| 5ug | 0 | 30secs | 0.003% |
| 5ug | 0 | 60secs | 0.025% |
| 5ug | 0 | 180secs | 0.008% |
| 5ug | 0 | 300secs | 0.01% |
| 0 | 0 | 120secs | 0 |

**Table 4.1 Frequency of transformation of Gal 73 cells to $G418^R$ with pSV2neo under various conditions of calcium phosphate precipitation.**

For each set of conditions, 3 flasks containing 2 x $10^5$ cells were transfected by calcium phosphate precipitation (Chapter 2, section 45(i)) and the mean frequency of transformation to $G418^R$ ($800ugml^{-1}$) calculated.

Column 1 concentration of pSV2neo used in ug

Column 2 concentration of V79 carrier DNA used in ug

Column 3 length of glycerol shock in secs.

Column 4 frequency of transformation per ug pSV2neo.

mediated transfer approach, but without co-transfection with a selectable marker it could not be rigorously demonstrated that this was not a result of the reversion of the cell-lines. Nor can second-site suppression or insertional mutagenesis be ruled out, in the absence of a marker for the integrated DNA. Failure to transform Gal 73 cells to respiratory competence successfully in these experiments could be due to a number of reasons, (i) an inability to achieve sufficiently high level expression, as a result of the gene being sensitive to negative regulatory sequences at insertion sites or being inserted at too great a distance from essential positive regulatory sequences, (ii) due to a general low transfection efficiency of linear high molecular weight genomic DNA in Gal 73 cells, or (iii) because the entire gene was not contained on the integrated genomic fragment, which is highly probable if the gene happens to extend over a large area of the genome.

#### 4.2.3.1 CONSTRUCTION OF A CHINESE HAMSTER COSMID LIBRARY.

As the co-transfection method for complementing the mutation in Gal 73 cells was unsuccessful, a cosmid library was constructed. Using a cosmid library has a major advantage over using linear high molecular weight DNA. The integrating molecule is circularised and supercoiled, and the genomic DNA is actually linked to the gene which encodes the selectable marker and is in close proximity to a strong enhancer in *cis* which enhances the expression of any genes in the genomic DNA insert. This means that integration and expression of the marker gene would indicate that genomic DNA had also been integrated. This was not the case in the co-transfection strategy, where integration of the plasmid does not necessarily mean that unlinked genomic DNA had been integrated also.

A cosmid library is preferable to a lambda library, as cosmids are capable of carrying inserts of up to 45 kb, wereas lambda libraries generally have inserts of at most about, 20 kb. This is useful if the gene is large. For a very large gene, even a cosmid vector would be unable to carry the gene in its entirety and a cDNA library would need to be constructed.

However, there are potential disadvantages to using cDNA libraries for complementing mutations in mammalian cell-lines. If the gene of interest is poorly represented in the mRNA, there would be too few cDNA copies of the desired gene in the library making the complementation frequency too low to be observed without screening a very large number of transfected, $G418^R$ Gal 73 cells.

As mentioned previously, many mammalian genes have been successfully isolated via complementation when the gene has been linked to plasmid marker DNA sequences (Perucho et al, 1980; Lowry et al, 1980 and Goldfarb et al, 1982) and so a cosmid wildtype genomic DNA library was constructed.

**4.2.3.1 PREPARATION OF HIGH MOLECULAR WEIGHT V79 CELL DNA.**

The molecular weight of the DNA that is used is probably the most critical component of the success of any library construction. 35-45 kb Sau3AI partially digested DNA fragments, isolated from molecules of starting size 100 kb, have only about a third of the molecules with the defined restriction sites at both ends. The sheared (i.e. blunt ended or partially blunt ended) DNA molecules compete in the ligation reaction and greatly reduce the efficiency of library construction. Therefore care has to be taken to reduce as much as possible the shearing of the DNA.

High molecular weight V79 cell DNA was prepared from approximately 6 x $10^7$ cells (Chapter 2, section 38). The resulting DNA was very viscous and difficult to pipette, and on a 0.3% TAE agarose gel (Fig. 4.3, tracks 3 and 4), resolved as a tight band with a significantly higher molecular weight than the 48.5Kb lambda DNA (Fig. 4.3, track 2) used as a molecular weight marker.

**4.2.3.2 Sau3A I PARTIAL DIGESTION OF V79 CELL DNA.**

**4.2.3.2.1 Pilot experiments.**

Pilot experiments were performed using V79 cell genomic DNA

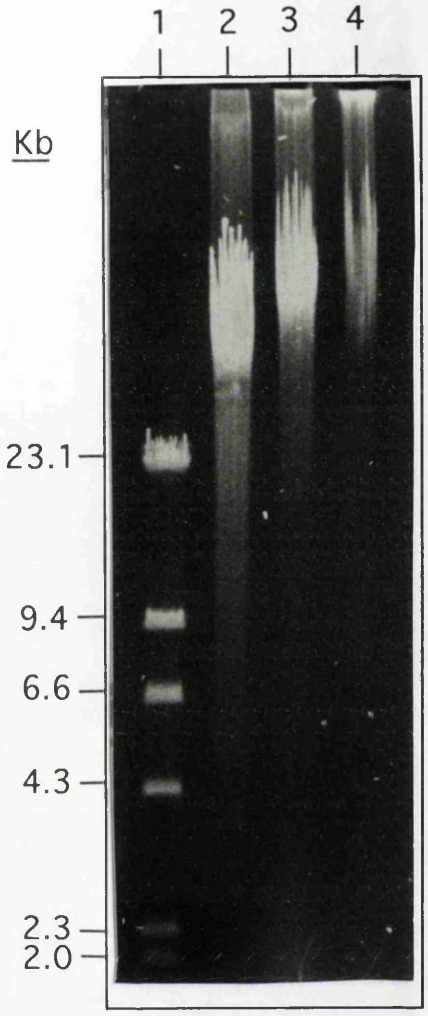



**Fig. 4.3 An ethidium bromide stained 0.3% TAE agarose gel, showing the mobility of isolated high molecular weight V79 DNA.**

High molecular weight genomic DNA was isolated from V79 cells as described in Chapter 2, section 2.20. 1 ug of genomic DNA from two separate isolates underwent gel electrophoresis at 30V for 16 hr, at 4$^{o}$C. The molecular weight of the V79 genomic DNA (tracks 3 and 4) was compared to the molecular weight of phage lambda DNA digested with HindIII (track 1) and undigested phage lambda DNA which has a molecular weight of 48.5Kb (track 2).

and serial dilutions of Sau3AI restriction enzyme to determine the DNA:enzyme ratio which would be optimal to acheive the highest concentration of Sau3AI digested genomic DNA fragments in the 35-45Kb size range, to be ligated to the cosmid vector for library construction. In these experiments, the method of Seed et al was used to identify the correct size of partially digested DNA and the optimal conditions were found to be 1ug V79 genomic DNA digested with between 0.125 and 0.25 units of Sau3AI for 30mins. at 37°C (Fig. 4.4, tracks 7 and 8).

#### 4.2.3.2.2 Large scale digestion of V79 DNA.

The optimal conditions for generating the highest concentration of 35-45Kb fragments of V79 genomic DNA with Sau3AI 'sticky' ends were scaled up 100 fold, i.e. 100ug of V79 DNA digested with 12.5 and 25 units of Sau3AI for 30mins. at 37°C. Due to the 100 fold increase in the volume of the reaction the V79 genomic DNA was prewarmed to 37°C prior to the addition of the Sau3AI enzyme so that optimal restriction digest conditions were present when the enzyme was added.

When following this proceedure the resulting V79 cell DNA was repeatedly over-digested (Fig. 4.5a, tracks 1-4). Due to this the viscosity of the V79 cell genomic DNA was used as an indication of the extent of the digestion of the DNA by Sau3AI.

As the viscosity of the V79 cell genomic DNA started to drop, digestion was stopped by the addition of 2ul 0.5M EDTA pH8 and heating to 80°C for 10mins. The extent of the digestion of the V79 cell genomic DNA was visulaised by agarose gel electrophoresis (Fig. 4.5b) which showed that this proceedure yeilded enough genomic DNA fragments in the desired size range to purify on sucrose density gradients.

### 4.2.3.3 SUCROSE DENSITY GRADIENT-FRACTIONATION OF V79 DNA PARTIAL DIGESTION PRODUCTS.

Sau3AI partially digested V79 DNA fragments in the size range 35-45Kb were purified using 10-40% sucrose gradient

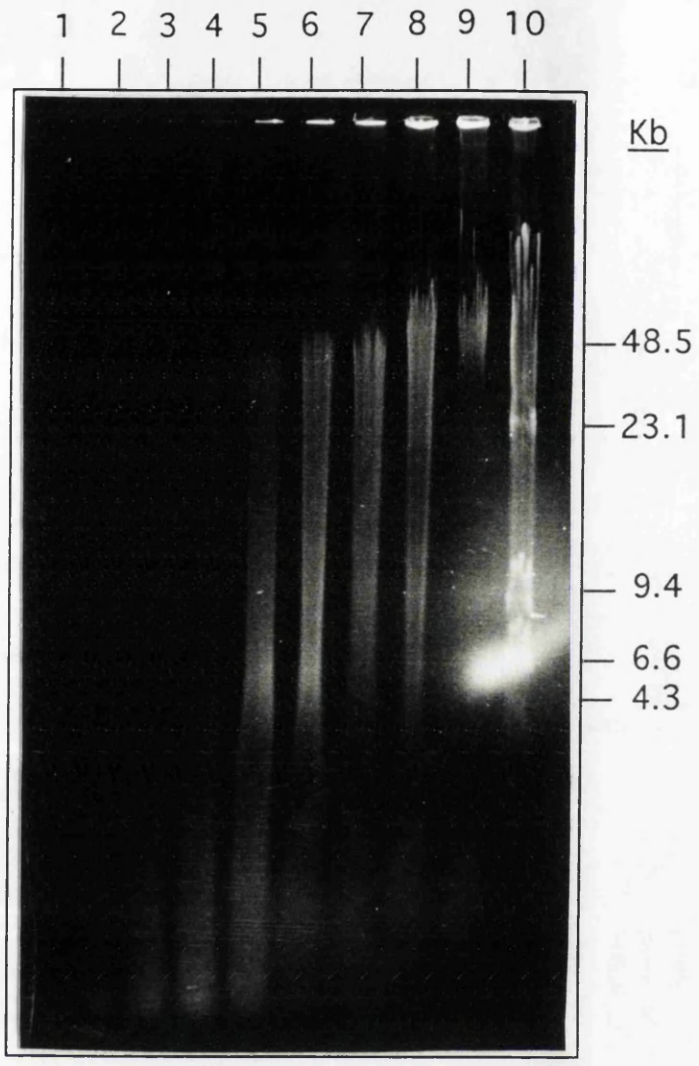



**Fig. 4.4 Small-scale Sau3AI digestion of V79 genomic DNA.** 1 ug aliquots of V79 genomic DNA were digested for 30 mins. at 37°C with the following concentrations of Sau3AI: 16 units (track 1); 8 units (track 2); 4 units (track 3); 2 units (track 4); 1 unit (track 5); 0.5 units (track 6); 0.25 units (track 7) and 0.125 units (track 8). The restriction enzyme was inactivated by the addition of 2ul 0.5M EDTA and heating at 80°C for 10mins. The extent of digestion was visualised by electrophoresis on a 0.3% TAE agarose gel and stained with EtBr. The molecular weight size markers used were uncut phage lambda DNA (track 9) and phage lambda DNA digested with HindIII (track 10).

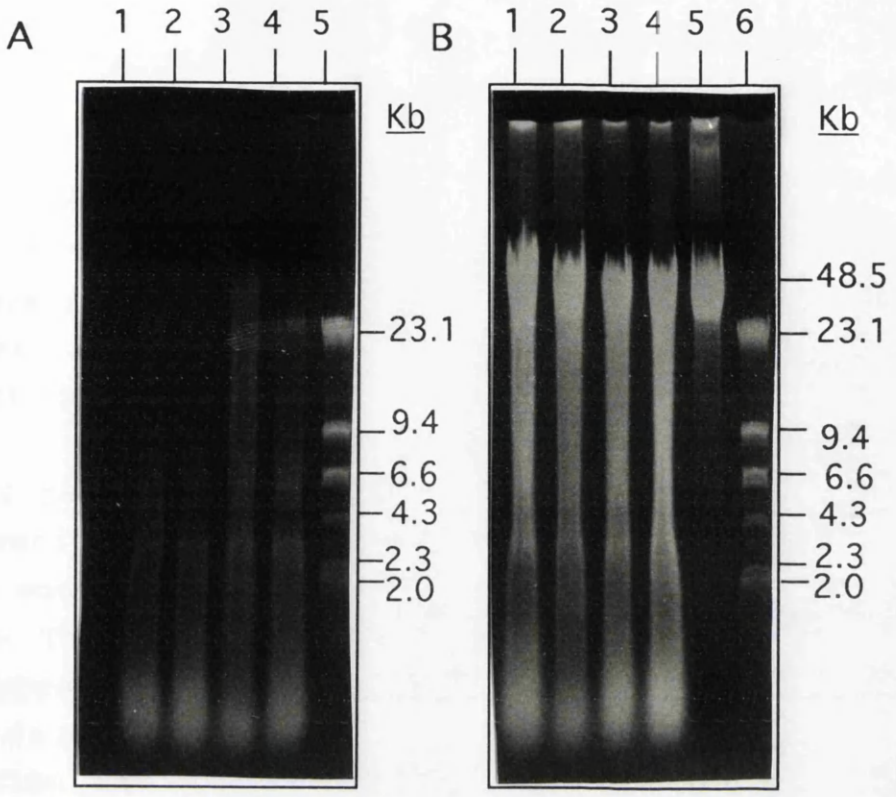



**Fig. 4.5 Large-scale digestion of V79 genomic DNA with Sau3AI.**
**a.** 100ug aliquots of V79 genomic DNA were prewarmed at 37°C for 30 mins. prior to the addition of 12.5 units of Sau3AI (tracks 1 and 2) and 25 units of Sau3AI (tracks 3 and 4). The restriction enzyme was inactivated and the extent of digestion visualised as for Fig 4.4. The molecular weight size marker used was phage lambda DNA digested with HindIII (track 5).
**b.** 100ug aliquots of V79 DNA digested with 4 units of Sau3AI (track 1), 6 units of Sau3AI (track 2), 8 units of Sau3AI and 10 units of Sau3AI. The reactions were checked by pipetting every 5 mins. until the viscosity of the DNA had started to drop, at which point the Sau3AI was inactivated and the extent of digestion visualised as for Fig. 4.4. Molecular weight markers are as for Fig. 4.5a.

centrifugation. Once the sucrose gradients were fractionated, a sample from every third fraction was subjected to agarose gel electrophoresis. Most of the aliquots from the bottom half of the gradients contained DNA fragments which were too large and would be of no further use in the production of the library (fig. 4.6, tracks 1-8). The DNA which was used in the subsequent steps, was that which was shown by electrophoresis to have undergone digestion but was still migrating between the two markers used i.e. uncut phage lambda DNA which is 48.5 kb, and the largest HindIII fragment of phage DNA (Fig. 4.6) which is 23.1Kb.

The Sau3AI partially digested genomic DNA was purified by ethanol precipitation from all the sucrose density gradient fractions containing partially digested DNA in the correct size range. The resulting DNA was again checked by agarose gel electrophoresis (Fig. 4.7) and all those fractions of DNA which underwent no mechanical shearing during the precipitation and resuspension proceedure were subsequently pooled.

**4.2.3.4 PREPARATION OF THE VECTOR pWE15 DNA.**

The cosmid vector which was chosen for the construction of the V79 cell genomic library was pWE15 (Fig. 4.8). This is a vector based on pBR322, which carries the $Amp^R$ marker gene. The vector also carries the $G418^R$ gene under the control of the SV40 promoter, which can act as a selectable marker when stably integrated into mammalian DNA. It was hoped that the SV40 sequences contained in pWE15 would also enhance the expression of any genes that were contained in the insert of the clone once transfected and stably integrated into the genome of the recipient cell.

Cosmid pWE15 DNA was digested with BamHI to generate restriction ends compatible with the restriction ends of the Sau3AI partially digested V79 genomic fragments. Subsequent calf intestinal phosphatase treatment was performed to dephosphorylate the 5' restriction ends of pWE15 to prevent its recircularisation and promote ligation between pWE15 DNA

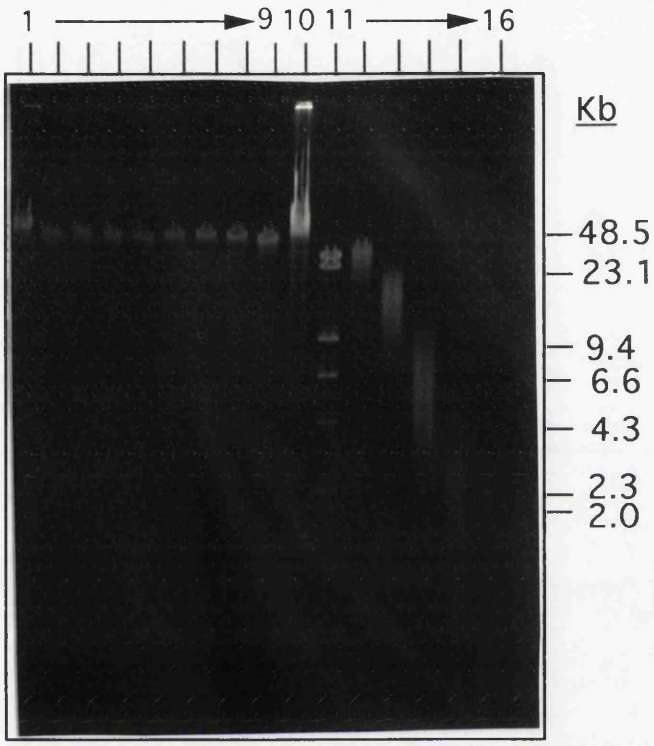



**Fig. 4.6 Separation of Sau3AI V79 genomic DNA fragments by sucrose density gradient centrifugation.**

Sau3AI digested V79 genomic DNA fragments were separated by size on 10-40% sucrose gradients (Chapter 2, section 2.24.2). The gradients were fractionated into 1ml aliquots, 20ul of every 3rd fraction (numbered 1-16 at the top of the gel, with no.1 being the fraction at the bottom of the gradient and no. 16 the fraction at the top) was visualised on an EtBr stained 0.3% TAE agarose gel (30V, 16hrs, 4$^{o}$C). The molecular weight of the V79 DNA fragments was compared to uncut phage lambda DNA (track 10) and HinDIII digested phage lambda DNA (track 11). The molecular weight markers were run in the middle of the gel to over come any potential mobility artifacts, frequently observed at the side of the gel.

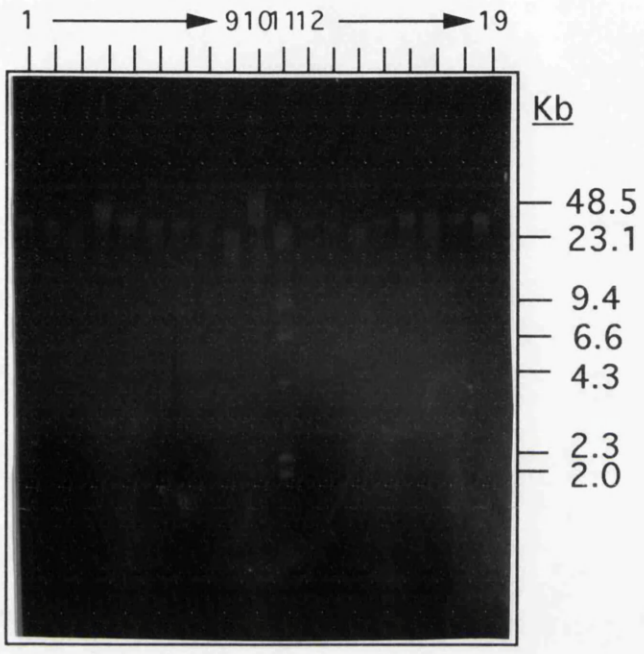



**Fig. 4.7 Isolation of V79 35-45Kb genomic DNA fragments from sucrose by ethanol precipitation.**

35-45Kb Sau3AI V79 genomic DNA fragments was purified from sucrose fractions of six sucrose gradients (track 1-9 and 12-19) by ethanol precipitation (Chapter 2, section 2.24.3). The DNA pellets were resuspended in 50 ul TE and 5 ul of each underwent electrophoresis on a 0.3% TAE agarose gel (30V, 16hrs, 4$^{o}$C) and were then visualised by EtBr. The molecular weight size markers used were uncut phage lambda DNA (track 10) and HindIII digested phage lambda DNA (track B).

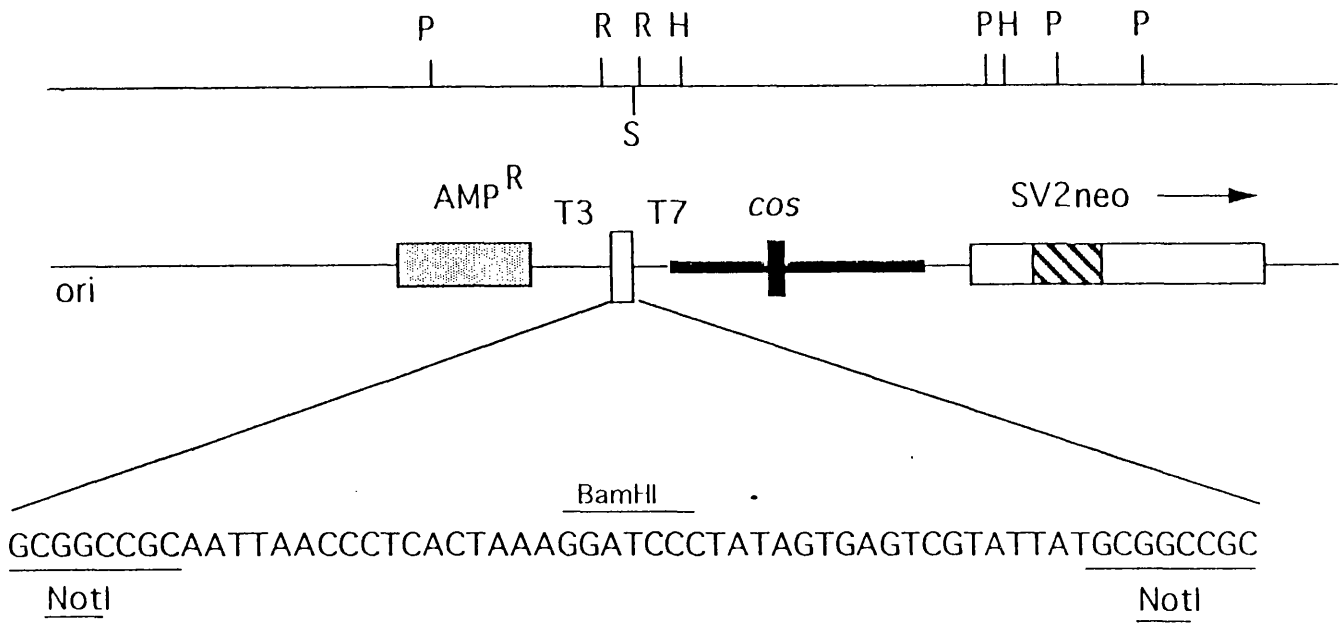



Fig. 4.8 **The structural map of pWE15**

The cosmid pWE15 contains the G418$^R$ gene like pSV2neo. AMP$^R$ denotes the antibiotic ampicillin resistance gene: T3 and T7, the bacteriophage promoters for the synthesis of end-specific probes: *cos* the cohesive termini from phage Charon 4A: and ori, the plasmid origin of replication. The hatched boxes represent tn5 Kanamycin/neomycin phosphotransferase (G418$^R$), and open boxes represent SV40 sequences which includes the SV40 promoter. Restriction sites are denoted as: B- BamHI: R- EcoRI; H- HindIII; P- PstI and S- SstI.

This is a linear representation of a circular plasmid.

and V79 Sau3AI partially digested genomic DNA fragments. BamHI digested, dephosphorylated pWE15 was ligated to itself and to BamHI digested pUC8 (Fig. 4.9) to test for the occurrence of recircularization of pWE15 DNA after dephosphorylation, and the ability of pWE15 DNA to ligate to DNA with compatible sticky ends. An extensive ladder of ligation products was produced from the second reaction, while no ligation products were observed when pWE15 was ligated to itself. Results from DNA transformation of competent *E. coli* C1400 cells using an aliquot from both ligation reactions (i.e. pWE15 ligated to pUC8 and pWE15 ligated to itself), confirmed the above results (Table 4.2). pWE15 ligated to pUC8 gave nearly as many transformants per ug of DNA as the uncut plasmid control and pWE15 ligated to itself gave no transformants.

**4.2.3.5 LIGATION AND PACKAGING OF V79 GENOMIC LIBRARY.**

For the ligation of pWE15 to V79 DNA a two-fold molar excess of vector to insert DNA was used. The concentration of each of the DNA molecules in such a ligation is dependent on their size. The pWE15 molecule was 8Kb and the V79 DNA taken to be 40Kb in length , a two-fold molar excess of vector over insert required 2ug of V79 insert DNA to 0.8ug of pWE15 DNA in the ligation. A parallel reaction, containing 375ng of V79 DNA was carried out to assess the condition of the Sau3AI restriction ends and their ability to ligate to the vector DNA. The ligation products were subjected to pulse-field gel electrophoresis. The results in Fig. 4.10 indicated that all the V79 DNA concatemerised (track 2) as very little DNA was left in the size range of unligated DNA (shown in track 1). Although a sample of pWE15 ligated to V79 DNA was not run on a gel every indication of the test ligations implies that the ligation should have worked well. This was confirmed by the number of recombinants in the cosmid library, which were obtained from packaging and plating (see below).

Competent *E. coli* HB101 cells were transformed with packaged pWE15/V79 ligation products as described in Chapter 2 (section 2.9.5) and the size of the library was assessed by colony titration.

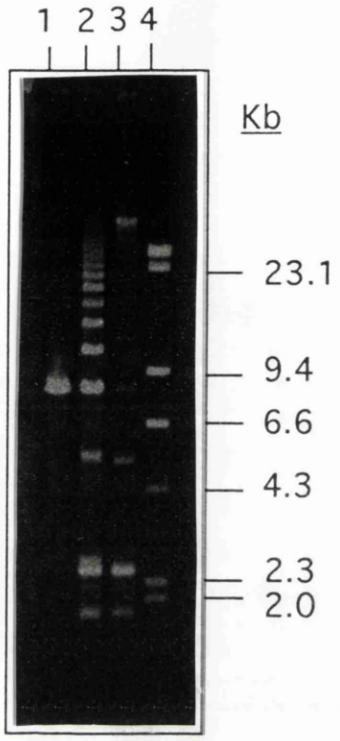



**Fig. 4.9 Dephosphorylation and ligation of pWE15 to pUC8.**
The vector pWE15 (20 ug) was completely digested with 24 units BamHI and its ends dephosphorylated using 20 units calf intestinal phosphatase (Chapter 2, section 2.16). To test the efficiency of the dephosphorylation and ligation techniques, 1 ug of dephosphorylated pWE15 was ligated (see Chapter 2, section 17) to itself (track 1), or ligated to 1 ug BamHI digested pUC8 (track 2). As a positive control for the ligation reaction 1 ug pUC8 was ligated to itself (track 3). The dephosphorylation and ligations were then assessed by electrophoresis on a 0.5% TAE agarose gel and visualised using EtBr. HindIII digested phage lambda DNA was used as molecular weight markers (track 4).

| Sample | 10ng | 1ng | 0.1ng |
|---|---|---|---|
| 1 | 814 | 88 | 5 |
| 2 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 |
| 4 | 620 | 56 | 2 |
| 5 | 0 | 0 | 0 |
| 6 | 943 | 58 | 8 |

**Table 4.2 Transformation of *E. coli* C1400 cells with pWE15/pUC8 ligation products.**

Numbers of colonies observed after transformation of competent *E. coli* C1400 cells (Chapter 2, section 2.9.1 and section 2.9.3) with 10 ng, 1 ng and 0.1 ng of pWE15/pUC8 ligation products from experiment shown in Fig. 4.9 and control DNAs selected for $AMP^R$. Sample 1 - pWE15, undigested, a positive control for transformation; Sample 2 - pWE15 digested with BamHI, to control for undigested plasmid; Sample 3 - pWE15, digested with BamHI, dephosphorylated with calf intestinal phosphatase, and treated with DNA ligase, a control to analyse the efficiency of dephosphorylation; Sample 4 - pWE15, digested with BamHI, dephosphorylated as above and ligated to BamHI digested pUC8; Sample 5 - pUC8 digested with BamHI, again a control for the efficiency of digestion as in sample 2; Sample 6 - pUC8 digested with BamHI and treated with DNA ligase, a control for the ability of pUC8 to self-ligate and hence to ligate to pWE15.

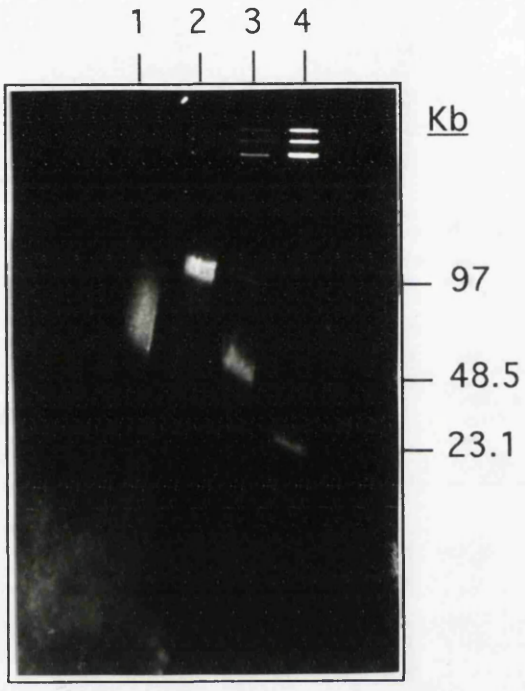



**Fig. 4.10 Ligation of 35-45Kb Sau3AI V79 restriction fragments.** Pulse-field gel electrophoresis (Chapter 2, section 2.11.4) of 1 ug of 35-45 kb Sau3AI partially digested V79 DNA after self-ligation (track 2). 1 ug of the same DNA, unligated is shown in track 1. Markers used were phage lambda DNA (track 3) and HindIII digested phage lambda DNA (track 4). Their sizes are shown in kilobases.

Self-ligation was carried out as follows: 1ug 35-45Kb Sau3AI partially-digested V79 DNA, 1ul 10 x Ligation buffer, 1ul 10mM ATP, 1ul $T_4$ DNA ligase made up to a final volume of 10ul with $dH_2O$ and incubated overnight at $16^0C$.

The result from the initial packaging reaction implied a library size of 1.2 x $10^5$ clones. This was not as good as expected or required. To be 99% sure that the whole repertoire of sequences are represented, a library size of 2.5 x $10^5$ clones would be required. After repeating the ligation and packaging, a total library of 5 x $10^5$ clones was obtained.

To examine the diversity of the library, 12 clones were picked at random from which cosmid DNAs were prepared and restriction analysis was performed using the restriction enzymes NotI and EcoRI. NotI was used as this enzyme cuts at two sites in pWE15, either side of the BamHI cloning site, and would therefore cut out the insert with very little flanking sequence attached, allowing insert size to be assessed (Fig. 4.11). All the clones contained inserts (panel A, Fig. 4.11) as shown by the NotI digests. The EcoRI digests (panel B, Fig. 4.11) showed that the inserts were all different, indicating the expected diversity of the library.

#### 4.2.3.6 LARGE-SCALE PLATING OF THE V79 GENOMIC COSMID LIBRARY.

The library was plated out on L-agar plates (24.5 x 24.5 cm) containing 100 ug.$ml^{-1}$ ampicillin, in groups of 20 000 clones. The cells were washed off the plates with 20 ml of L-broth containing 100 ug.$ml^{-1}$ ampicillin. 2 x 1 ml aliquots from each group of clones were stored in glycerol at -20$^{o}$C. DNA was prepared from the rest of the cells resulting in 24 aliquots of DNA representing the whole library, to use in subsequent transfection experiments.

### 4.2.4 PHENOTYPIC RESTORATION OF GAL 73 BY COSMID LIBRARY DNA TRANSFECTION.

#### 4.2.4.1 OPTIMIZATION OF ELECTROPORATION CONDITIONS FOR GAL 73 CELLS.

In order to try and complement the mutated gene in Gal 73 cells, aliquots of DNA prepared from pools of 20 000 clones of the V79 genomic cosmid library were introduced into Gal 73 cells by electroporation.

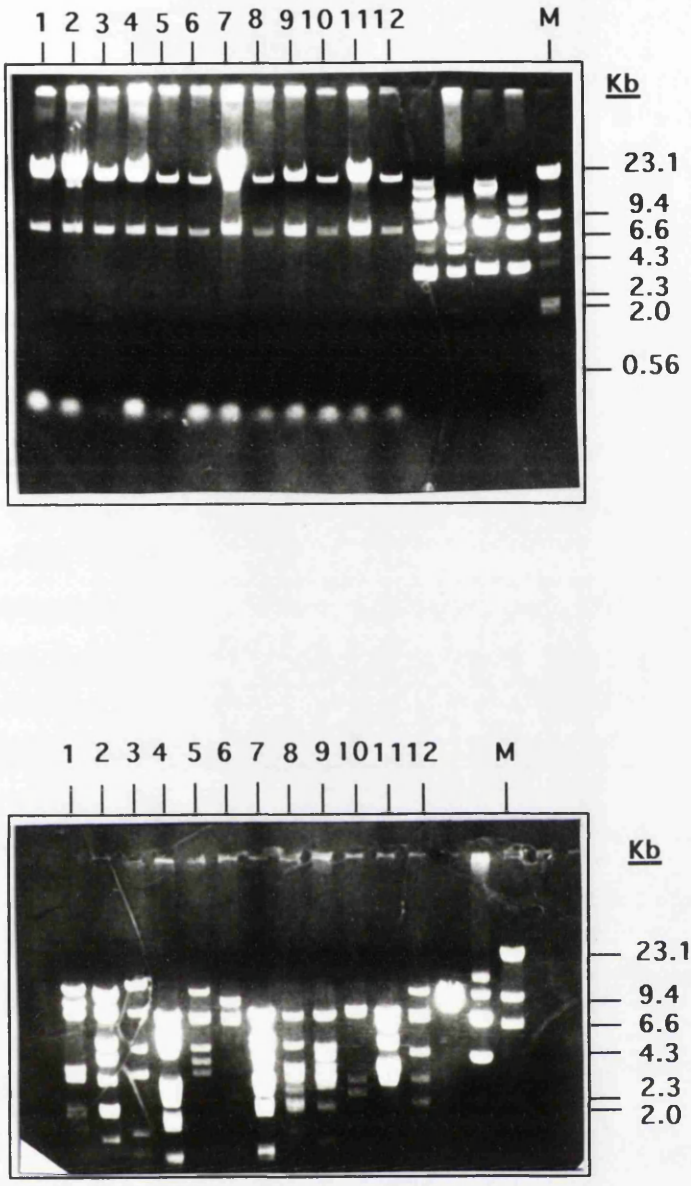



**Fig. 4.11 Restriction analysis of 12 putative cosmid clones.** Twelve putative recombinant cosmid (pWE15/V79) clones were picked at random and DNA prepared and subjected to restriction enzyme analysis. The top panel shows 1ug of each of the 12 clones digested with NotI (8 units/ul) to cut out the V79 genomic DNA insert (tracks 1-12) and the bottom panel, the same DNAs digested with EcoRI (10 units/ul). Phage lambda DNA digested with HindIII was used as molecular weight markers (M).

When a cell is exposed to an electric field, the membrane components become polarized and a voltage potential develops across the membrane. If the potential difference exceeds a threshold level, the membrane breaks down in localized areas and the cells become permeable to exogenous macro-molecules. The permeability induced is reversible provided the magnitude and duration of the electirc field do not exceed critical limits, otherwise the cell is irreversibly damaged (Neumann et al, 1982; Toneguzzo et al, 1986; Chu et al, 1987; Andreason and Evans, 1988; Shigekawa and Dower, 1988; Winterbourn et al, 1988). In a trial experiment, different electroporation conditions were tested, for the introduction of pWE15 DNA into Gal 73 cells. Both the percentage of surviving cells and the number of transformants to $G418^R$ were scored in each of these trials. In this experiment the percentage of cell death indicates the amount of irreversible membrane damage caused by the size and pulse duration of the electric field applied to the cell suspension. Relative cell viability was measured by plating cells exposed and not exposed to the electric field on parallel plates, allowing them to grow for 72 hours and then counting them directly using a haemocytometer. The results from test transfections performed showed that the highest frequencies of transformation were obtained when 40-50% cell death was recorded.

The parameters used to generate the electric pulse which gives optimal membrane permeability without damage are different for each cell-line and were therefore worked out individually for each of the Chinese hamster respiratory deficient cell-lines, using supercoiled pWE15 DNA, prior to transfection with DNA from the cosmid library.

Aliquots of $2 \times 10^6$ Gal 73 cells were resuspended in Hepes buffered saline as described in the materials and methods section 2.23.2 and transfected by electroporation with supercoiled pWE15 DNA under various voltage and capacitance levels (Table 4.3). The conditions which gave the highest frequency of transformation (0.3%) were 300 V with a capacitance of 960 uFD. This gave a time constant for the duration of the electric field through the cell suspension of

| Sample | Voltage (volts) | Capacitance (uFD) | % cell death | % transformants per ug DNA |
|---|---|---|---|---|
| 1 | 100 | 125 | 0 | 0 |
| 2 | 100 | 500 | <1% | 0.00005% |
| 3 | 100 | 960 | <1% | 0.0001% |
| 4 | 300 | 125 | 25% | 0.043% |
| 5 | 300 | 500 | 35% | 0.1% |
| 6 | 300 | 960 | 50% | 0.3% |
| 7 | 400 | 500 | 30% | 0.092% |
| 8 | 400 | 960 | 85% | 0.005% |
| 9 | 500 | 25 | 10% | 0.012% |
| 10 | 500 | 3 | <1% | 0.00005% |
| 11 | 500 | 1 | 0 | 0 |
| 12 | 1000 | 25 | 25% | 0.12% |
| 13 | 1000 | 3 | 5% | 0.003% |
| 14 | 1000 | 1 | <1% | 0.00005% |

**Table 4.3 Electroporation of Gal 73 cells with pWE15.**
Aliquots of 2 x $10^6$ Gal 73 cells were prepared for electroporation (Chapter 2, section 2.23.2) with 5ug supercoiled pWE15 DNA under the voltage and capacitance levels noted above. The cell death due to electroporation was calculated (Chapter 2, section 2.23.2) and the viable cells placed into selective medium (glucose medium plus 800 $ug.ml^{-1}$ G418) for 3 weeks. $G418^R$ colonies were visulised by staining with Leishman's stain (Chapter 2, section 2.23.1) and counted.

12-14 ms. The higher voltage, smaller capacitance conditions gave much shorter durations of electric field, which resulted in lower transformation efficiencies.

The conditions of voltage and capacitance subsequently used in the experimental transfection of cosmid library DNA into the cell-lines Gal 73 and Gal 13 were 300 volts and 960uFD and although not tested specifically, the transfection efficiency with a cosmid recombinant clone of 50Kb was expected to be lower than for an 8Kb plasmid due to the increase in size of the in-going DNA molecule. Approximately 2 x $10^8$ exponentially growing cells from each cell-line Gal 73 and Gal 13 were grown and prepared for electroporation according to the protocol described in section 2.23.2. Exponentially growing cells were defined as cells which had reached 50-60% confluency in the tissue culture flasks used, before harvesting for electroporation. Exponentially growing cells are known to give an increase in the frequency of transformation over cells used which have reached stationary growth phase (Chu et al, 1987).

10ug of DNA prepared from 13 of the pools of 20 000 clones of the V79 genomic cosmid library was used in each of the electroporations. Stable (chromosomal) integration of cosmid library DNA was selected for, by resistance to G418. Growth on the non-fermentable substrate, galactose, was used to select for respiratory competence. Using DNA from the first 13 pools of 20000 clones, only DNA from pool 2 generated Gal 73 cells which were both $G418^R$ and could grow on galactose-containing medium ($gal^+$).

A dilution series of $10^3$, $10^4$ and $10^5$ cells was plated out in glucose-containing medium containing G418, for each transfection, to obtain the overall frequency of stable integration of cloned DNA from the library. The frequency of transformation to $G418^R$ obtained with the cosmid library DNA was lower than with the vector pWE15 DNA alone, at 0.1-0.2%. This implies that approximately 200 000 independent transformants were generated in each electroporation. Assuming that only one cosmid clone was integrated in each

transformant, the pool size of 20 000 different cosmid clones would imply, that if each clone were randomly represented in the mixture, about 10 transformant colonies would be produced from a pool of clones containing one cosmid capable of complementing the Gal 73 mutation. However, only two $G418^R$, $gal^+$ colonies were observed when transfected with cosmid library DNA from pool 2, indicating that the cosmid clone capable of complementing the mutation in Gal 73 cells is either represented in the library at a low frequency, or that major rearrangements of the cosmid clone DNA has occurred upon integration, effectively lowering the frequency of observable $G418^R$, $gal^+$ cells.

### 4.2.5 ANALYSIS OF INTEGRATED DNA SEQUENCES IN RESPIRATORY COMPETENT GAL 73 CELL CLONES.

The two $G418^R$, $gal^+$ Gal 73 colonies, denoted as $Gal^+$73A and $Gal^+$73B, that were isolated when Gal 73 was transfected with DNA from pool 2 of the V79 genomic cosmid library were cloned (Chapter 2, section 2.23.4), after being allowed to grow in selective medium for three weeks. In all subsequent experiments $Gal^+$73A and $Gal^+$73B were maintained and grown in selective medium (galactose-containing medium with 800 $ug.ml^{-1}$ G418). Aliquots of $Gal^+$73A and $Gal^+$73B cells were also grown in complete medium for 3 weeks before being put back under selection (galactose medium containing G418) to test for the stable maintenance and expression of the integrated DNA sequences. No loss of viability was observed.

Genomic DNA was prepared from $Gal^+$73A and $Gal^+$73B cells, and the pattern of integration of pWE15 DNA in each clone was analysed by southern blotting of this DNA after digestion with restriction endonucleases, and probed with pWE15 DNA radiolabelled by nick translation (Chapter 2, sections 2.18.1 and 2.19.1).

The results of the southern blots (Fig. 4.12) showed that the pattern of integration of pWE15 sequences in the genomic DNA of $Gal^+$73A and $Gal^+$73B was different, based on the pattern of restriction fragments which hybridised to radiolabelled pWE15

DNA, indicating that the clones were not siblings. Respiratory competence must therefore have been restored, in these two clones, by independent integration events. The pattern of fragments detected by radiolabelled pWE15 DNA in Gal$^+$73B DNA cut with various restriction enzymes would suggest that the pWE15/V79 recombinant cosmid clone had perhaps integrated tandemly, with one of the copies undergoing some rearrangment upon integration. This is most strongly suggested by the EcoRI digest of Gal$^+$73 DNA. EcoRI cuts pWE15 DNA on either side of the BamHI cloning site which would effectively cut out the insert from the vector DNA. Two pWE15 reactive restriction fragments were generated in Gal$^+$73B DNA cut with EcoRI, one of which, at 8.5Kb could possibly represent the complete vector sequences. This band was approximately 3-4 times more intense than the other band observed which was 10.5Kb in length. The lower intensity of the 10.5Kb EcoRI fragment suggests there is a lower number of copies of this fragment and possibly represents a copy of pWE15 that has been rearranged upon integration so as to move or remove one of the EcoRI sites and make a larger fragment. This is supported by the pattern of pWE15-related restriction fragments observed when the DNA was digested with SstI and XbaI (Fig.4.12, panel B, tracks 7 and 8). There is no XbaI site within pWE15 so when Gal$^+$73B DNA was digested with this enzyme and probed with pWE15, two fragments were observed. One intense band with a molecular weight of 16.5Kb and a less intense band of 11Kb, again indicating probably 3-4 copies of the integrated cosmid clone and one copy which was rearranged to give a fragment containing pWE15 sequences of a different size. For the SstI digest, the pattern can only be explained in the same way if the single SstI site that occurs in pWE15 is located close to the BamHI cloning site used in the preparation of the library. The SstI restriction site is approximately 40bp away from the BamHI site and supports this hypothesis. The pattern of pWE15 reactive fragments generated from the HindIII and PstI digests of Gal$^+$73B DNA also conform to this hypothesis. However, the pattern observed for the BamHI digest does not, and is difficult to explain unless one of the BamHI sites in the integrated recombinant clone was reconstituted when ligated to the Sau3AI digested V79 genomic DNA during the construction of

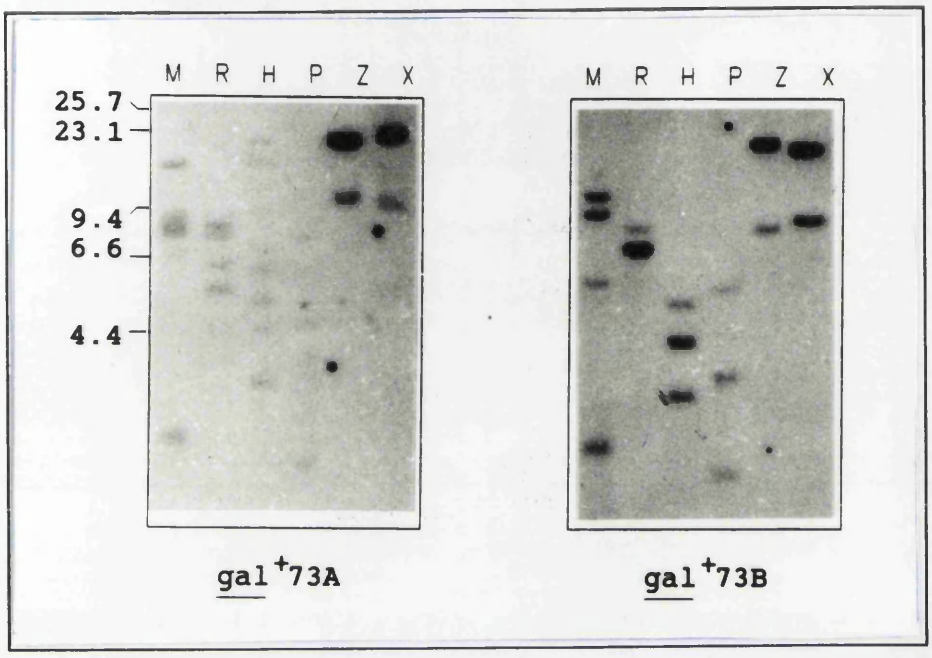



**Fig. 4.12 Southern blot analysis of Gal$^+$73A and Gal$^+$73B DNA.** Restriction enzyme digests of Gal$^+$73A genomic DNA (panel A) and Gal$^+$73B genomic DNA (panel B) were analysed by southern blotting (Chapter 2, section 2.18.1). The filters were probed with nick-translated pWE15 DNA (Chapter 2, section 2.19.1). Digests are indicated as B (BamHI), R (EcoRI), H (HindIII), Z (SstI) and X (XbaI). The molecular weight of the fragments were compared to phage lambda DNA digested with HinDIII and are shown in kilobases.

the V79 genomic cosmid library (see Fig. 4.13), of which there is a 1/4 chance.

The 2.75Kb BamHI fragment observed in both Gal$^+$73A and Gal$^+$73B DNA, was also observed in the V79 DNA negative control and was found to be a contaminant in the BamHI enzyme and was ignored.

Although there is evidence for tandemisation in Gal$^+$73A DNA also from the SstI and XbaI digests (Fig. 4.12, panel A, tracks 7 and 8), the remaining digests show a confusing pattern of integration of pWE15 DNA, which is hard to reconcile with a simple hypothesis of this kind. However, there are pWE15 reactive fragments common to both Gal$^+$73A and Gal$^+$73B DNA which would not seem to be internal fragments of pWE15 eg. a 5.2Kb HindIII fragment and a 4.25Kb PstI fragment, which would suggest that the same cosmid clone had integrated in both Gal$^+$73A and Gal$^+$73B DNA. However, the greater number of pWE15 reactive fragments generated in Gal$^+$73A DNA for each digest (except SstI and XbaI) compared to Gal$^+$73B DNA is difficult to explain with the information known. To give a more detailed explanation of what is happening in Gal$^+$73A DNA the area of integration would have to be isolated and analysed by subcloning and mapping.

### 4.2.6 PHENOTYPIC RESTORATION OF GAL 13 BY TRANSFECTION WITH DNA FROM THE V79 GENOMIC COSMID LIBRARY.

The protein synthesis analysis of Gal 13 cells referred to in Chapter 3, indicated that the nuclear mutation in Gal 13 cells would seem to directly affect the protein synthesis of a subset of mitochondrial DNA encoded polypeptides, possibly comprising the mitochondrially encoded NADH dehydrogenase subunits of Complex I, unlike the mutation in Gal 73 cells which does not seem to affect mitochondrial protein synthesis. To find out the function of the Gal 13 mutant gene, attempts were made to complement the mutation using DNA from the V79 genomic cosmid library, in the same way as performed for Gal 73 cells. Transfection of Gal 13 cells with DNA from 12 pools of 20 000 V79 genomic cosmid clones generated no G418$^R$, Gal$^+$ colonies. Due to the low frequency of transformation of Gal 73

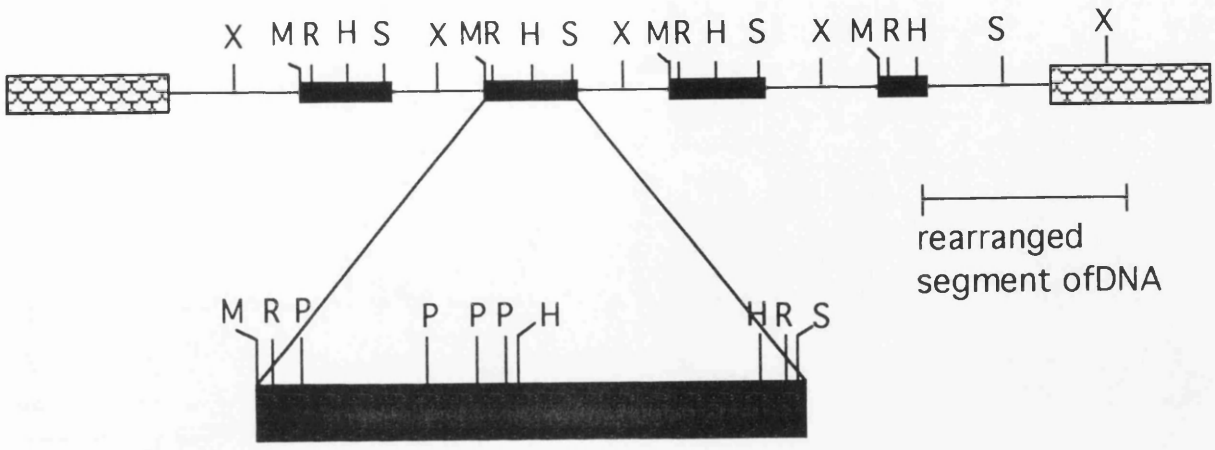



**Fig. 4.13 Possible map of integration of the pWE15/V79 recombinant cosmid clone into Gal$^+$73B DNA.**

Deduced from the southern blot analysis of Gal$^+$73B DNA (Fig. 4.12, panel B) a possible schematic representation has been drawn up of the integration and tandemisation of the pWE15/V79 recombinant clone in the Gal$^+$73B cell-line. The black boxes represent pWE15 cosmid sequences; the hatched boxes Gal 73 genomic DNA into which the cosmid recombinant clone has integrated and the thin black line V79 genomic DNA from the insert of the pWE15/V79 recombinant clone. Restriction sites are indicated as: B - BamHI, R - EcoRI, H - HindIII, P - PstI, S - SstI and X - XbaI. The positions of the restriction sites within the V79 genomic sequences have been arbitrarily placed. Those shown in pWE15 sequences are placed in their correct position, although the BamHI site is thought to have been regenerated after ligation to V79 genomic DNA. Not drawn to scale.

cells to $G418^R$, $gal^+$ phenotype, a high frequency of transformation of Gal 13 cells would have been unexpected and it was not disheartening at this point not to have isolated $G418^R$, $gal^+$ Gal 13 colonies, as DNA from 12 other pools of 20 000 cosmid clones could be screened for its ability to restore respiratory competence. However, this work was not pursued further, in favour of attempting to define more precisely the nature of the complementing DNA in the $Gal^+73A$ and $Gal^+73B$ transformants.

## 4.3 DISCUSSION.

Although several $gal^-$ cell-lines have been restored to respiratory competence (Garnett et al, 1985), by transfection with high molecular weight genomic DNA, I was not successful in stably transforming Gal 73 by this method. Two $gal^+$ clones which were isolated when over $10^6$ $G418^R$ clones were screened in co-transfection experiments of high molecular weight HeLa DNA and pSV2neo, appear ed unstable and died prior to cloning. As the integration of HeLa DNA into Gal 73 cells could not be demonstrated, it is possible that although pSV2neo had integrated, HeLa DNA had not, and the $gal^+$ phenotype was due to the spontaneous reversion of the mutation in these two unstable clones, as is known to occur in the $gal^-$ cell-lines (Whitfield et al, 1981). However, the spontaneous reversion frequency was tested for Gal 73 cells under the experimental conditions employed here and was found to be less than 1 in 2 x $10^9$ cells, implying that these two $gal^+$, $G418^R$ colonies would have been most unlikely to have arisen by spontaneous reversion.

Another possible explanation could be that upon integration of pSV2neo, a suppressor mutation was generated. Suppressor mutations have been shown to occur spontaneously in respiratory deficient yeast, in which the deletion of a region of mitochondrial DNA restores respiratory ability (Muller et al, 1984). The deletion of a portion of mitochondrial DNA could not be shown due to the premature death of the two colonies.

The two gal$^+$ G418$^R$ clones, isolated when V79 genomic DNA was integrated into Gal 73 as recombinant cosmid clones, could also, conceivably have been generated due to the suppression of the mutant phenotype by the insertion of the cosmid recombinant clone at a particular chromosomal site i.e. a nuclear suppression. However, because two G418$^R$, gal$^+$ clones were generated when transfected with DNA from the same pool of V79 genomic DNA cosmid clones (pool 2) and from no others, it would seem much more likely that Gal$^+$73A and Gal$^+$73B were isolated due to a V79 genomic DNA cosmid clone within this pool, which has the ability to restore respiratory competence in the Gal 73 mutant cell-line.

The failure to see a higher frequency of gal$^+$ clones by transfection with DNA from the V79 genomic cosmid library could be due to a number of reasons. Southern blot analysis of Gal$^+$73A and Gal$^+$73B genomic DNA shows evidence in support for the integration of tandem copies of pWE15 sequences but also for the rearrangement of some of those copies. The pattern of pWE15 reactive fragments in Gal$^+$73A DNA could be interpreted as showing extensive rearrangement of the integrated DNA in contrast to Gal$^+$73B, although there are pWE15 reactive fragments common to both cell-lines. These restriction fragments do not have the same molecular weight as the 'internal' PstI and HindIII pWE15 restriction fragments i.e. when supercoiled plasmid DNA alone is digested with these enzymes, taking into account that one PstI and one HindIII restriction fragment would have been disrupted due to the cloning of the V79 genomic DNA into the BamHI cloning site. These restriction fragments are therefore probably composed of both pWE15 and V79 genomic DNA which suggests that both Gal$^+$73A and Gal$^+$73B were generated due to the integration of the same recombinant cosmid clone, and that the integrated DNA in Gal$^+$73A DNA underwent more rearrangement than took place in Gal$^+$73B DNA. There is the possibility that if Gal$^+$73A and Gal$^+$73B represent differing degrees of rearrangment of the integrated recombinant cosmid clone, that in some integrations of this clone, major rearrangements could have occurred which have disrupted the gal$^+$ gene within the cosmid clone resulting in the maintenance of the gal$^-$ phenotype and

contributing to the low frequency of transformation of Gal 73 cells to $gal^+$.

Nothing was known prior to this project about the gene which carries the mutation within Gal 73, it could be a very large gene with many introns spread over a large area. In this case it would be very difficult to transform this cell-line to respiratory competence unless the DNA covering the mutated region is integrated by homologous recombination, in which the whole $gal^+$ gene would not be necessary. However, the frequency of homologous recombination in mammalian cells is relatively low. In gene-targeting experiments (which score for homologous recombination) where the modification of the Chinese hamster *aprt* gene (Adair et al, 1989) was attempted, the ratio of homologous to non-homologous integrations was approximately 1:1000. The frequency of homologous recombination can depend on the cell-line being used. In one study with human cells, the ratio of homologous to non-homologous events varied from 1:500 to 1:76 between two cell-lines (Song et al, 1987) and the gene being targeted can also effect the frequency of homologous recombination. The need for homologous integration of this type may have contributed to the low frequency of transformation obtained.

Under double selection for $G418^R$, $gal^+$, the possibility of isolating a spontaneous $gal^+$ revertant is low but cannot be discounted absolutely. However the probability of isolating 2 revertant clones from one pool when 12 other pools have been tested in two cell-lines with no success, is statistically extremely improbable. By using the equation for the Poisson Distribution, the probability of isolating 2 $gal^+$, $G418^R$ clones due to spontaneous reversion in 1 genomic DNA pool and none in 12 other genomic DNA pools can be worked out, assuming that the spontaneous reversion of $gal^-$ cells occurs randomly. The probability of $Gal^+$73A and $Gal^+$73B having been isolated as a result of spontaneous reversion works out to be 0.06%. Therefore it was believed that two independent integration events had resulted in the transformation to respiratory competence of the mutant phenotype in $Gal^+$73A and $Gal^+$73B cells. Whether the same cosmid clone has been responsible in

both cases is not yet known because of the extensive rearrangements within $Gal^{+}73A$. To study this, the integrated DNA in both cell-lines would have to be isolated and analysed. However, it is a reasonable working hypothesis that pool 2 of the library contains a single recombinant clone capable of complementing the mutation in Gal 73 cells.

# CHAPTER 5

## THE ISOLATION AND CHARACTERISATION OF INTEGRATED DNA SEQUENCES FROM GAL 73 RESTORANT CELL CLONES BY MARKER RESCUE.

## 5.1 INTRODUCTION

As described in Chapter 4, DNA from a V79 genomic DNA cosmid library was used in an attempt to restore Gal 73 cells to respiratory competence in DNA transfection experiments. As a result of performing this task two cell-lines, $Gal^+73A$ and $Gal^+73B$ were isolated which showed the required phenotype.

The next step in the process of isolating the wildtype gene which is mutated in Gal 73 was to attempt to characterise the wildtype genomic DNA sequences which had integrated into the genome of these two $gal^+$ cell-lines. To do this, a marker rescue scheme was devised (Fig. 5.1) making use of the selectable marker of ampicillin resistance expressed in bacterial cells, carried by pWE15. As direct packaging of genomic DNA prepared from $Gal^+73A$ and $Gal^+73B$ using high efficiency packaging extracts was not successful in isolating the integrated wildtype DNA sequences, a cosmid genomic library was prepared from $Gal^+73B$ DNA. The vector Lorist 6 was chosen (Gibson *et al*, 1987). Lorist 6 is a phage-lambda origin-containing vector which does not confer ampicillin resistance on its bacterial host and has little sequence homology to pWE15, except the tn5 kanamycin/neomycin transferase sequences it contains. The idea behind using a cosmid vector which carries an antibiotic resistance marker different from the ampicillin resistance marker expressed in bacteria by pWE15, is that plating out the $Gal^+73B$ cosmid library on ampicillin would allow only those clones containing ampicillin resistance sequences to grow. Any recombinant Lorist 6 cosmid clones isolated by this method should therefore contain portions of V79 genomic DNA originally integrated as a pWE15 recombinant clone into Gal 73 to create the $Gal^+73B$ restorant, and if lucky, might contain intact the $gal^+$ gene responsible for restoring wildtype phenotype in Gal 73 cells. Four such cosmid clones were isolated by this method. After some initial characterisation, four were analysed further by southern blotting; which confirmed that they contained overlapping segments of $Gal^+73B$ DNA, containing pWE15 and associated genomic sequences.

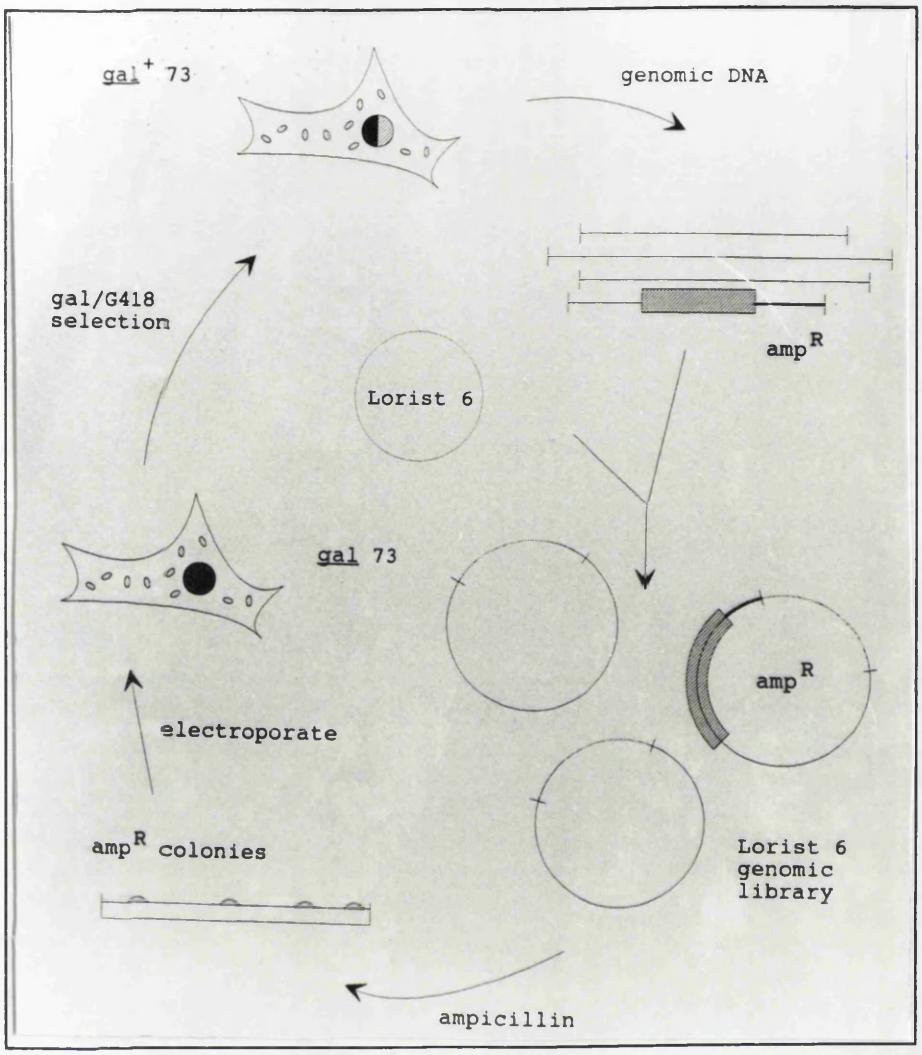



Fig. 5.1 Marker rescue scheme for isolating from the genomic DNA of Gal$^+$73 transforants the integrated pWE15 library sequences which have conferred gal$^+$ phenotype. The genomic sequences of interest are shown as a shaded box, and the pWE15 and Lorist 6 vector sequences are depicted by bold lines and broken lines respectively. Nuclei containing both the wildtype and mutant allele are shown by being half filled in (mutant allele) and half shaded (wild type allele).

Transfection of Gal 73 cells using DNA from these four Lorist 6 recombinant clones did not however, result in the restoration of wildtype phenotype, suggesting that individually the Lorist 6 clones do not contain an entire copy of the $gal^+$ gene of interest. However, they could contain parts of the gene as transfection of Gal 73 cells using DNA from all four Lorist 6 clones together, did result in the isolation of three $gal^+$ clones. To try to identify sequences in the Lorist 6 clones which could restore $gal^+$ phenotype, single copy V79 sequences were obtained by subcloning Sau3AI fragments of the Lorist 6 clones into M13. Sequencing single copy clones isolated could potentially identify the gene responsible for restoring respiratory competence in Gal 73, but if not, could at least be used as probes in an attempt to isolate the original recombinant clone from the pool of 20 000 pWE15 clones whose DNA was used to restore Gal 73 to respiratory competence, or to use as probes to screen a wildtype genomic or cDNA Chinese hamster library.

## 5.2 RESULTS

### 5.2.1 ISOLATION OF INTEGRATED SEQUENCES BY IN VITRO PACKAGING OF $GAL^+73A$ AND $GAL^+73B$ GENOMIC DNA.

In Aspergillus, tandemly repeated cosmid sequences integrated into the genome have been isolated by directly extracting the cosmid sequences from genomic DNA, using the ability of the phage head construction proteins and enzymes found in packaging extracts to cut and encapsulate concatermerised phage DNA into infective phage heads. This was attempted with genomic DNA from $Gal^+73A$, and $Gal^+73B$, as the results from the southern blots of genomic DNA from these two cell-lines probed with nick-translated pWE15 (Chapter 4, fig. 4.14) suggested the possibility that the pWE15 recombinant clones had integrated tandemly in the genome of each of the cell-lines.

5 ug of $Gal^+73A$ and $Gal^+73B$ DNA, which each resolved as a discrete band above lambda DNA on a 0.3% agarose gel, in a final volume of 4 ul TE was packaged using the high efficiency Giga- Pack Gold packaging kit from Stratagene. HB101 cells

infected with the packaged DNA were plated out in the presence of 50 ug.ml$^{-1}$ ampicillin. No ampicillin resistant colonies were observed after incubation at 37$^{0}$C for 48 hours. However, no positive control was performed to test the effectiveness of the packaging extract, so failure to recover the integrated sequences could be due to lack of activity of the packaging extracts used.

If the packaging extracts were performing to the manufacturer's stated efficiency, then other reasons why the in vitro packaging of the integrated cosmid sequences failed could be: (i) the low number of tandemized copies of the *cos* sequence separated by the appropriate length (50 kb) of DNA integrated into the cell-lines. The recommended concentration of DNA which can be used in each packaging reaction is up to 5 ug per extract. For the 5 ug of Gal$^{+}$73A and Gal$^{+}$73B genomic DNA used in the reaction only a small percentage was concatermised cosmid and was probably swamped by the surrounding genomic DNA.

(ii) Genomic DNA with an average strand length exceeding 100Kb had to be isolated to acquire two uninterrupted tandemised copies of a recombinant cosmid clone. Possible shearing of the DNA to shoryter lengths, (as the DNA was precipitated prior to packaging to obtain 5ug of DNA in the volume required for packaging), could account for the failure to package in vitro the integrated V79 sequences from Gal$^{+}$73A and Gal$^{+}$73B DNA.

(iii) To be able to package cosmid DNA into phage heads and recover it, a number of conditions must be met. A plasmid origin of replication, an intact selectable marker, and two lambda *cos* sites separated by 40-50Kb of intervening sequence must all be present. Disruption of any one of these by rearrangment would eliminate the possibility of isolating the integrated sequences by this method.

(iv) The interpretation of the results discussed in Chapter 4 may possibly be incorrect and no tandemization of the integrated pWE15 recombinant clones occurred. In this case, in vitro packaging of Gal$^{+}$73A or Gal$^{+}$73B DNA would not result in

the recovery of the integrated sequences. Further analysis of the region of Gal$^+$73A and Gal$^+$73B DNA in which the integration had occurred would clarify this.

### 5.2.2 CONSTRUCTION OF A COSMID GENOMIC LIBRARY OF GAL$^+$73B DNA IN LORIST 6.

Due to the failure of the attempts to isolate the integrated pWE15 recombinant clones from Gal$^+$73A and Gal$^+$73B genomic DNA by direct *in vitro* packaging, rescue of the amp$^R$ gene of pWE15 and adjacent sequences from Gal$^+$73B was attempted by preparing a genomic library from this cell-line in a second cosmid vector, Lorist 6, following the scheme shown in Fig. 5.1. Lorist 6 contains the kanamycin resistance marker (Fig. 5.2), but not the ampicillin resistance marker which is expressed by pWE15 .

A cosmid library was constructed in Lorist 6 of *Sau*3AI partially digested Gal$^+$73B genomic DNA fragments in the same manner as the pWE15 wildtype genomic library (see Chapter 4, section 4.4). 35-45 kb *Sau*3AI Gal$^+$73B DNA fragments were isolated by sucrose gradient centrifugation. 2 ug of this DNA was ligated to 1 ug of *Bam*HI digested Lorist 6 DNA which had been dephosphorylated using calf intestinal phosphatase. Of the 10 ul ligation reaction, 5 ul was packaged using Stratagene's Giga-Pack Gold packaging kit, following their protocol. Titration (protocol by Stratgene) on 25 ug.ml$^{-1}$ kanamycin plates of the packaging reaction indicated a library size of 3 x $10^5$ clones. The majority of the packaging reaction was then used to infect competent HB101 cells, which were then plated on 50 ug.ml$^{-1}$ ampicillin.

Seven ampicillin resistant colonies L73B1-7 were isolated of which five L73B1-5 were also kanamycin resistant upto 25 ug.ml$^{-1}$. The other two, L73B6 and L73B7, were kanamycin sensitive down to concentrations of lower than 5 ug.ml$^{-1}$, produced low yields of cosmid DNA, and underwent no further analysis, so that analysis of L73B1-5 could be concentrated on.

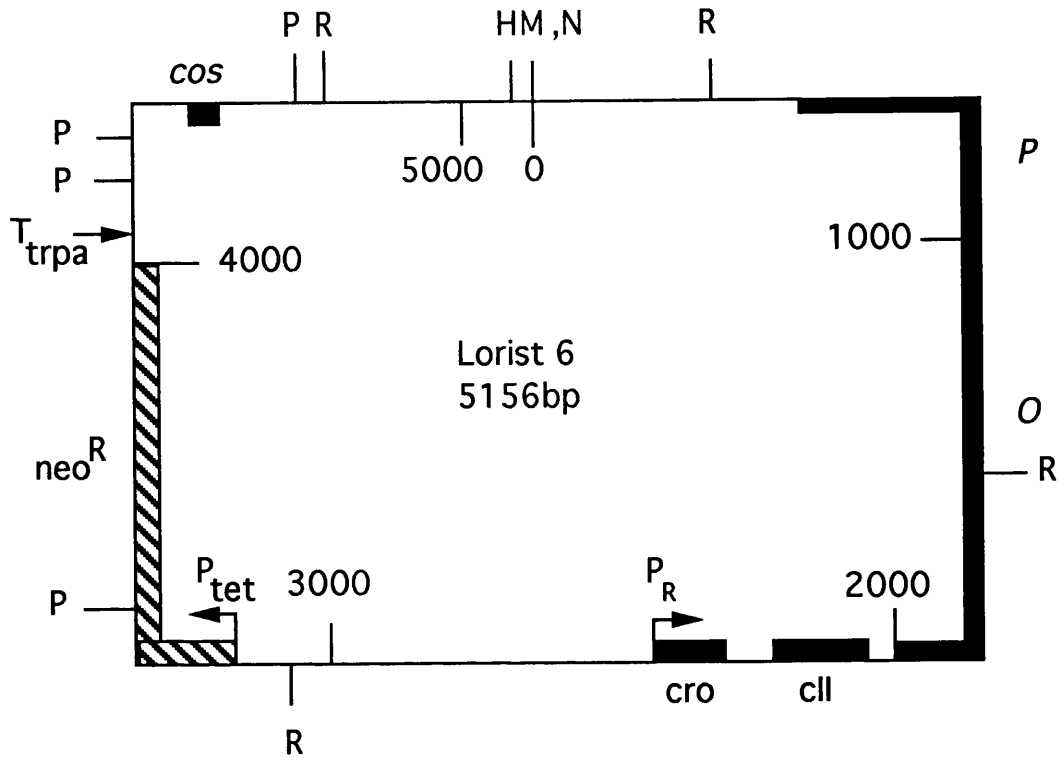



Fig.5.2 Molecular map of Lorist 6 vector. The vector genome is represented by a square with thick sections indicating protein coding regions: neo, neomycin phosphotransferase; cro, cII, *O* and *P*, bacteriophage lambda genes. Promoters ($P_{tet}$ and $P_R$) are denoted as hooked arrows and $T_{trpa}$ (terminator) as a downward arrow. *Cos* is the lambda cohesive end point for cleavage. R, H, N, P and M indicate the restriction sites EcoRI, HindIII, NotI, and BamHI.

The same procedure was followed for rescuing pWE15 and adjacent sequences from Gal$^+$73A DNA. A library of approximately 4 x $10^5$ clones, as determined by titration on 25 ug.ml$^{-1}$ kanamycin, was subsequently plated on 50 ug.ml$^{-1}$ ampicillin, but no amp$^R$ clones were isolated. This could have been due to the high rearrangement frequency inferred for the integrated pWE15 recombinant clone in Gal$^+$73A DNA.

### 5.2.3 CHARACTERIZATION OF COSMID CLONES OF GAL$^+$73B DNA OBTAINED BY MARKER RESCUE.

Restriction digests of DNA isolated from each of the 5 amp$^R$, kan$^R$ Lorist 6 clones, L73B1, L73B2, L73B3, L73B4 and L73B5 showed that apart from L73B1 and L73B5 (which were identical and subsequently referred to as L73B1), all the clones were different from each other to varying degrees (Fig. 5.3).

(i) L73B1 and L73B3

Southern blotting analysis along with the restriction digest results allowed for the restriction fragment typing of the four L73B clones. L73B1 and 3 were most similar to one another, with only a few fragments occurring which were not present in both clones. Southern blots probed with nick-translated pWE15, Lorist 6 and pUC8 (pUC8 was used to help distinguish between pWE15 and Lorist 6-derived sequences, although fragments containing only neo$^R$ or kan$^R$ sequences or *cos* site DNA could have derived from either vector and would not hybridise to pUC8) showed that L73B1 and 3 did not contain an intact, complete copy of Lorist 6 (Fig. 5.4 and 5.5). The three fragments generated when Lorist 6 is digested with EcoRI (1238bp, 1592bp and 1610bp) which should have hybridised to radiolabelled Lorist 6 DNA (Fig. 5.2) were absent. This indicates that the sequences lying between the *cos* sequences which were packaged in these clones contained either pWE15 sequences only or a chimeric product of ligation of Lorist 6 and pWE15,including no complete copies of either vector (Fig. 5.6). The amp$^R$ gene from pWE15 must be contained within the insert of these clones, and therefore the portion of pWE15 present would be from the *cos* site through to beyond the amp$^R$

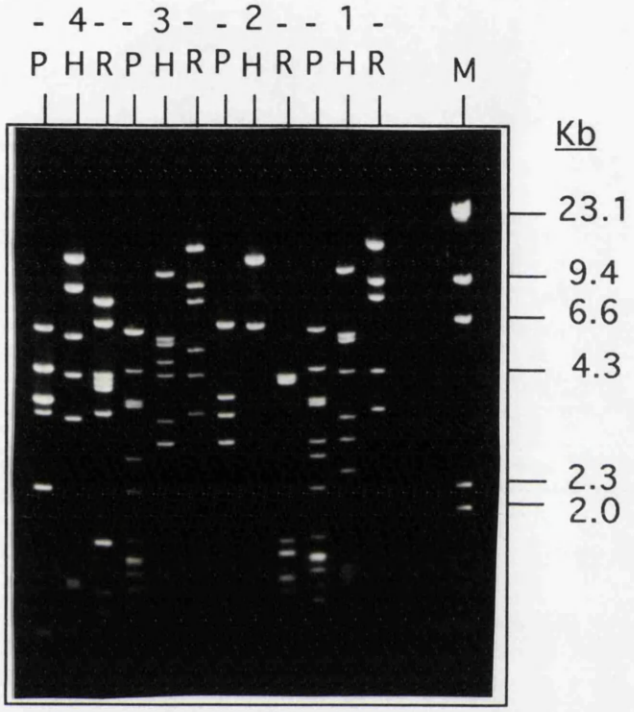



Fig. 5.3 0.7% agarose gel electrophoresis of 1ug aliquots of L73B1, 2, 3 and 4 DNAs having undergone restriction digestion with EcoRI (R), HindIII (H) and PstI as indicated. Phage lambda DNA digested with HindIII was used as a size standard (M). The DNA was visualised by ethidium bromide staining.

Fig. 5.4

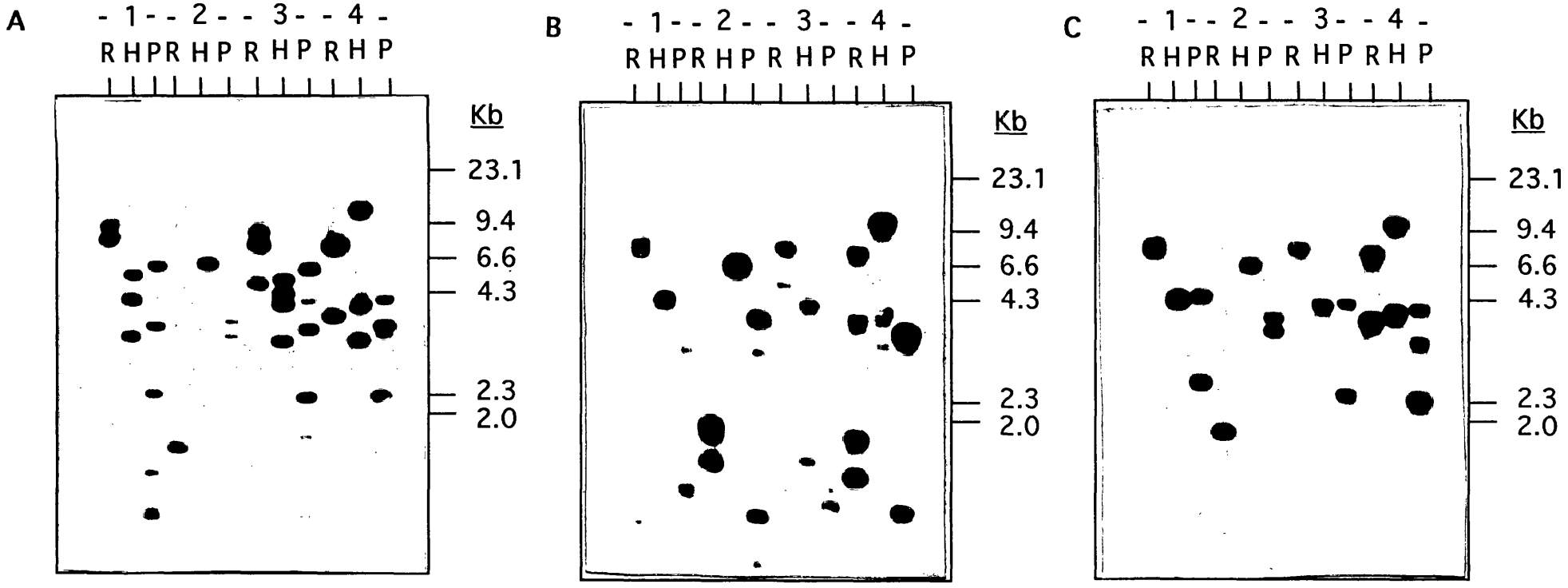

A
- 1 - - 2 - - 3 - - 4 -
R H P R H P R H P R H P
Kb
23.1
9.4
6.6
4.3
2.3
2.0
B
- 1 - - 2 - - 3 - - 4 -
R H P R H P R H P R H P
Kb
23.1
9.4
6.6
4.3
2.3
2.0
C
- 1 - - 2 - - 3 - - 4 -
R H P R H P R H P R H P
Kb
23.1
9.4
6.6
4.3
2.3
2.0

Fig. 5.4 Southern blot analysis of L73B1, 2, 3 and 4 DNAs probed with nick-translated pWE15 (A), Lorist 6 (B) and pUC8 (C). 1ug of L73B1 (1), L73B2 (2), L73B3 (3) and L73B4 (4) were digested with EcoRI (R), HindIII (H) and PstI (P) prior to gel electrophoresis on a 0.7% agarose gel, and southern blotted overnight. Hybridisation was carried out at 65$^{o}$C using nick-translated pWE15, Lorist 6 and pUC8 DNAs as probes. The filters were washed in 0.1 x W buffer (Chapter 2, 2.18.6) at 65$^{o}$C. The autoradiograph was exposed for 24 hours. Phage lambda DNA digested with HindIII was used as size standards and is shown in kilobases.

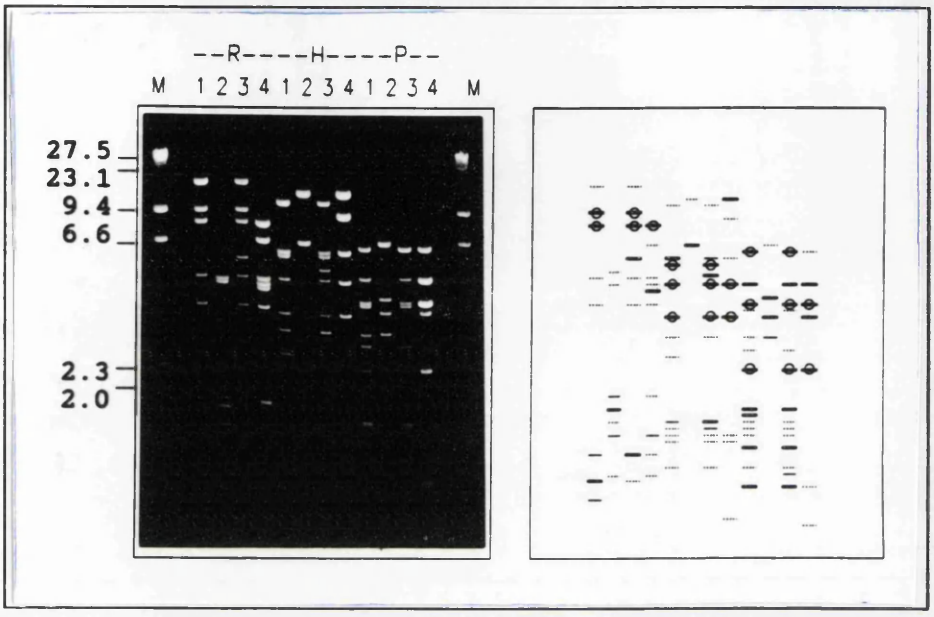



Fig. 5.5 A diagramatical representation of the results of the southern blots performed in Fig. 5.4. The left-hand panel shows restriction fragments visualised by EtBr-fluoresence, from EcoRI (R), HinDIII (H) and PstI (P) digests of clones L73B1, L73B2, L73B3 and L73B4, denoted 1, 2, 3and 4 respectively. The sizes of phage lambda HindIII markers (M) are as shown in kilobases. The right-hand panel shows a representation of the same gel, indicating the bands which hybridised in the southern blots of Fig. 5.4 probed with nick-translated pWE15 (bold lines), or Lorist 6 (but not pWE15) DNA (solid lines). Bands which correspond with those previously detected in $Gal^{+}$73B genomic DNA by southern blotting (see Chapter 4, Fig. 4.8) are indicated by circles.

gene and so would also contain the $neo^R$ gene and the SV40 sequences upstream, whereas only a portion of Lorist 6 from the *cos* site to the cloning site would be present (Chapter 4, Fig.4.8 and Chapter 5, Figs. 5.2 and 5.6).

Based on this reasoning, the EcoRI fragment containing pUC sequences would be predicted to be less than the size of linearized pWE15, at approximately 7.6Kb. To this, 280bp of DNA from Lorist 6 would be added, from the *cos* sequence up to the EcoRI site near the cloning site, giving a total fragment size of about 7.9Kb in length that would hybridise to both pWE15 and Lorist 6. An EcoRI fragment of around 7.8Kb was indeed observed to hybridise to all three probes used (Fig. 5.4). An additional EcoRI fragment and HindIII fragment in L73B3 hybridised to Lorist 6 and pWE15 but not to pUC8, and probably represents additional copies of either the *cos* or $neo^R$ sequences. Both L73B1 and L73B3 contain two EcoRI fragments which only hybridised to pWE15, and not to the other two probes. These probably represent fragments of SV40 sequences ligated to genomic DNA and indicates a complex pattern of rearrangement of the pWE15 recombinant clone upon integration into Gal 73.

L73B1 and 3 also have a number of genomic DNA-derived restriction fragments in common i.e. restriction fragments which did not hybridise with any of the vector DNA probes (Fig. 5.5 - broken lines), some of which are also present in L73B4. These presumably derive from the V79 genomic sequences contained within the original cosmid which integrated into the genome of Gal 73 restoring it to respiratory competence.

(ii) L73B4

L73B4, along with L73B1 and L73B3, contains the 7.9Kb EcoRI fragment that hybridised to all three probes (Figs. 5.4A-C), but in this case the southern blot analysis suggests that the clone contains a complete (which from further analysis was shown not to be the case) or almost complete copy of Lorist 6. The three EcoRI fragments which do not contain the cloning site in Lorist 6 are present in L73B4 when hybridised with

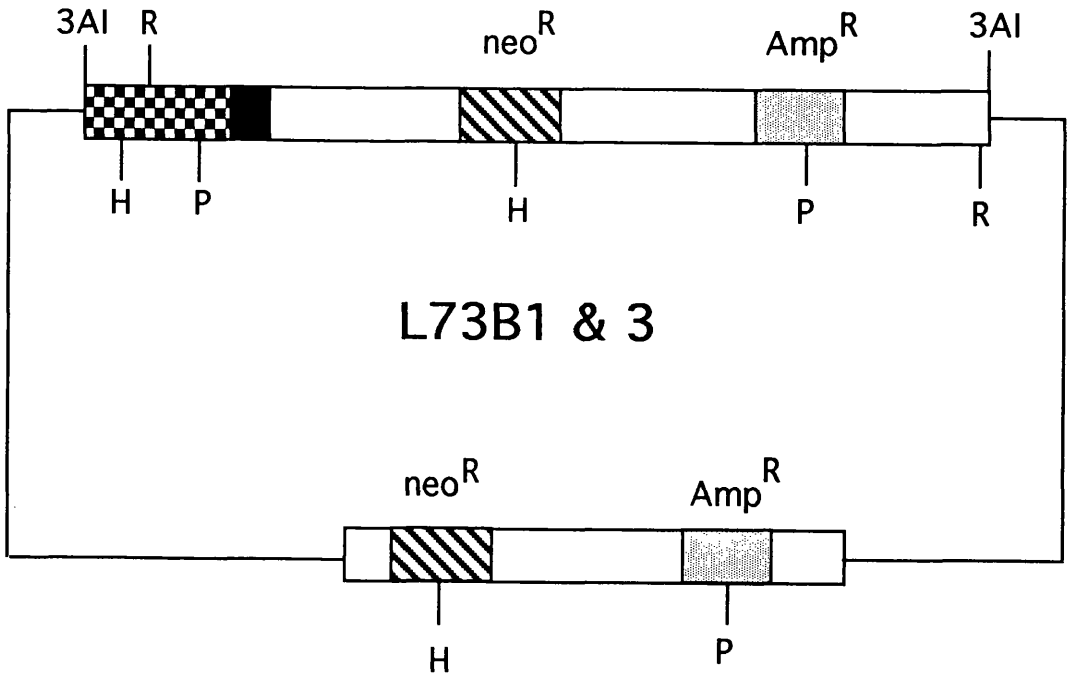



Fig. 5.6 A crude map of L73B1 and L73B3, deduced from restriction digest and southern blot analysis. Restriction sites are shown as follows: EcoRI (R), Sau3AI (S), HindIII (H) and PstI (P). The Lorist 6 vector sequences are portrayed as the checkered box, pWE15 sequences as the open box, the phage lambda *cos* sequences as a black box and the $amp^r$ and $neo^R$ genes as dotted and stripped boxes respectively. Chinese hamster genomic DNA is depicted as a single continuous line. The diagram is not to scale.

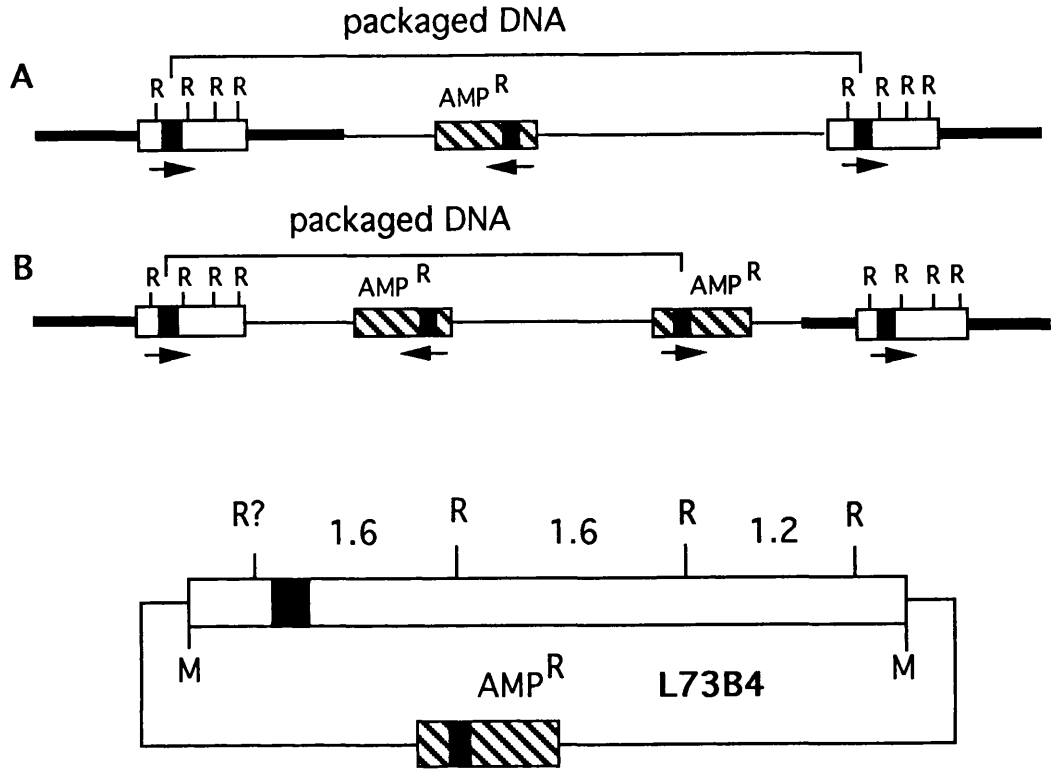



Fig. 5.7 Diagramamatic representation to show the possible *cos* site ligations to produce L73B4.

Lorist 6 sequences are portrayed as open boxes, pWE15 sequences as diagonally striped boxes. The phage lambda *cos* sequences are indicated as black boxes with their directionality shown by arrows. EcoRI sites are indicated as R.

(A) Ligation between *cos* sites in Lorist 6.

(B) Ligation between a *cos* site in Lorist 6 and a *cos* site in pWE15.

Lorist 6 (Fig. 5.4B). This would indicate that L73B4 has resulted from packaging between two *cos* sites from Lorist 6 with a copy of pWE15 and adjacent integrated V79 genomic DNA within the insert to give a complete copy of Lorist 6 and $amp^R$, or, between a *cos* site within Lorist 6 and a *cos* site within pWE15 to give a nearly complete copy of Lorist 6. However, due to the directionality of the staggered break produced in the *cos* site by the packaging extract, if a nearly complete copy of Lorist 6 is present then only a small fragment of pWE15 would be ligated to Lorist 6. Therefore, to render the clone $amp^R$ there must be additional pWE15-derived sequences in the insert (Fig. 5.7).

Of the Lorist 6 EcoRI fragments not disrupted by the cloning site, two (1.6 and 1.2 kb) would only hybridise to Lorist 6 and not to pWE15 or pUC8. The other fragment which also migrates at 1.6 kb on agarose gels would hybridise to pWE15 as well (Fig. 5.2) as this fragment spans the *cos* site and the $neo^R$ gene of Lorist 6. However the 1.6 kb EcoRI restriction fragment in L73B4 only hybridises to Lorist 6 indicating that only one of the fragments is present, and the copy of Lorist 6 in L73B4 is therefore not complete. Based on this Option b in fig. 5.7 is possibly the more correct representation of the structure of this clone.

Based on restriction enzyme and southern blot analysis L73B4 shows an overlap with L73B1 and L73B3, but also contains a number of unique restriction fragments, some deriving from genomic DNA alone and some which hybridise to Lorist 6 (Fig. 5.6). Although these fragments are not found in L73B1 and L73B3, some are found in L73B2.

(iii) L73B2

L73B2 also has a partially intact copy of Lorist 6 but its restriction fragment typing is further complicated by obvious deletions which occurred during growth. Faint higher molecular weight bands can be seen in restriction digests of the DNA (Fig. 5.5, left-hand panel), some of which correspond with fragments seen in digests of L73B4 DNA. This was later

confirmed when an undeleted version of L73B2 was isolated. However, the deleted or undeleted form of L73B2 have almost nothing in common with L73B1 and L73B3, and must therefore overlap L73B4 on the opposite side to L73B1 and L73B3.

Although some indication of what sequences are present within the four $amp^R$, Lorist 6 clones was obtained from the restriction digest and southern blot analysis, detailed restriction mapping was not performed. However this analysis has revealed that L73B1-4 represent a group of overlapping cosmid clones covering a region of $Gal^+$73B DNA, with a low number of integrated copies of pWE15, some intact and some rearranged. A number of the restriction fragments in L73B1, L73B3 and L73B4 were also observed in the original southern blot of $Gal^+$73B DNA probed with nick translated pWE15 (Fig. 5.5, right-hand panel, circled fragments), confirming that the region of $Gal^+$73B DNA which contains a low number of copies of an integrated pWE15 recombinant clone is represented by L73B1-4 in a way shown in Fig. 5.8. Even so, it is possible that the L73B clones do not contain the intact wildtype gene ($gal^+$) responsible for restoring respiratory competence to Gal 73. However, they should contain sequences derived, at least in part, from the $gal^+$ gene or adjacent DNA, unless restoration of respiratory competence in Gal 73 was due not to the content of the pWE15 recombinant clone but to the location in which it integrated into the genome of Gal 73, i.e. creating an insertional second site suppressor mutation. To distinguish between these two possibilities, the genomic DNA of $Gal^+$73B around the insertion site would have to be analysed in detail along with the genomic DNA from the insert of the pWE15 recombinant clone present in $Gal^+$73B DNA.

### 5.2.4 SUBCLONING OF SINGLE COPY SEQUENCES FROM OVERLAPPING COSMID CLONES OF $GAL^+$73B DNA.

Based on the above results, I set out to attempt to identify and subclone fragments from the L73B clones representing genomic DNA from both the integration site where the pWE15 recombinant clone inserted into the genome of Gal 73, and genomic fragments derived from the insert of the pWE15

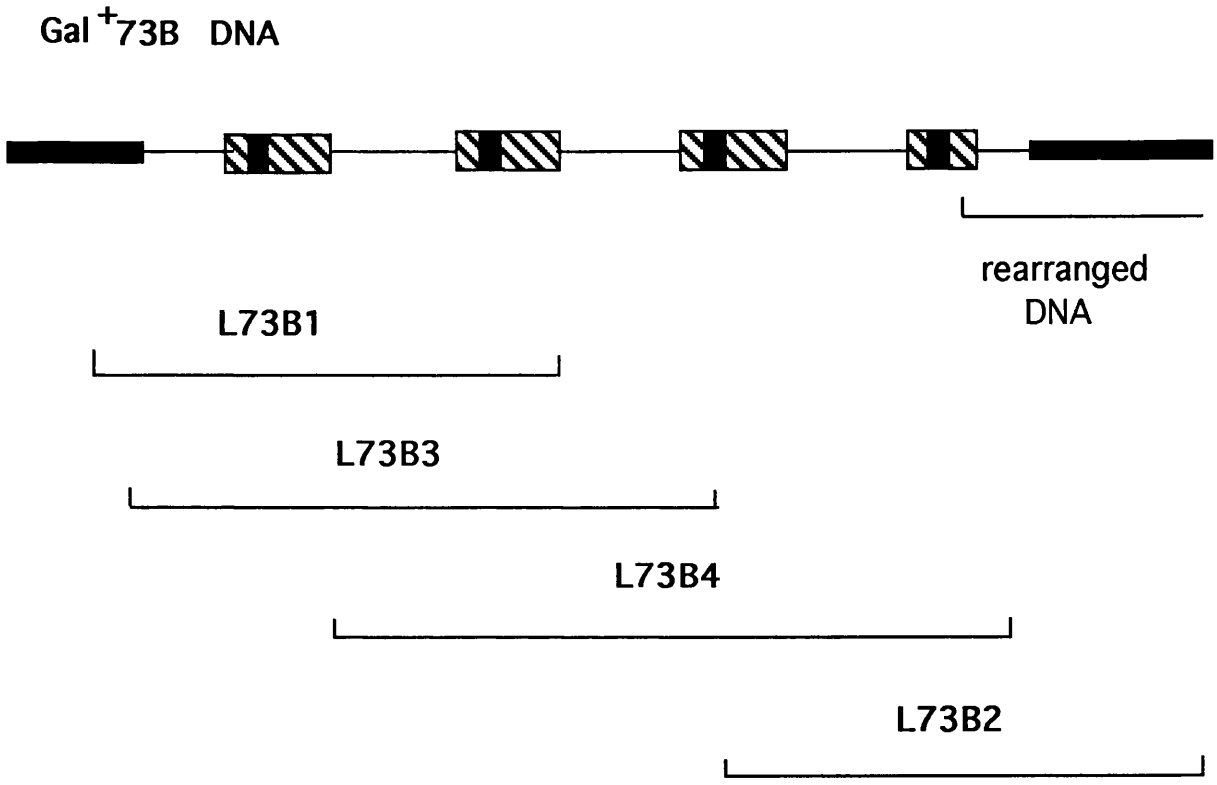



Fig. 5.8 Schematic diagram of the approximate relative positions of the L73B1-4 clones in Gal$^+$73B genomic DNA.
In tegrated pWE15 sequences are depicted as striped boxes and the phage lambda *cos* sequences as black boxes. Integrated V79 DNa is shown as a thin continuous line and insertion site Gal 73 DNA as a thick line. This diagram is not drawn to scal, but the four L73B clones could span approximately 100-150 kb of Gal$^+$73B DNA.

recombinant clone itself. Any fragments obtained could then be used as probes to screen libraries to isolate and characterise (i) the genomic DNA which was inserted into Gal 73 and (ii) genomic DNA from the insertion site.

### 5.2.4.1 CLONING L73B2 AND L73B3 SAU3AI FRAGMENTS INTO M13.

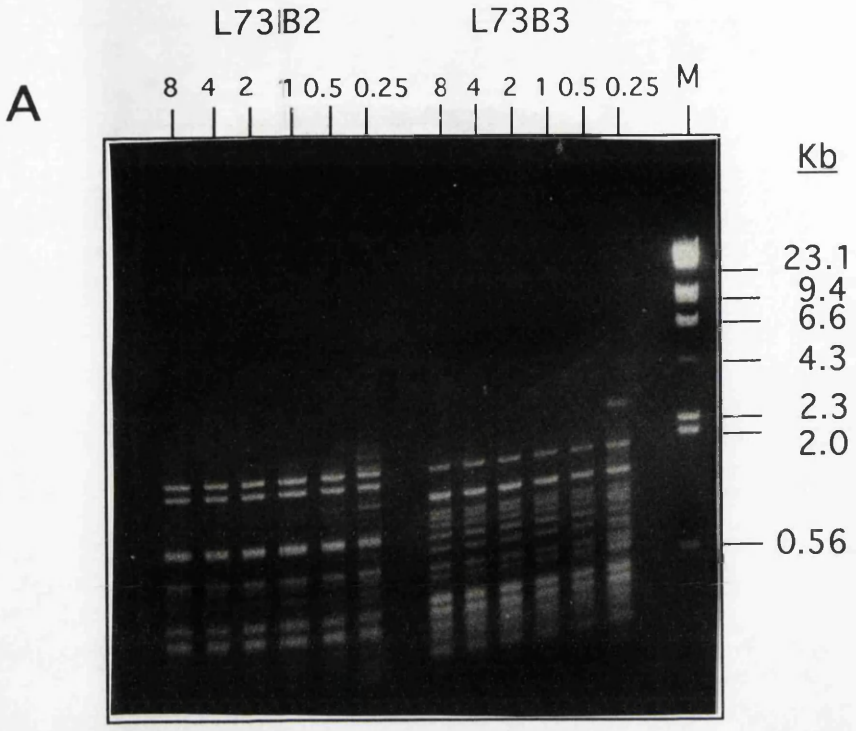
To isolate such probes single copy genomic DNA sequences from two of the Lorist 6 clones were subcloned into M13. L73B2 and L73B3 were chosen for the apparent lack of homologous sequences they contain, based on restriction enzyme and southern blot analysis (Fig. 5.8). Hopefully these two cosmids would then cover not only the pWE15 recombinant DNA integrated into $Gal^{+}73B$ but also the junctions between the pWE15 recombinant DNA and genomic DNA of the insertion site.

DNA from L73B2 and L73B3 was completely digested with Sau3AI (Fig. 5.9A) to give small fragments with a higher probability of containing only single copy sequences with no attached pWE15 or Lorist 6, or any repetitive (e.g. Alu) sequences. These were ligated to BamHI digested dephosphorylated M13mp18 DNA (Fig. 5.9B) in three different vector:insert ratios, to find the most efficient vector:insert concentrations to be used for the ligations. Fig. 5.9B shows that the pattern of ligation products was not substantially different for vector:insert ratios of 2:1, 5:1 and 10:1.

Competent JM109 cells were transformed with a dilution series of the ligation mix containing a vector:insert ratio of 10:1. Transformants were plated out in the presence of X-gal and IPTG to score for the disruption of the B-galactosidase gene when the M13 clones contain inserts. Three transformations were carried out altogether and phage from the white recombinant plaques were isolated and kept in microtitre dishes to ease the screening for single copy inserts.

### 5.2.4.2 SCREENING M13 CLONES FOR SINGLE COPY SEQUENCES.

Supernatants from the isolated M13 clones were spotted onto biodyne hybridization membrane and screened for the presence


A
L73B2
L73B3
8 4 2 1 0.5 0.25 8 4 2 1 0.5 0.25 M
Kb
23.1
9.4
6.6
4.3
2.3
2.0
0.56

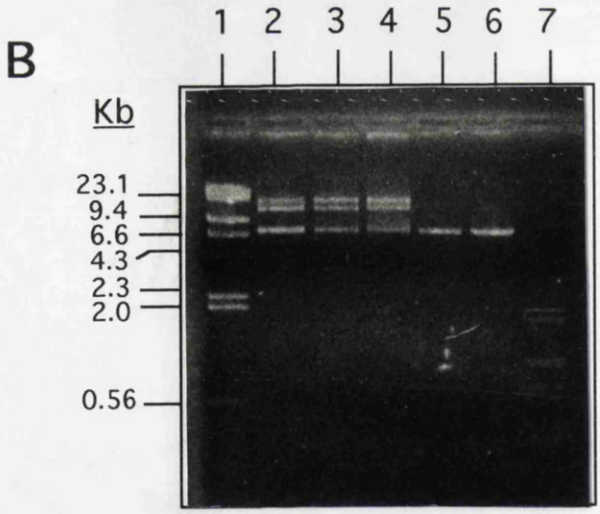
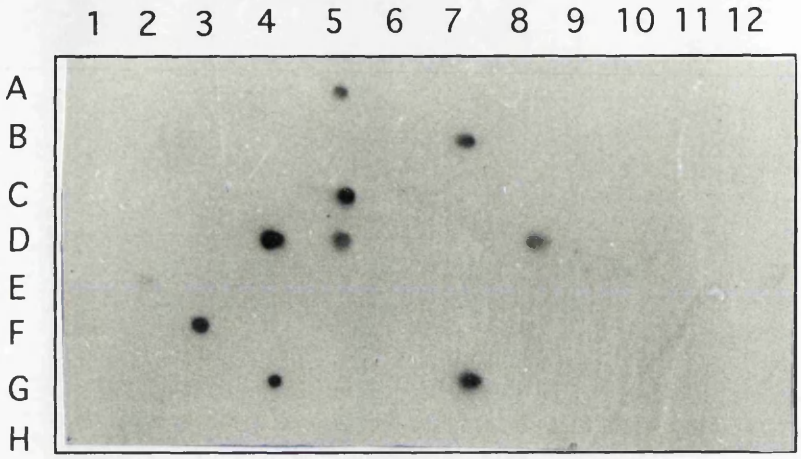

B
1 2 3 4 5 6 7
Kb
23.1
9.4
6.6
4.3
2.3
2.0
0.56

Fig. 5.9 0.7% agarose gel electrophoresis of Sau3AI digested L73B2 and L73B3 DNA (A) and the products of ligation of this DNA to BamHI digested, dephosphorylated M13mp18 DNA (B). HindIII-digested phage lambda DNA was used as a size standard (M).

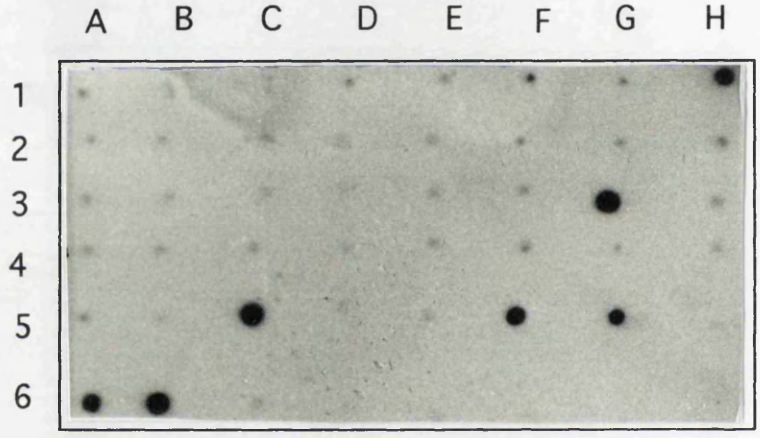
A: 5ug of each DNA sample was split into 6 aliquots of 17ul and digested at 37°C for 30 mins. with a dilution series of Sau3AI from 8 to 0.25 units as noted.

B: Three ratios of M13 to L73B2 DNA were tried, 2:1 (500ng:250ng), 5:1 (500ng:100ng) and 10:1 (500ng:50ng), shown in tracks 2, 3, and 4 respectively. A ligation of BamHI digested, dephosphorylated M13 DNA without L73B2 DNA was carried out as a control for the dephosphorylation of the M13 DNA (track 6). HindIII digested phage lambda DNA (track 1) and Sau3AI digested L73B2 DNA (track 7) were used as size standards.

A


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

B


A B C D E F G H
1
2
3
4
5
6

C

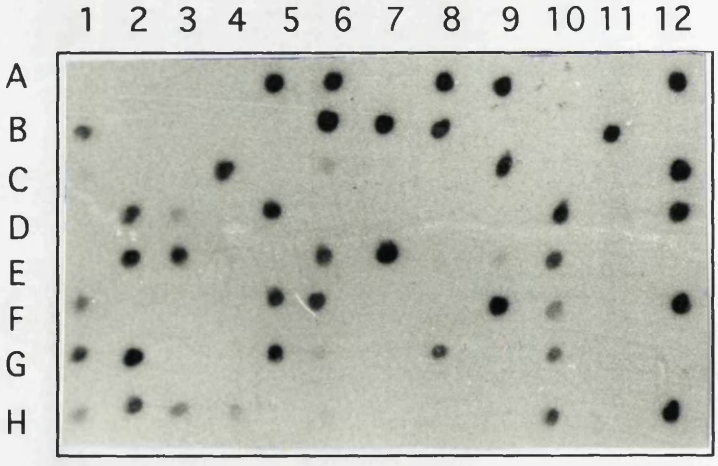
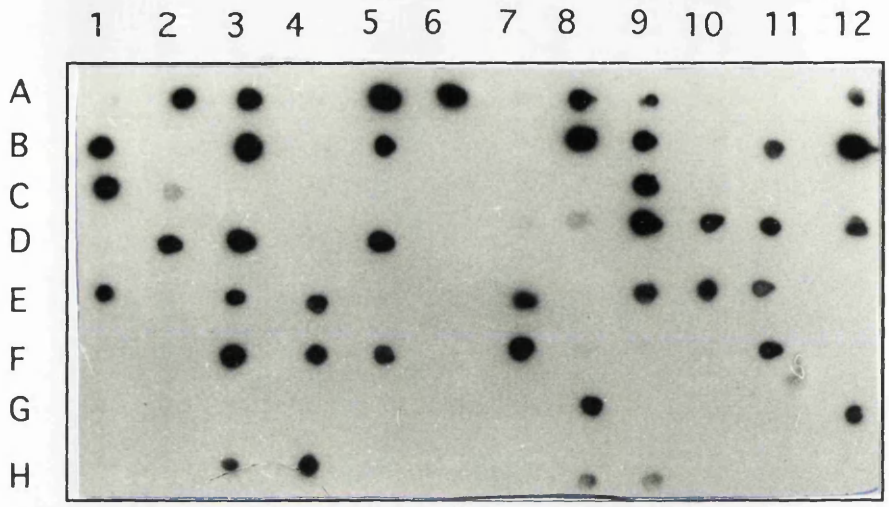
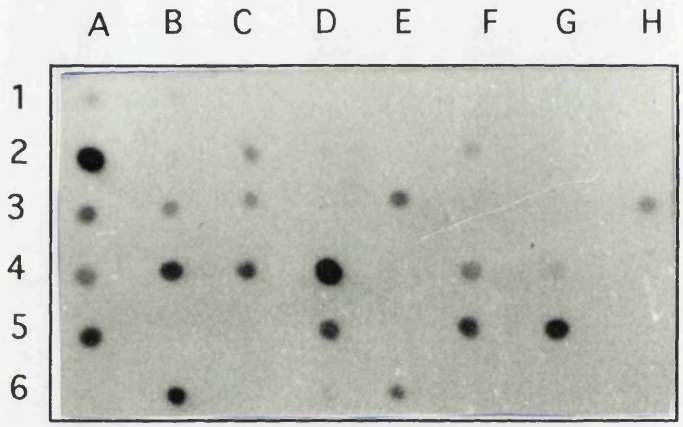

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Fig. 5.10 L73B2 and L73B3 M13 subclones screened with pWE15 DNA. 2ul of each phage supernatant was spotted onto a biodyne filter, denatured for 5 mins. on a piece of 3MM paper saturated with 0.5 x D solution and then neutralised twice for 3 mins. on 3MM paper soaked in 1 x N solution and washed in 2 x SSC. The filters were then baked for 2 hours at 80$^{o}$C prior to hybridisation at 65$^{o}$C with nick-translated pWE15. The filters were washed at 65$^{o}$C in 0.1 x W buffer. Positives were visualised by overnight autoradiography.
(A) subclones resulting from the 1st transformation of 10ng, 1ng and 100pg of the L73B2/M13 ligation mix.
(B) subclones resulting from the 2nd transformation of 100 ng and 150 ng of the L73B2/M13 ligation mix.
(C) subclones resulting from transformation of 100ng and 150 ng of the L73B3/M13 ligation mix.

A


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

B


A B C D E F G H
1
2
3
4
5
6

C

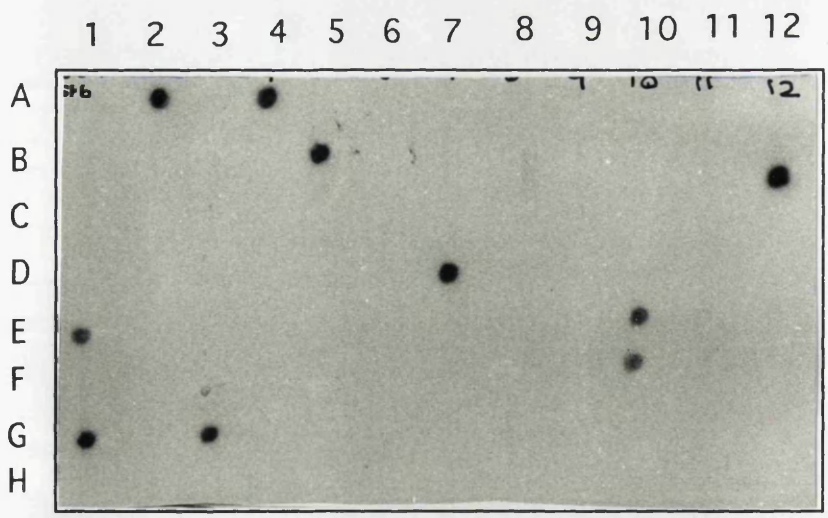

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Fig. 5.11 L73B2 and L73B3 M13 subclones probed with nick-translated Lorist 6 DNA.
(A), (B) and (C) are the same as for Fig. 5.10. The filters were prepared and hybridised under the same conditions as for Fig. 5.10.

of cosmid vector sequences by probing with nick translated pWE15 and Lorist 6 DNA (Fig. 5.10 and Fig. 5.11 respectively). Those which gave positve results were discarded. Some phage clones which hybridised weakly to Lorist 6 (i.e. at the same level as negative controlswhich were 2 ul aliquots of fresh JM109 cells) were not discarded, but underwent further rounds of screening.

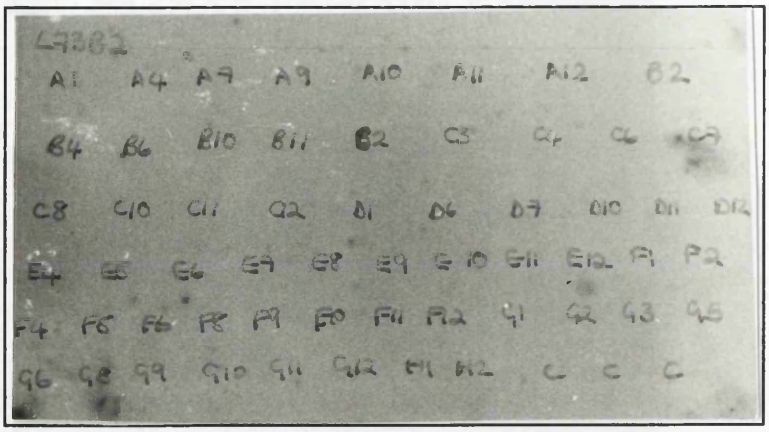
To be sure the remaining M13 subclones contained inserts derived from the two cosmids, they were probed with nick translated L73B2 or L73B3, resulting in a possible 70 out of 128 and 58 out of 96 M13 subclones containing genomic DNA sequences from L73B2 and L73B3 respectively.

To narrow down this number of subclones even further, they were each screened for sequences that are expressed in V79, Gal 73 and $Gal^{+}$73B cells. To do this, radiolabelled total cDNA was prepared by reverse transcription of mRNA from these three cell-lines and used to probe the resulting M13 subclones (Fig. 5.12) in the hope to identify highly expressed sequences which were single copy and exonic. However the results indicated otherwise. To all three types of radiolabelled cDNA there were three faintly hybridising clones (E4, G9 and H6) and one strongly hybridising clone (H8) each derived from L73B3, and after a long exposure of the autoradiograph one weakly hybridising clone (E12) derived from L73B2.

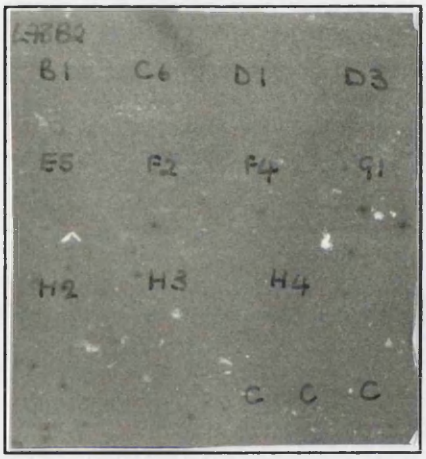
The next step in obtaining single copy DNA clones was to discard any clones containing highly or intermediately repetitive DNA elements. This was acheived in two ways. Firstly, L73B2 subclones were probed with nick translated L73B3 and vice versa, as L73B2 and L73B3 have very few, if any, genomic restriction fragments in common (Data not shown). However, it is more than likely that they will both contain the highly repetitive Alu sequence. Therefore any subclone hybridising to DNA from the opposing cosmid may contain Alu sequences. The second approach taken was to prepare an 'Alu rich' probe from V79 DNA and screen the subclones with this.

The results from these experiments (Fig. 5.13) show that those

A


L9382
A1 A4 A7 A9 A10 A11 A12 B2
B4 B6 B10 B11 B2 C3 C4 C6 C7
C8 C10 C11 C2 D1 D6 D7 D10 D11 D12
E4 E5 E6 E7 E8 E9 E10 E11 E12 F1 F2
F4 F6 F6 F8 F9 F10 F11 F12 G1 G2 G3 G5
G6 G8 G9 G10 G11 G12 H1 H2 C C C

B


L9382
B1 C6 D1 D3
E5 F2 F4 G1
H2 H3 H4
C C C

C

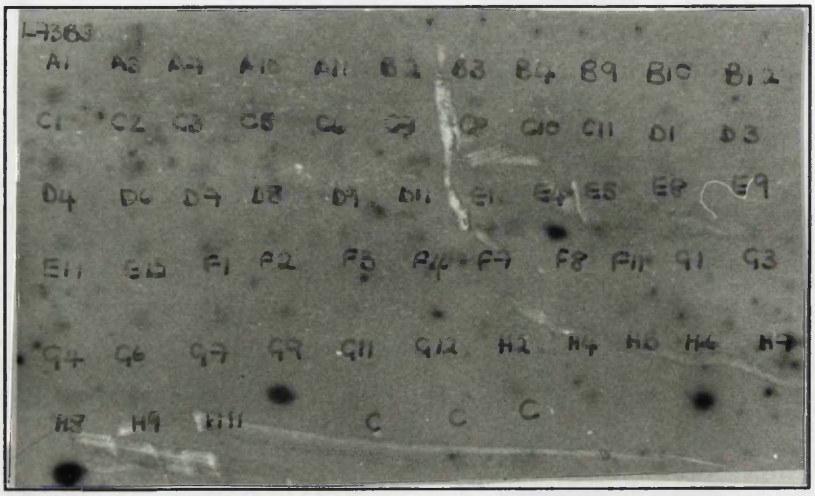
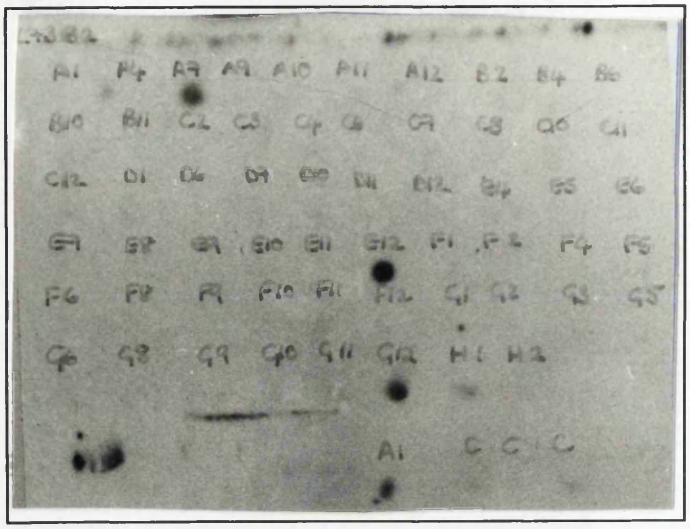

L9383
A1 A2 A4 A10 A11 B2 B3 B4 B9 B10 B12
C1 C2 C3 C5 C6 C7 C8 C10 C11 D1 D3
D4 D6 D7 D8 D9 D11 E1 E4 E5 E8 E9
E11 E12 F1 F2 F5 F6 F7 F8 F11 G1 G3
G4 G6 G7 G9 G11 G12 H2 H4 H5 H6 H7
H8 H9 H11 C C C

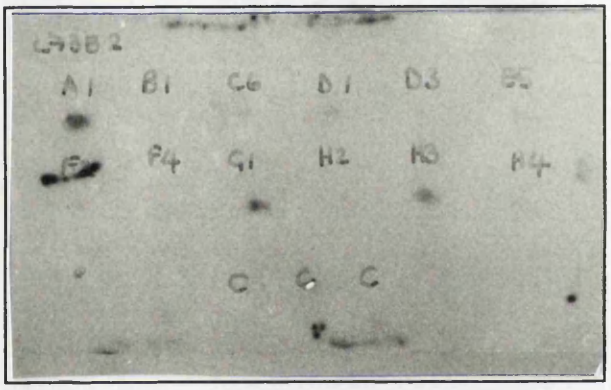
Fig. 5.12 L73B2 and L73B3 M13 subclones probed with $^{32}P$ labelled cDNA derived by reverse transcription of V79 cell poly(A)$^+$ RNA. The filters were prepared as for Fig. 5.10, but hybridised at 42$^{o}$C in 5 x SSC, and 50% formaldehyde as for Norhtern blot analysis. The filters were washed at 55$^{o}$c in 0.1 x W buffer. The filters are designated as for Fig. 5.10, but are re-arranged, excluding those clones containing pWE15 of Lorist 6 sequences.

The results obtained when the same M13 subclones were screened with $^{32}P$ labelled cDNA derived by reverse transcription of Gal 73 and Gal$^+$73B cell poly(A)$^+$ RNA were the same (not shown).

A



B



C

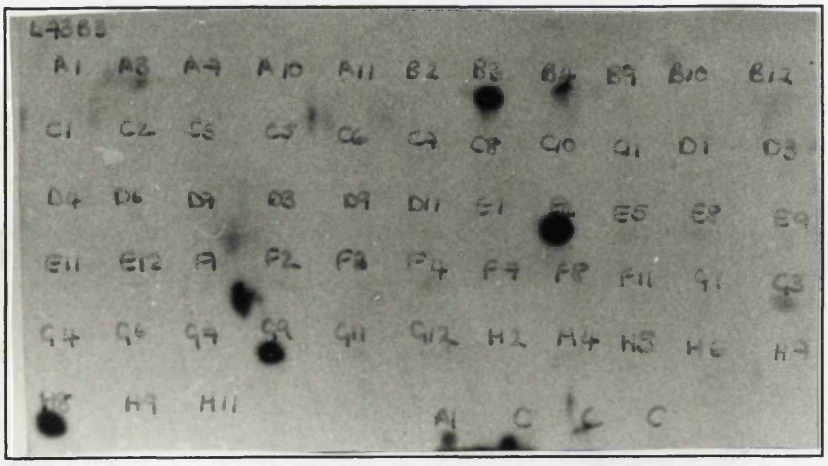


Fig. 5.13 Screening the L73B2 and L73B3 M13 subclones with an "Alu" rich probe.
The "Alu" rich probe was prepared by the mechanical shearing (pushing the DNA through an 18 gauge needle 5-6 times) of V79 cell genomic DNA, denaturation by heating at 100$^{o}$C for 10 mins. and subsequent renaturation upon cooling. The DNA was then nick-translated and used to screen the L73B2 and L73B3 M13 subclones as in Fig. 5.10. Hybridisation and washing conditions were also as for Fig. 5.10. Positive clones were visualised by autoradiography for 24 hours. Filters and clones are arranged as in Fig. 5.12.

subclones which gave a positive result when probed with the opposing cosmid, also hybridised to the 'Alu rich' probe and supports the hypothesis that L73B2 and L73B3 have little single copy genomic DNA sequences in common. These results also showed that those M13 subclones which gave a positive result with the three radiolabelled cDNAs (E4, G9, H6, H8 and E12) all hybridised to the opposite cosmid DNA. When screened with the 'Alu' rich probe H6 gave no hybridisation, whereas, the other four were all positive. However, H6 when sequenced did in fact turn out to contain Chinese hamster repetitive DNA when compared to sequences in the Embl genebank. Therefore H6 was discarded along with the others and resulted in 63 L73B2 M13 subclones and 53 L73B3 M13 subclones remaining that contained potential single-copy sequences. None of them appeared, based on cDNA screening (Fig. 5.12), to be highly expressed.

### 5.2.5 IDENTIFICATION OF M13 SUBCLONES REPRESENTING INTEGRATED DNA AND INSERTION SITE DNA.

The single-copy genomic DNA sequences represented by the M13 subclones derived from L73B2 and L73B3 fall into two categories; i) genomic DNA integrated into Gal$^+$73B DNA as part of the pWE15 recombinant clone and, ii) genomic DNA at the site into which the pWE15 recombinant clone inserted. These two types of genomic DNA will subsequently be referred to as integrated DNA and insertion site DNA respectively.

To distinguish between the two, southern blot analysis of V79, Gal 73 and Gal$^+$73B genomic DNA was performed using radiolabelled probes prepared from the M13 subclones, and a simple hypothesis was followed.

Those M13 subclones which carry single copy genomic DNA sequences, which hybridised to one or two restriction fragments in V79 and Gal 73 DNA but which hybridised to the same fragments in Gal$^+$73B DNA as in V79 and Gal 73 DNA plus additional restriction fragments, would be classed as integrated DNA. This includes the caviat that, depending on the restriction enzyme used in the digestion of the DNA prior to southern blotting, an increased copy number of the existing

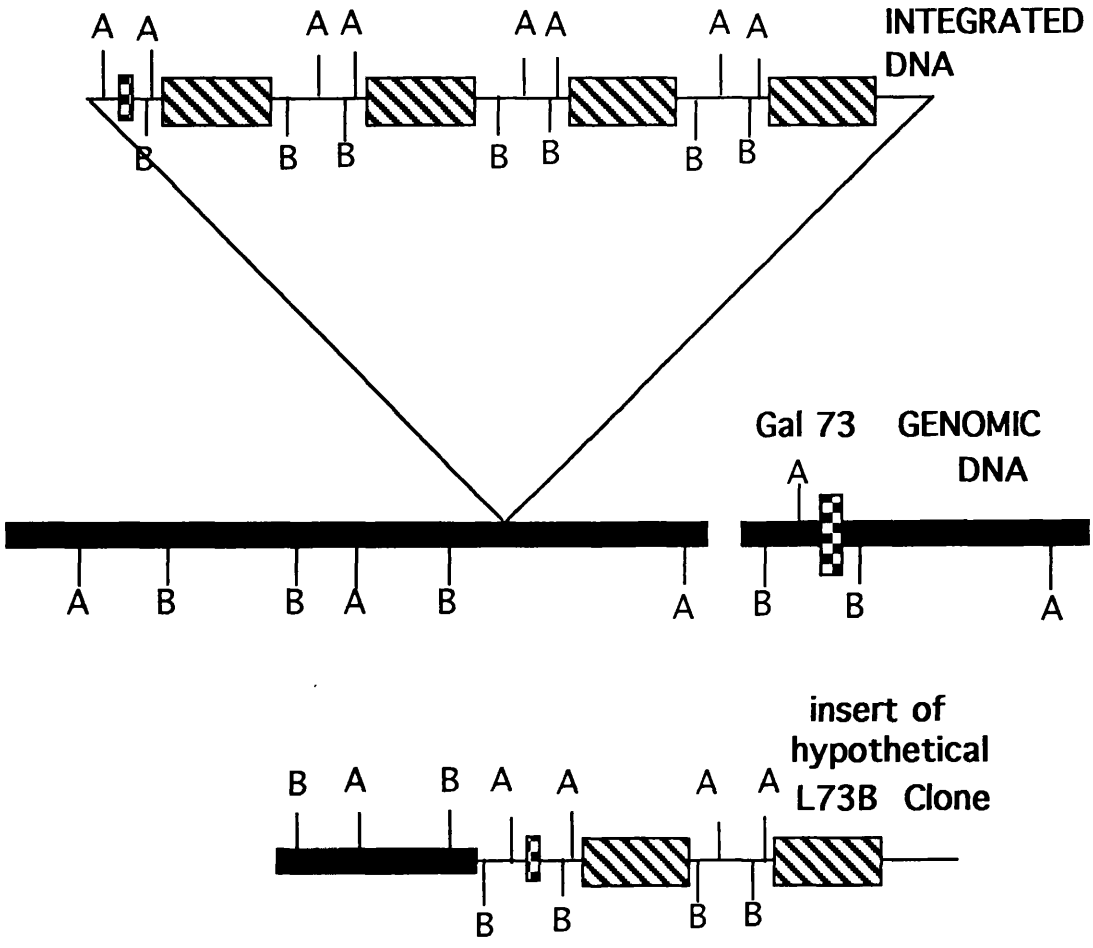
fragments might be observed instead of hybridization to a new restriction fragment (Fig. 5.14). Conversely, clones carrying insertion site DNA would either show a disruption in the pattern of hybridization of wildtype V79 DNA or show no detectable difference at all, again depending on which restriction enzymes are used (Fig. 5.15).

### 5.2.5.1 GENOMIC DNA PROBED WITH B1, C6 and E5, THREE M13 SUBCLONES DERIVED FROM L73B2.

In order to determine whether the M13 subclones contained integrated DNA or integration site DNA aliquots of V79, Gal 73 and Gal$^+$73B genomic DNA were digested with EcoRI and BamHI for screening with B1, and with EcoRI and PstI for screening with E5 and C6. The digested genomic DNAs underwent agarose gel electrophoresis, were blotted on to a nylon membrane, and probed with $^{32}$P-labelled probes (Chapter 2, section 2.19.2), prepared from the single-stranded DNA of M13 subclones B1, C6 and E5. The multiple bands detected in the autoradiographs for each of the M13 subclones (Fig. 5.16, 5.17 and 5.18) show either (i) that there are internal EcoRI site(s) and/or BamHI sites in the inserts of B1, C6 and E5 or (ii) that there is more than one copy of these sequences present in the Chinese hamster genomic DNAs analysed. The probability of more than one site for enzymes recognising 6 basepair restriction sites within a small (approx. 2 kb) fragment is small but not zero.

In Gal$^+$73B DNA, subclone B1 detected a 2.75 kb BamHI fragment with increased intensity relative to the same fragment in V79 and Gal 73 DNA (Fig. 5.16). It also detected a novel 2.75 kb EcoRI fragment which is partially obscuring the signal from the 2.85 kb EcoRI fragment. Following the above arguments (see previous section 5.6) the results seem to indicate that the insert in B1 originated from integrated DNA: new fragments hybridiseing with B1 having been created by the integration of V79 wildtype genomic DNA, as part of a pWE15 recombinant cosmid (Fig. 5.14). Due to the very similar sizes of the intense BamHI and EcoRI fragments in Gal$^+$73 DNA hybridising to B1, it was considered a possibility that they were an artifact due to contamination in the Gal$^+$73B DNA, the

A


INTEGRATED DNA
Gal 73 GENOMIC DNA
insert of hypothetical L73B Clone

B

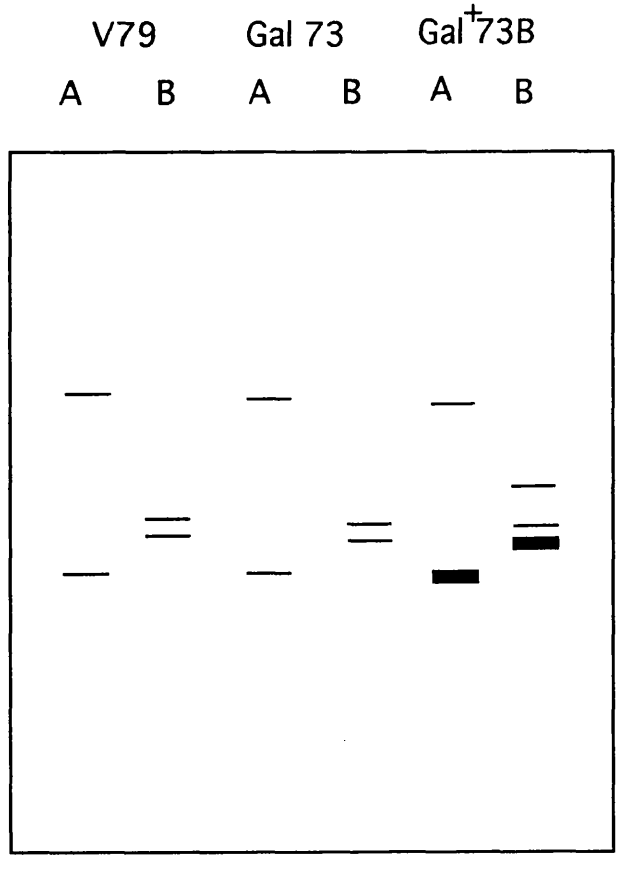
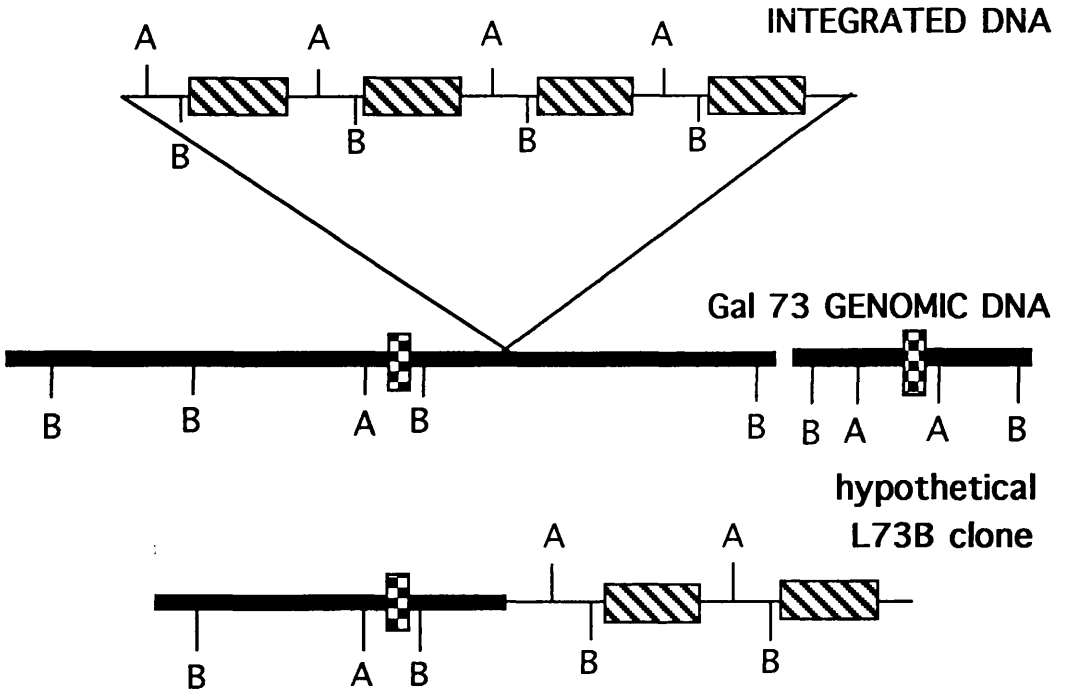

V79
Gal 73
Gal+73B
A B A B A B

Fig. 5.14 (A) Hypothetical schematic diagram of the predicted southern blot hybridisation pattern which would result from probing genomic DNA of $Gal^+73B$ cells cut with different enzymes, with an M13 subclone of L73B2 or 3 containing integrated DNA. $Gal^+73B$ DNA and Lorist 6 DNA are as labelled. Integrated pWE15 sequences are depicted as striped boxes and the hypothetical-sequences contained within the M13 subclone as a checkered box. Integrated V79 genomic DNA sequences are shown as a continuous thin line and insertion site Gal 73 genomic DNA as a thick Continuous line. Hypothetical sites for two different restriction enzymes are marked A and B.
(B) Schematic diagram of a southern blot of V79, Gal 73 and $Gal^+73B$ genomic DNA digested with enzymes A and B at the sites indicated above, and probed with the M13 subclone above.

A


INTEGRATED DNA
A
A
A
A
B
B
B
B
Gal 73 GENOMIC DNA
B
B
A
B
B
B
A
A
B
hypothetical
L73B clone
A
A
B
A
B
B
B

B

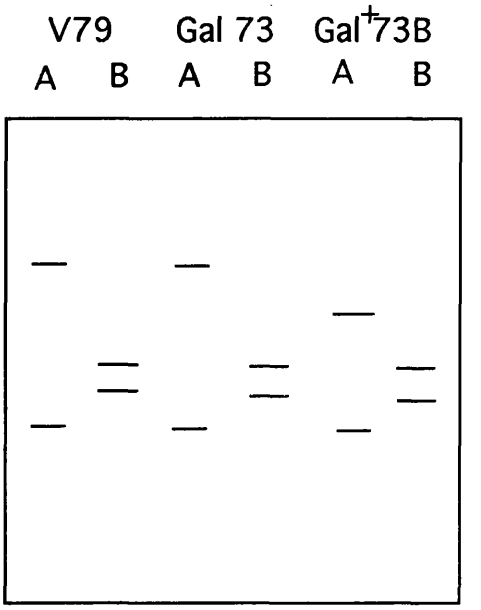

V79
Gal 73
Gal+73B
A
B
A
B
A
B

Fig. 5.15 (A) Hypothetical schematic diagram of the resulting southern Blot hybridisation pattern when probin genomic DNA of $Gal^+73B$ cells, cut with different enzymes, with an M13 subclone containing insertion site DNA. Symbols are as in Fig. 5.14.

(B) Schematic diagram of a southern blot of V79, Gal 73 and $Gal^+73B$ genomic DNA digested with enzymes A and B at the sites indicated, and probed with the M13 subclone above.

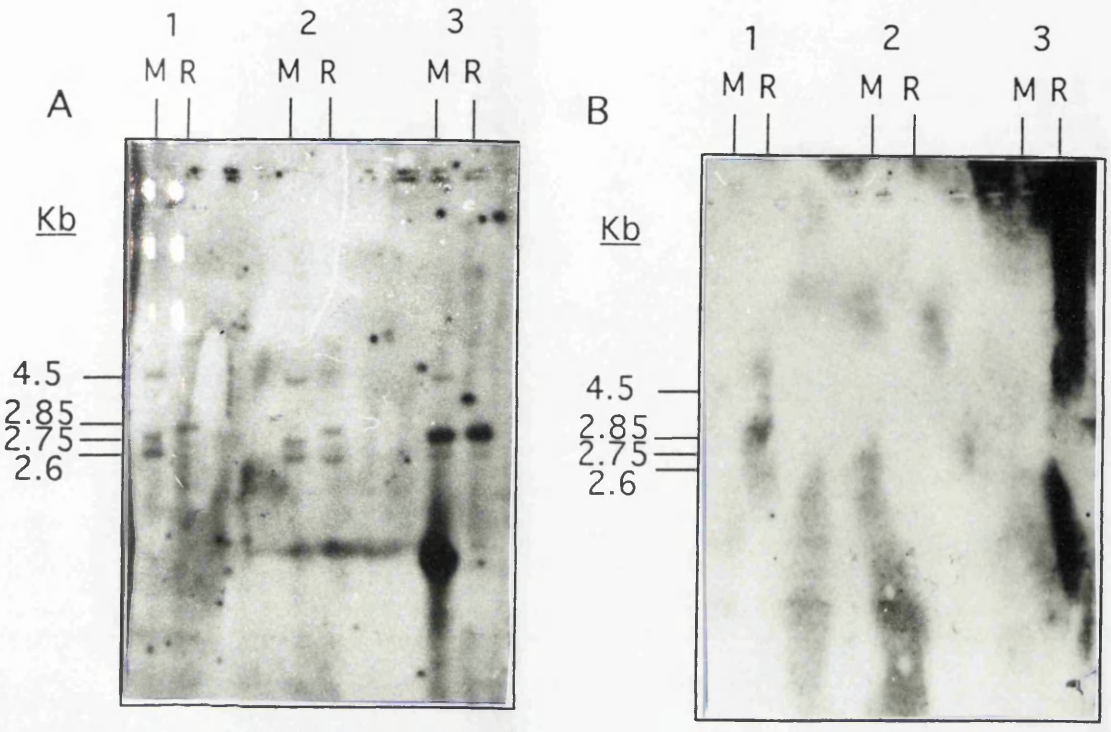



Fig. 5.16 Southern blot analysis of V79, Gal 73 and Gal$^+$73B DNA probed with (A) B1, an M13 subclone containing genomic DNA derived from L73B2 and (B) M13mp18 DNA, as a negative control. (A) 2 ug aliquots of V79 (1), Gal 73 (2) and Gal$^+$73B (3) DNA were digested with BamHI (M) and EcoRI (R) prior to southern blotting and hybridisation at 65$^o$C with a $^{32}$P-labelled probe prepared from single strand DNA of B1 (Chapter2, section 2.19.2). The filter was washed in 0.1 x W at 65$^o$C.
(B) The same filter striped by boiling for 5 mins. in $dH_2O$ and 0.1% SDS and probed with nick-translated M13 RF DNA. Hybridisation was visualised by autoradiography.
The sizes of the reactive fragments are shown in kilobases.

most likely being related to M13, although BamHI and EcoRI linearise wildtype M13 giving a 7Kb fragment, whereas the fragments detected were around 2.75 kb. The filter was stripped by boiling in $dH_2O$ and 0.1% SDS for five mins. and reprobed with nick-translated M13 RF DNA. The results were totally negative (Fig. 5.16B), i.e. there was no hybridisation to M13, although no positive comtrol was carried out.

300bp of B1 was sequenced (appendix I) and this failed to reveal any significant similarity when compared to sequences in the EMBL sequence database. The computer analysis also failed to identify any open reading frames, suggesting that B1 does not contain coding sequence.

The initial autoradiographs of the genomic blots probed with $^{32}P$ labelled E5 and C6 DNA (i.e. washed in 0.1 x W at 65°C) detected multiple copies of sequences within the genomic DNAs hybridising to these subclones (Fig. 5.17A and 5.19A). The filters were therefore rewashed at a higher temperature (0.1 x W, 80°C), resulting in a reduction in the number of hybridising DNA fragments (Fig. 5.17B and 5.19B).

Due to the low concentration of PstI-digested V79 DNA, on these filters, the comparison of PstI digests of V79 and Gal 73 DNA was not informative. However, other digests yielded important data.

Assuming that the pattern of E5-reactive PstI fragments is the same for V79 as for Gal 73 DNA, the results indicate that like B1, E5 contains integrated DNA. EcoRI digests of V79, Gal 73 and $Gal^+$73B DNAs showed little difference in the pattern of hybridisation to E5, except that the upper 4.2 kb and lower 1.3 kb bands were more intense in $Gal^+$73B than in Gal 73, indicative of an increase in the relative copy number of these restriction fragments. By contrast, when V79, Gal 73 and $Gal^+$73B DNAs were digested with PstI, a new fragment was generated in $Gal^+$73B, and the 3.1 kb fragment increased in relative intensity. However, there was no loss of any E5-reactive EcoRI or PstI restriction fragments in $Gal^+$73B DNA compared to V79 and Gal 73 DNA.

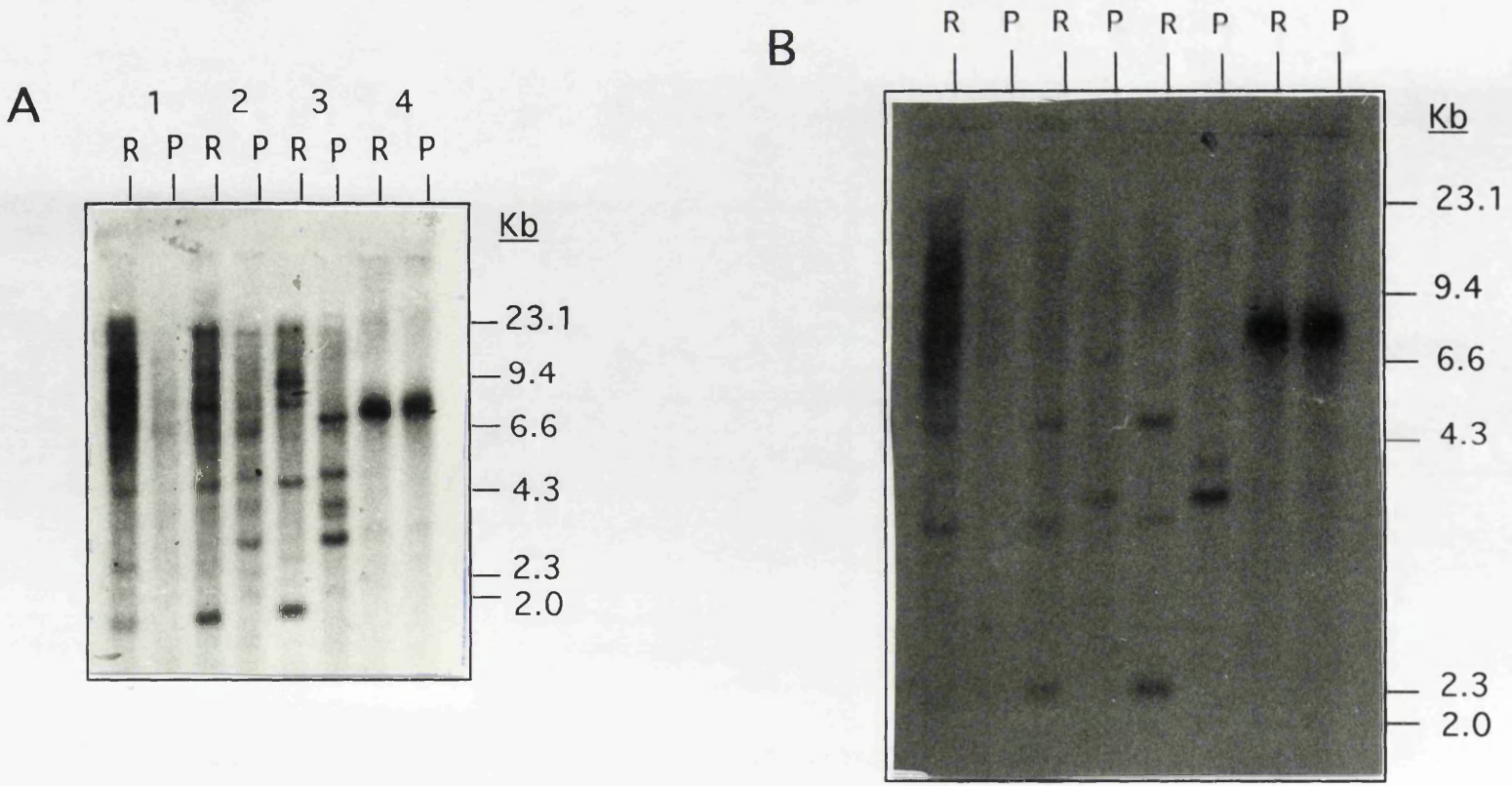

A
1
2
3
4
R
P
R
P
R
P
R
P
Kb
23.1
9.4
6.6
4.3
2.3
2.0
B
R
P
R
P
R
P
R
P
Kb
23.1
9.4
6.6
4.3
2.3
2.0

Fig. 5.17 Southern blot analysis of V79, Gal 73 and Gal$^+$73B DNA probed with E5, an M13 subclone containing genomic DNA derived from L73B2.

(A) 2ug aliquots of V79 (1), Gal 73 (2) and Gal$^+$73B (3) DNA and 50pg aliquots of M13 DNA (4) were digested with EcoRI (R) and PstI (P) and blotted onto nylon membrane. Overnight hybridisation at 65$^o$C took place with $^{32}$P-labelled E5 prepared from the single-strand DNA of the M13 subclone. The filter was washed in 0.1 x W at 65$^o$C for 20 mins. and then visualised by autoradiography after 72 hours.

(B) The above filter rewashed in 0.1 x W at 80$^o$C for 30 mins. Hybridisation was visualised by autoradiography for 8 days.

E5 was generated by subcloning Sau3AI fragments of L73B2 into M13. When DNA from the four L73B clones was digested and probed with radiolabelled E5, only fragments present in L73B2 and L73B4 hybridised to E5 (Fig. 5.18). Although E5 sequences are present in both L73B2 and L73B4, the pattern of E5-reactive fragments in these two cosmids is not the same. This could indicate that E5 derives from the region of L73B2 which does not overlap with L73B4, and that two copies of E5 are found in this region, one in L73B2 and one in L73B4, possibly as a result of rearrangment during cloning or integration. Another possible explanation could be that E5 is derived from the region of overlap between L73B2 and L73B4 and hybridises to junctional fragments in every case. To distinguish between these two possibilities this region of $Gal^+$73B DNA and L73B2 and L73B4 would have to be characterised further: this was not considered useful at this point in the study, as E5 (like B1) could be used to probe wildtype genomic libraries for clones representing an unrearranged copy of the genomic DNA which integrated into $Gal^+$73B and restored the cell-line to a wildtype phenotype.

Sequencing single-stranded E5 DNA revealed a growth deletant in the DNA of the clone, which remained after two rounds of plaque purification. However, approximately 130bps of DNA sequence from E5 was obtained and compared to sequences in the EMBL sequence database. Like B1, E5 was not significantly similar to anything in the database and no open reading frames were identified.

In contrast to B1 and E5, the southern blot data obtained with subclone C6 suggests that it contains insertion site DNA. Again the filter was washed in 0.1 x W at 80°C to eliminate additional signals detected when it was washed at 65°C (Fig. 5.19A). The main feature which stood out from the rewashed filter (Fig. 5.19B) was a 3.5 kb EcoRI fragment present in Gal 73 DNA but not in V79 DNA, and also absent in $Gal^+$73B DNA. Unfortunately, because very little PstI digested V79 DNA was on the filter, the presence of a new PstI fragment in Gal 73 which might correspond with the 3.5 kb EcoRI fragment could not be ascertained. The pattern observed could result from an

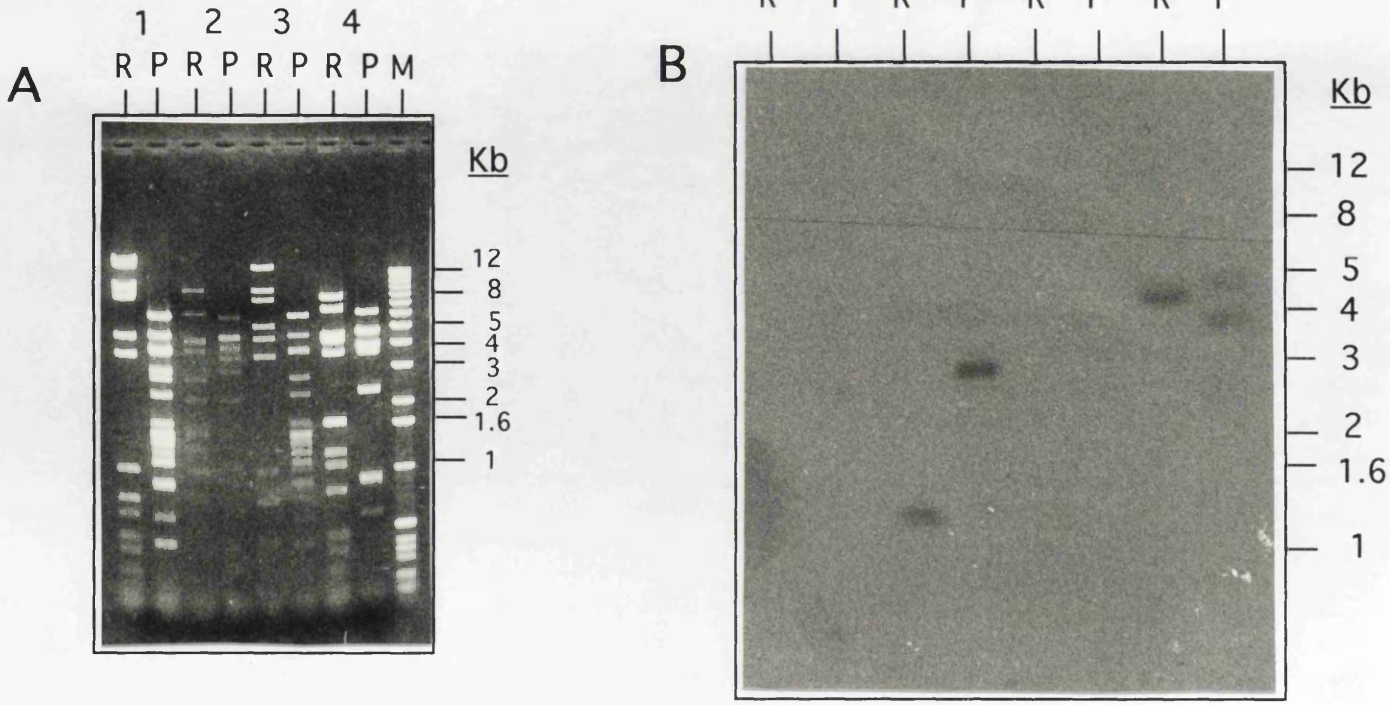
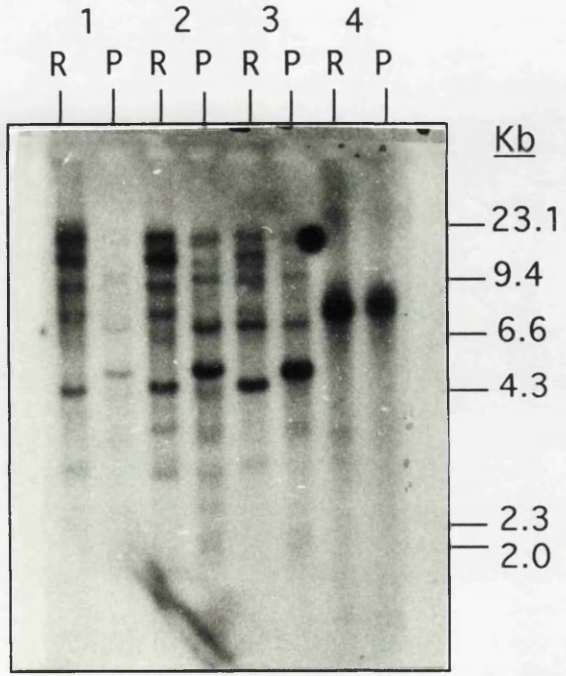

A
1 2 3 4
R P R P R P R P M
Kb
12
8
5
4
3
2
1.6
1
B
1 2 3 4
R P R P R P R P
Kb
12
8
5
4
3
2
1.6
1

Fig. 5.18 (A) 0.7% agarose gel electrophoresis of EcoRI (R) and PstI (P) digests of 1ug each of L73B1, 2, 3 and 4 DNA (denoted 1, 2, 3 and 4), visualised by staining with EtBr prior to southern blotting. BRL 1 kb DNA ladder (M) was used as size marker.
(B) Southern blot analysis of the DNA from L73B1, 2, 3 and 4 in the agarose gel in (A), probed with $^{32}$P-labeeled E5 DNA. Marker sizes are shown in kilobases.

A


1 2 3 4
R P R P R P R P
Kb
23.1
9.4
6.6
4.3
2.3
2.0

B

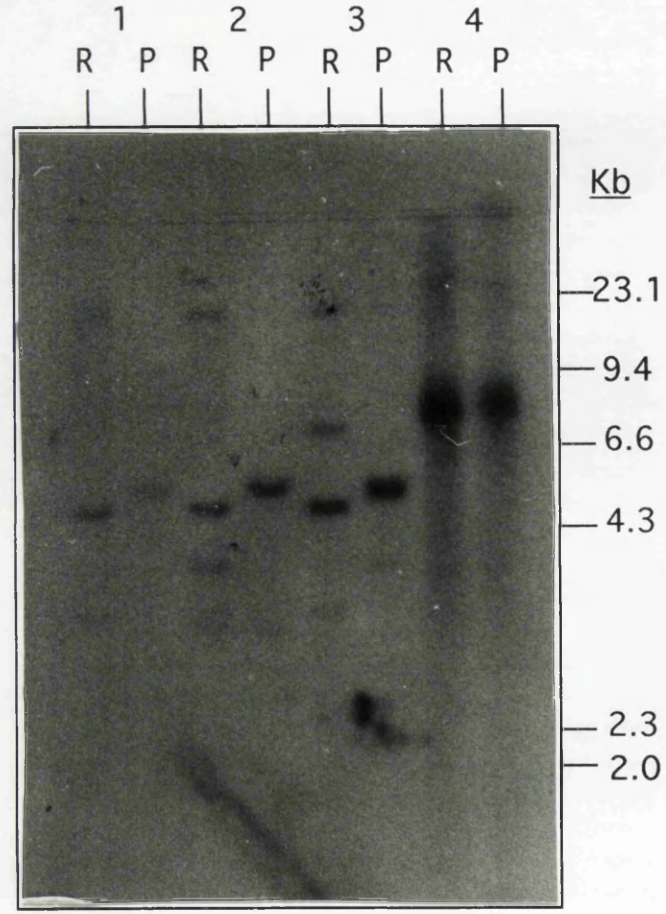

1 2 3 4
R P R P R P R P
Kb
23.1
9.4
6.6
4.3
2.3
2.0

Fig. 5.19 Southern blot analysis of genomic DNA probed with $^{32}$P-labelled C6.

(A) 2ug aliquots of V79 (1), Gal 73 (2) and Gal$^{+}$73B (3) DNA and 50pg aliquots M13 DNA were digested with EcoRI (R) and PstI (P) and subjected to southern blot analysis. Overnight hybridisation at 65$^{o}$C took place with $^{32}$P-labelled C6 prepared from single-strand DNA. The filter was washed in 0.1 x W at 65$^{o}$C for 20 mins. The duration of autoradiography was 72 hours. Marker sizes are shown in kilobases.

(B) the same filter as in (A) rewashed in 0.1 x W at 80$^{o}$C for 30 mins. The duration of autoradiography was 8 days.

insertion or deletion event in the genome of Gal 73, that appears to have been corrected or further altered in Gal$^+$73B, as a result of the integration event which also restored respiratory competence.

In Gal$^+$73B DNA probed with C6 there is a new 7 kb EcoRI fragment associated with the loss of the 3.5 kb EcoRI fragment and in the PstI digest, there is also a new fragment. Along with this there is another copy of the 5.1 kb fragment, as this has doubled in intensity, relative to Gal 73 DNA.

The above results strongly suggest that phenotypic restoration of Gal 73 involved a homologous integration event. The presence of the new C6-reactive EcoRI fragment in Gal$^+$73B DNA, plus the loss of the additional EcoRI fragment detected by this probe in the genome of Gal 73 strongly suggests that C6 contains not only insertion site DNA, but also the 'breakpoint' of the genomic rearrangement which has occurred in Gal 73, be it insertion or deletion.

In conclusion, two M13 subclones from L73B2, B1 and E5 have been shown to contain DNA integrated into Gal 73 upon transfection of DNA from the pWE15 wildtype genomic library. In contrast, C6 contains DNA from the region of the Gal 73 genome into which the pWE15 cosmid clone integrated. Along with this, C6 also contains DNA from a region in Gal 73 with a different EcoRI restriction map to V79, and upon integration of the pWE15 cosmid clone, a further rearrangement has occurred, probably a gene conversion by homologous integration, thereby complementing the mutant phenotype.

Approximately 380bp of C6 was sequenced (Appendix III) and found to consist of a mixed population with one clone being more abundant than the other. C6 underwent plaque purification and was resequenced to check for purity. Six plaques were chosen randomly out of a population of approximately 50. Single stranded DNA was prepared from each and sequenced. As all six were the same, the contaminant was assumed to be a deletion product of C6 produced during growth. Further data however, indicated that this assumption was incorrect.

Comparison of the sequence of C6 to sequences in the EMBL database showed no significant homology and did not identify any open reading frames.

### 5.2.6 LOCALIZATION AND CHARACTERISATION OF THE REARRANGEMENT BREAKPOINT IN GAL 73 DNA.

In an attempt to determine the nature of the molecular lesion present in Gal 73 cells, an analysis of the region within L73B2 from which C6 was derived was performed. Unfortunately, this did not enable the characterisation of the breakpoint, as further genomic blot analysis, using regions of the insert of plaque- purified C6 (C6-A) as probes, revealed that the original C6 probe must have comprised at least two different M13 subclones of L73B2 DNA.

EcoRI, HindIII and PstI digests of DNA from L73B2 were initially probed with non-plaque purified C6 (Fig. 5.20A) not knowing that it contained more than one M13 subclone. C6 DNA hybridised to two EcoRI fragments (7 kb and 4.2 kb) but to only one HindIII (8 kb) and one PstI fragment (4.5 kb), suggesting (without subsequent knowledge) either that there was an EcoRI site within the insert of C6, or else that C6 contained two non-contiguous L73B2-derived Sau3AI fragments.

The plaque-purified subclone designated C6-A was used for all subsequent experiments. Sequencing approximately the first 380 bp of C6-A revealed that there were indeed two Sau3AI fragments in the insert, the first being only 50bp long. Two Oligonucloeotides 50bp in length were therefore synthesized, representing the sequence either side of the internal Sau3AI site. These were used to reprobe the southern blot of L73B2 DNA. Oligo-I, representing the shorter Sau3AI fragment of C6-A, hybridised to a 1.85 kb EcoRI fragment (Fig. 5.21A) seen in the original blot of L73B2 DNA probed with C6 (Fig. 5.20A and B) only at lower stringency (Fig. 5.20C). Restriction analysis of double-stranded DNA isolated from C6-A showed that the larger Sau3AI fragment contained an internal EcoRI site (Figs. 5.22). The two Sau3AI-EcoRI sub-fragments of C6-A (0.7 and 1.1 kb) were then separately cloned into the $SK^+$

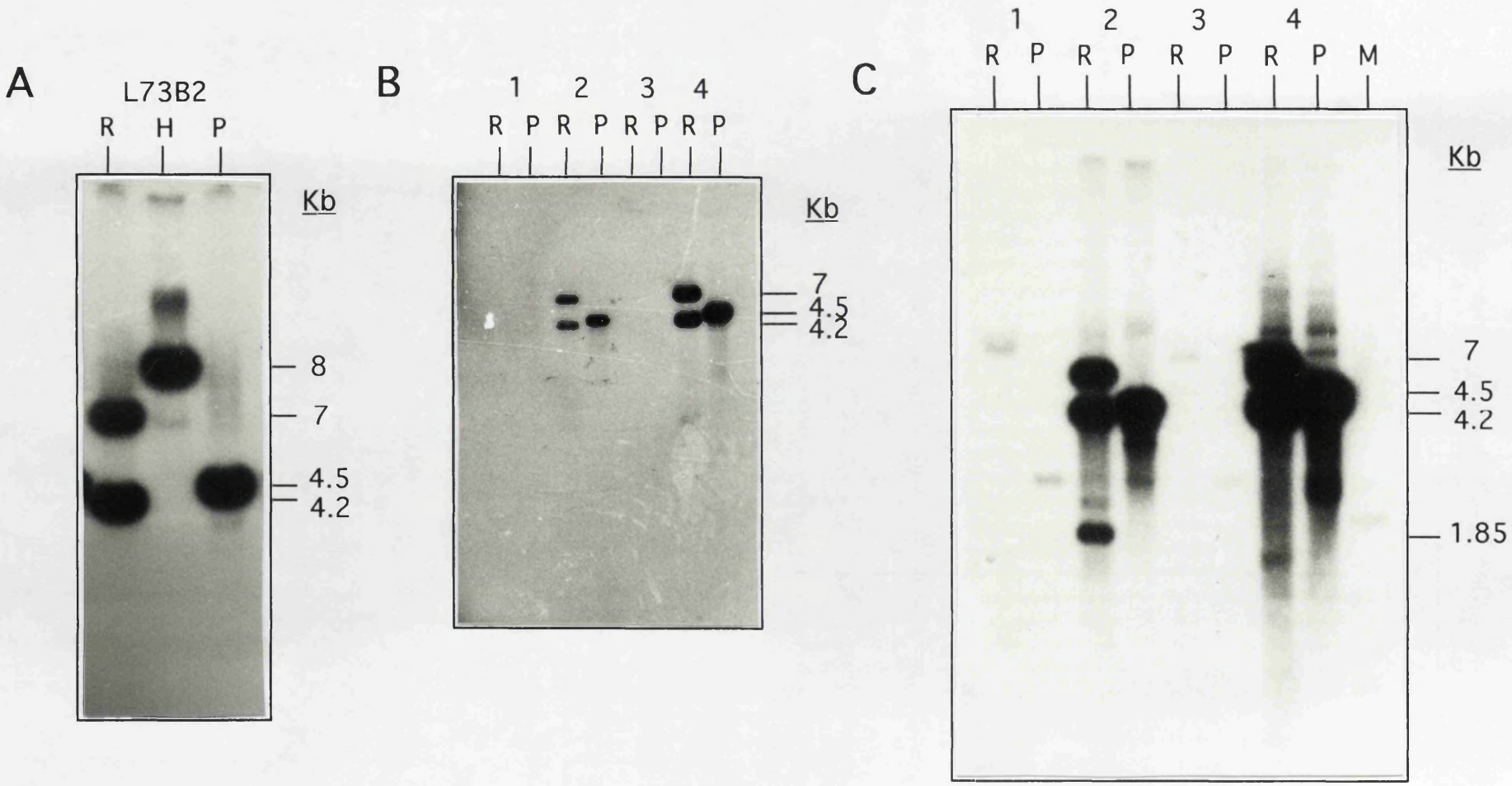

A
L73B2
R H P
Kb
8
7
4.5
4.2
B
1 2 3 4
R P R P R P R P
Kb
7
4.5
4.2
C
1 2 3 4
R P R P R P R P M
Kb
7
4.5
4.2
1.85

Fig. 5. 20 (A) Southern blot analysis of EcoRI (R), HindIII (H) and PstI (P) digests of L73B", probed with $^{32}$P-labelled C6 prepared from single-strand DNA (Chapter 2, section 2.19.2). Hybridisation and washing conditions are as for Fig. 5.19(B). Autoradiographywas carried out for 24 hours. The sizes of the fragments are shown in kilobases.
(B) Southern blot analysis of EcoRI (R) and PstI (P) digests of L73B1, 2, 3 and 4 DNA (shown as 1, 2, 3 and 4) probed with $^{32}$P-labelled C6 prepared from single-strand DNA. Hybridisation took place at 65$^{o}$C and washing at 80$^{o}$C in 0.1 x W buffer for 20 mins. Autoradiography was carried out for 24 hours. The sizes of the fragments are shown in kilobases.
(C) Southern blot analysis of EcoRI (R) and PstI (P) digests of L73B1, 2, 3 and 4 (shown as 1, 2, 3 and 4) hybridised with $^{32}$P-labelled C6 as for (B). This filter was washed in 0.1 x W buffer at 65$^{o}$C instead of 80$^{o}$C for 20 mins. Autoradiography was carried out for 24 hours.

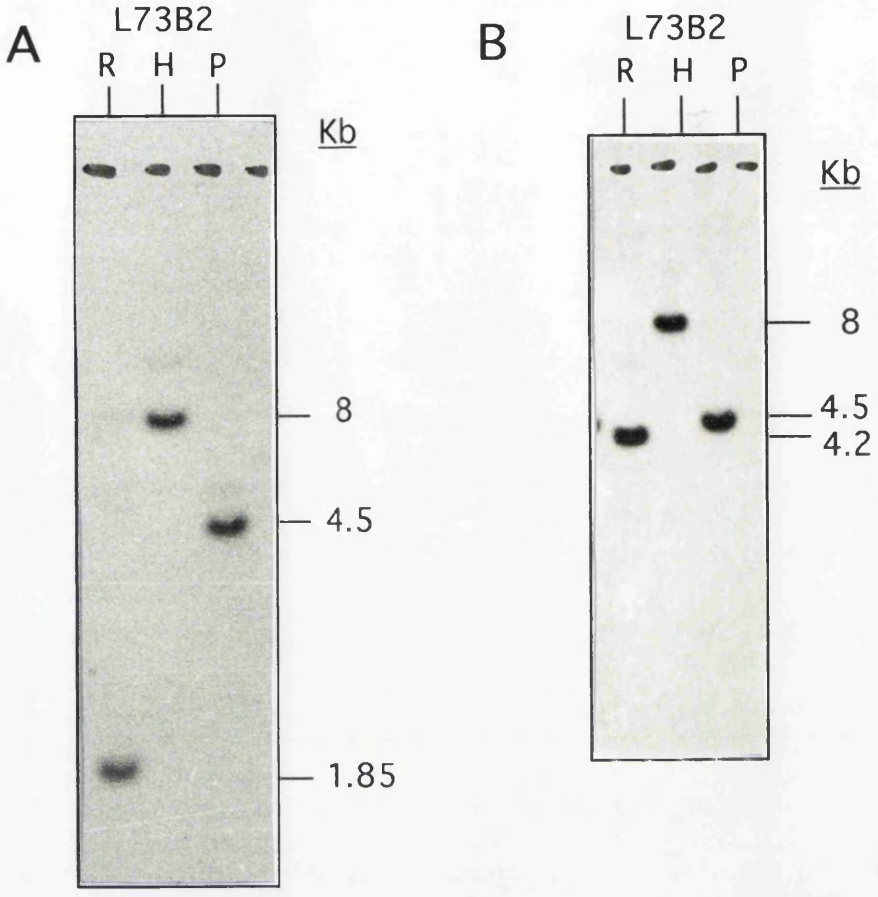



Fig. 5.21 (A) Southern blot analysis of EcoRI (R), HindIII and PstI (P) digests of L73B2 probed with $^{32}$P-labelled Oligo I. The filter from the experiment whose results are shown in Fig. 5.20(A) was striped of any radioactive probe by boiling for 10 mins. in $dH_2O$ containing 0.1% SDS and rehybridised overnight with Oligo I at 63°C and washed in 0.1 x W at 59°C for 20 mins.

(B) Southern blot analysis of L73B2 DNA digested as in (A) above, this time probed with $^{32}$-labelled Oligo II. The filter was hybridised with Oligo II overnight at 54°C and washed in 0.1 x W at 50°C for 20 mins.

Autoradiography in both cases was carried out for 24 hours. Fragment sizes are shown in kilobases.

bluescript vector. These two bluescript clones are subsequently referred to as pSK1.1 and pSK0.7 (Fig. 5.22B).

To determine whether the clone designated C6-A hybridised with the breakpoint region of Gal 73 genomic DNA, and if so, which portion of it detected the 3.5 kb EcoRI fragment in Gal 73 DNA, the genomic blots described above were repeated using as probes the 1.1 kb and 0.7 kb fragments isolated from pSK1.1 and pSK0.7 respectively. Both oligo-II, the isolated 1.1 kb Sau3AI-EcoRI fragment of C6-A, and pSK1.1, hybridised to the 4.2 kb EcoRI fragment of L73B2 (Fig. 5.21B) and to the 4.2 kb EcoRI fragment in the genomic southern blot (Fig. 5.23A). The 0.7 kb EcoRI-Sau3AI fragment of C6-A and pSK0.7, hybridised to the 7 kb EcoRI fragment of L73B2 (not shown) and the 7 kb EcoRI fragment in Gal$^{+}$73B DNA, along with a high molecular weight fragment of approx. 27 kb, present in all three types of genomic DNA (Fig. 5.23B). Neither region of C6-A hybridised to the 3.5 kb EcoRI fragment in Gal 73 DNA, nor to the new 3.6 kb PstI fragment in Gal$^{+}$73B DNA which had been detected in earlier blots probed with unpurified C6. The 2.9 kb EcoRI fragment found in all three genomic DNAs also remains unaccounted for. The most likely explanation for the loss of these bands is that the 'lost' hypothetical clone C6-B hybridised with these fragments, and that it is this clone which detected the breakpoint. Based on the criteria presented earlier clone C6-A would appear to represent only integrated DNA, and not the breakpoint or insertion site DNA. To clarify the results observed in Fig. 5.19, the two (or more) subclones in the original isolate of C6 would have to be independently recovered, and C6-B used as a probe in genomic blots. From earlier data it can be concluded that the sequence of C6-B must be located close to that of C6-A, in L73B2, as the restriction fragments in L73B2 to which the original C6 (mixed) clone hybridised (Fig. 5.20) were identical to those detected by pSK1.1 and pSK0.7 (data not shown). The 7 kb and 4.2 kb EcoRI fragments from L73B2 which hybridised to pSK0.7, pSK1.1 and C6 have subsequently been cloned into pBluescript.

The conclusion reached from the above experiments, i.e. that C6-A contains integrated DNA implies that C6-A could be used,

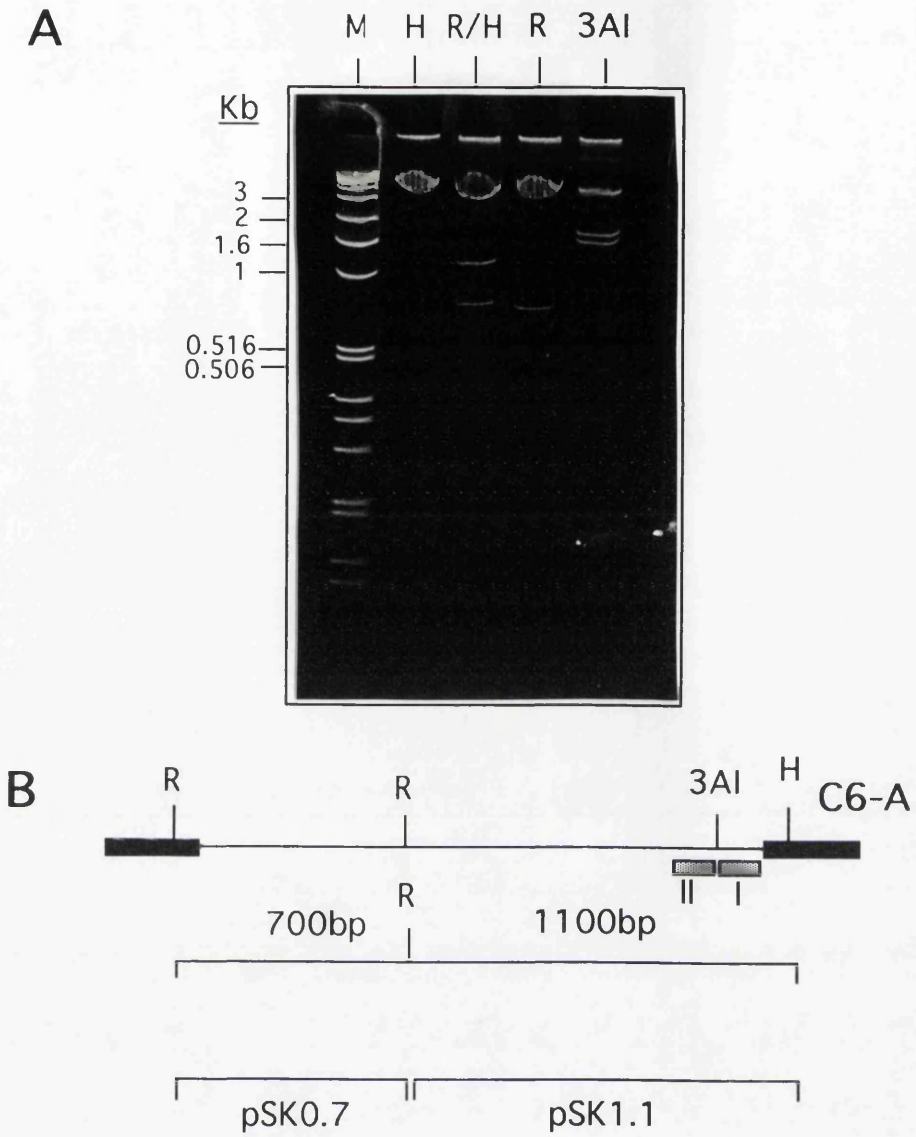



Fig. 5.22 Restriction mapping of the insert of C6-A.
(A) 5% polyacrylamide gel electrophoresis of digests of C6-A . 500 ng of double stranded C6-A DNA was digested with EcoRI (R), HindIII and Sau3AI (3AI). BRL 1 kb ladder (M) was used as a size marker and is shown in kilobases. The restriction pattern was visualised by staining with EtBr.
(B) Restriction map of the genomic DNA insert of C6-A.
M13 DNA is shown as thick lines, the L73B2 genomic DNA insert as a thin line. EcoRI, HindIII and Sau3AI restriction sites as R, H and 3AI respectively. Oligo I and Oligo II are shown as dotted boxes marked I and II.

like E5 and B1, as a probe to screen the pool of 20 000 original pWE15 cosmid clones used in the successful transfection in which Gal$^+$73A and Gal$^+$73B were originally isolated. Alternatively, they could be used to screen other genomic libraries to isolate clones containing DNA from this region in the wildtype genome. The results obtained from reverse northerns and sequencing indicate that B1, C6 and E5 do not contain coding sequence and are probably not represented in mRNA. However, northern blots of V79 cell RNA using B1, C6 and E5 as probes, should be performed to clarify this. If one or more of the M13 clones is found to contain DNA which is expressed at the RNA level, then isolation of a cDNA would hopefully result in speedy characterisation of the nuclear gene which is mutated in Gal 73 cells. This, along with the eventual isolation of C6-B, will aid the characterisation of the molecular lesion in Gal 73 cells.

### 5.2.7 TRANSFECTION ANALYSIS OF COSMID CLONES OF GAL$^+$73B DNA OBTAINED BY MARKER RESCUE.

One of the ways in which it would be possible to confirm that the integration of a pWE15 clone of wildtype (V79) genomic DNA had complemented the respiratory deficient phenotype of Gal 73, would be to show that the gal$^+$ phenotype could be successfully passaged by DNA transfection, using one or more of the Lorist 6 clones L73B1-4, rescued from the Gal$^+$73B genome.

As a result, transfection of Gal 73 cells was performed using DNA from L73B1-4 selecting for G418$^R$ and gal$^+$ phenotype. The transfection results showed that all four of the L73B clones were able to confer G418$^R$ on Gal 73 cells, This confirmed the observation that the L73B clones contained the region in Gal$^+$73B into which one or more intact copies of a pWE15 clone integrated, as only the copy of the neo$^R$ gene with the SV40 promoter sequences from pWE15 would confer such resistance on eukaryotic cell-lines such as Gal 73. 2 x $10^8$ Gal 73 cells were transfected with 10 ug of DNA separately from each of the L73B clones. Flasks containing $10^3$ and $10^4$ cells were

subsequently selected for $G418^R$. The remainder of the cells were selected for $G418^R$ and $gal^+$ phenotype. The frequency of $G418^R$ was 0.1%: therefore 200 000 $G418^R$ clones were effectively screened for $gal^+$ phenotype in each of the four transfections. No respiratory competent clones were obtained, suggesting that although the L73B clones contain integrated DNA, individually they do not carry the intact wildtype gene ($gal^+$) required for the efficient rescue of Gal 73. Alternatively, the $gal^+$ gene may have been rearranged during the subsequent cloning into Lorist 6. Although individually they do not contain the intact gene, it could be spread over 2 or more of the L73B clones, especially if it is a large gene. A further possibility, given that $Gal^+$73B appears to have arisen from homologous integration and/or gene conversion, is that the wildtype gene might be spread over a huge distance extending beyond the cosmid clones. However, in this case it might still be possible to passage the phenotype, albeit inefficiently, by further homologous integration events.

To test this possibility, further transfections were carried out using DNA from all four L73B clones mixed together as the frequency of experimental homologous recombination depends on the length of completely homologous DNA, and concatenation between the four L73B clones might facilitate this. $2 \times 10^8$ cells were transfected with a mixture of 2.5 ug of each of the four L73B clones. The frequency of transformation to $G418^R$ was 0.1% as before, and therefore 200 000 $G418^R$ cells were screened for $gal^+$ phenotype. From these, three $G418^R$, $gal^+$ colonies were isolated, $Gal^+1$, $Gal^+2$ and $Gal^+3$.

DNA from the three $G418^R$, $gal^+$ colonies $Gal^+1$, $Gal^+2$ and $Gal^+3$ was extracted and probed in genomic blots with pSK1.1 (Fig. 5.23A). There were no pSK1.1 hybridising fragments, other than those observed in the wildtype V79 cell-line, to indicate the integration of a new copy of this region of L73B2 into the genomes of $Gal^+1$, $Gal^+2$ or $Gal^+3$. This was also the case for $Gal^+$73A DNA probed with pSK0.7 (Fig.5.23B). These results strongly imply that a homologous integration event has occurred in each case (as in $Gal^+$73B). The low spontaneous

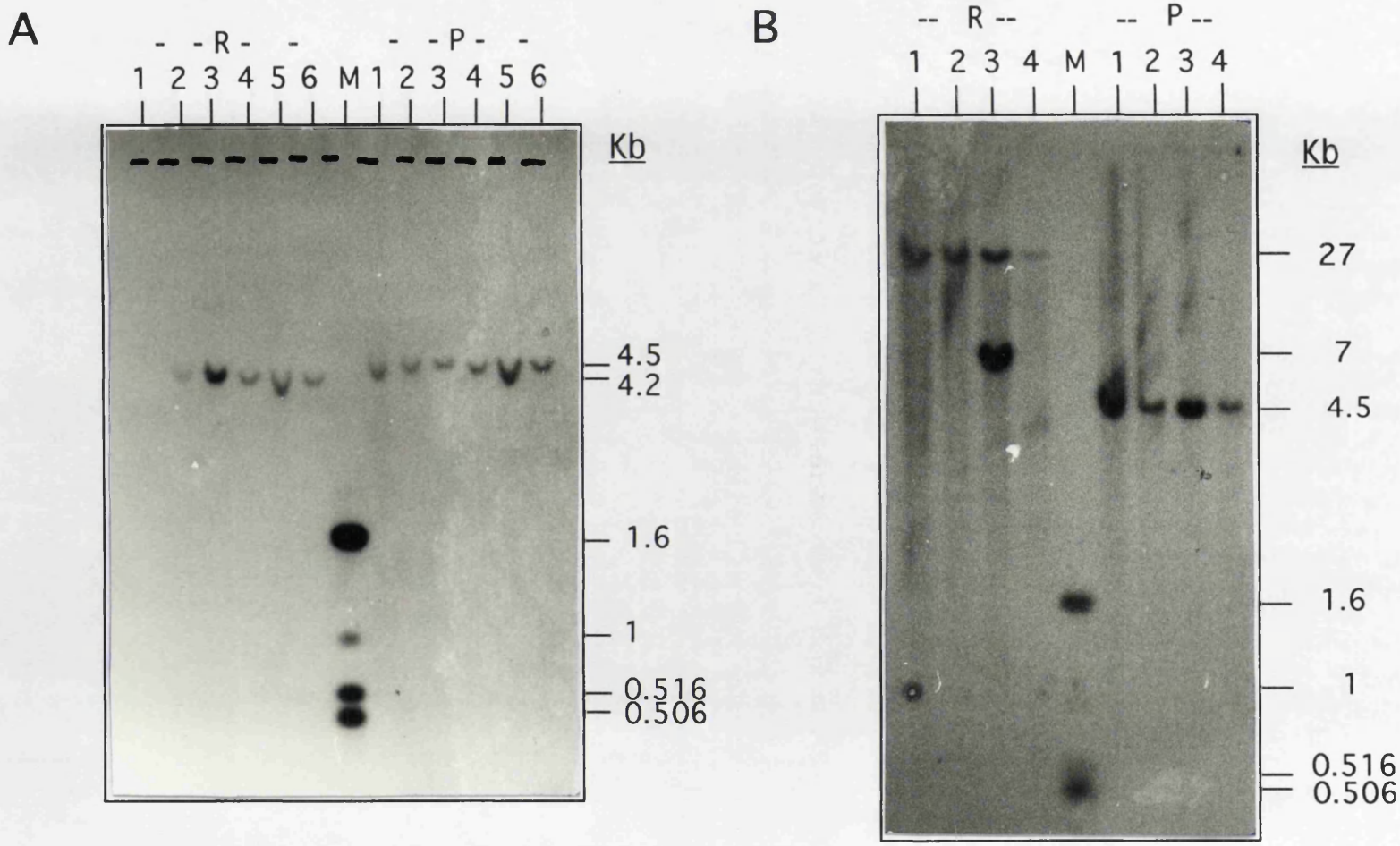

A
- - R - - - - P - -
1 2 3 4 5 6 M 1 2 3 4 5 6
Kb
4.5
4.2
1.6
1
0.516
0.506
B
-- R -- -- P --
1 2 3 4 M 1 2 3 4
Kb
27
7
4.5
1.6
1
0.516
0.506

Fig. 5.23 (A) Southern blot analysis of V79 (1), Gal 73 (2), $Gal^+73$ (3), $Gal^+1$ (4), $Gal^+2$ (5) and $Gal^+3$ (6) DNA digested with EcoRI (R) and PstI (P) and probed with nick-translated pSK1.1. 2ug of each DNA underwent digestion prior to gel electrophoresis and blotting. Hybridisation took place overnight at 65°C and the filter washed at 80°C in 0.1 x W for 20 mins. Autoradiography was carried out for 4 days. Fragment sizes are shown in kilobases.
(B) Southern blot analysis of V79 (1), Gal 73 (2), $Gal^+73B$ (3) and $Gal^+73A$ (4) DNA digested with EcoRI (R) abd PstI (P) and probed with nick-translated pSK0.7. The amount of DNA on the filter and the hybridisation and washing conditions are as for (A) above. Fragment sizes are shown in kilobases.

reversion frequency exhibited by Gal 73 cells indicates that the isolation of three $gal^+$ clones in this transfection was not due to such reversion. The low frequency of isolation of $gal^+$ colonies, plus the absence of new pSK1.1 or pSK0.7-reactive fragments in $Gal^+1$, $Gal^+2$, $Gal^+3$ (and $Gal^+73A$) DNAs, strongly suggests that the phenotype has been corrected by gene conversion, rather than by an ectopic integration event. To test definitively whether such an event had occurred, the nature of the mutation in Gal 73 would have to be fully ascertained and then this region analysed by PCR in $Gal^+1$, $Gal^+2$, $Gal^+3$ and $Gal^+73A$ genomic DNA for any alterations which restore the wildtype sequence.

The very low frequency of successful complementation by transfection with all four L73B clones together (3 positives out of an estimated 200 000 transformants) means that the failure to obtain any positives with the L73B clones individually is unlikely to be significant. One or more of the L73B clones may therefore be able to passage the phenotype by homologous integration, although this remains to be proven.

### 5.2.8 DISCUSSION.

Marker rescue was used, successfully, to isolate the region of $Gal^+73B$ DNA containing the integrated cosmid sequences which restored respiratory competence to Gal 73 cells. Although individually the four Lorist 6 cosmid clones L73B1-4, isolated by marker rescue, did not transform Gal 73 cells to respiratory competence, mixed together L73B1-4 successfully passaged the $gal^+$ phenotype, albeit inefficiently. These results suggest that the sequences necessary for the expression of the $gal^+$ wildtype gene may be split over two or more of the clones, or more likely that homologous integration is required to passage the phenotype.

Southern blot data of $Gal^+73B$ DNA showed that the integration of the pWE15 recombinant clone involved complex rearrangments of both integration and insertion site sequences, and based on the properties of low-copy number M13 subclones isolated from the region of integration, Gal 73 appears to have suffered a

mutational event involving sequences at or close to the integration site.

The mixed clone C6, detected a highly informative pattern of bands in southern blots, indicative of a rearrangement in the genomic DNA of Gal 73, which logically must be an insertion or deletion. Gal 73 is a respiration-deficient cell-line obtained after EMS treatment of V79 cells. EMS treatment predominantly causes single base pair changes within the DNA, or small deletions. As the respiration-deficient phenotype of the original clone of Gal 73 has a low spontaneous reversion rate i.e. less than 1 in $10^9$ cells, this would imply that a point mutation in Gal 73 cells, alone, is unlikely to be the cause of the $gal^-$ phenotype as point mutations typically revert at a frequency of $10^{-6}$ to $10^{-7}$. Being a diploid cell-line, however, the rearrangement would only give respiratory deficiency if the second copy of the gene was in some way inactivated, i.e. if the second copy was already deleted or rearranged. It is unlikely (though not impossible) that such a rearrangement occurred as a result of the EMS treatment and that, conceivably, both copies of the gene are affected by rearrangements. However, various regions of the genome are known to be present in Chinese hamster cell-lines in the haploid state, and this could be one reason why so few different complementation groups of respiration-deficient cell-lines have been isolated after mutagenesis. Nevertheless, upon restoration of respiration in $Gal^+$73B, the rearrangement seen in Gal 73 DNA, that was detected with subclone C6, is no longer present. Therefore, it is likely that a gene conversion event occurred upon integration of a pWE15-wildtype DNA recombinant, which also accounts for the low frequency of restoration of the $gal^+$ phenotype and of its subsequent passage by the L73B clones.

The clone originally designated C6 was shown by sequencing to be a mixed clone, and when plaque purified, one of the resulting subclones designated C6-A, did not hybridise to the 'breakpoint' restriction fragment in Gal 73 DNA. The other (hypothetical) subclone, designated C6-B, not yet characterised, must therefore represent the breakpoint region.

To purify C6-B from C6, plaque purification, with help from pSK0.7 and pSK1.1 to identify negatively hybridising subclones from C6, can be performed.

From the data already obtained we know that the sequences corresponding to the inserts of C6-A and C6-B must be relatively close to one another in clone L73B2, as DNA from both C6-A and C6-B (both presumed to be present in the original C6 DNA prep) were found to hybridise to the same EcoRI, HindIII and PstI fragments of L73B2 (Fig. 5.20). The two EcoRI fragments of L73B2 which hybridised to C6 DNA have subsequently been cloned into pBluescript $SK^+$ for sequencing. This should allow eventual characterisation of this region of L73B2, the location of C6-A in relation to C6-B, and therefore of the rearrangement breakpoint. Once characterised, probes could be produced which could be used as primers for the PCR analysis of this region in V79 and Gal 73 genomic DNA, i.e. to amplify the wildtype sequence and the corresponding region in Gal 73, and characterise the exact nature of the rearrangement in the mutant cell-line.

C6-A, along with B1 and E5, could be used to help isolate the wildtype gene represented by the integrated DNA in the $Gal^+73B$ genome. This could be done by using the three M13 subclones as probes to screen the original pool (i.e pool 2) of 20 000 cosmid recombinant clones containing V79 genomic DNA, that were used in the original transfection into Gal 73, which resulted in the isolation of $Gal^+73A$ and $Gal^+73B$. After the isolation of positive clones, these could be re-assayed for their ability to restore respiratory competence in Gal 73 cells in DNA transfection experiments. Candidate open reading frame(s) within any resulting positive cosmid clones could then be located and eventually characterised for function in mitochondrial biogenesis.

C6-A, B1 and E5 contain only small inserts of genomic DNA from $Gal^+73B$ cells. They would not necessarily be useful in isolating the coding region of the $gal^+$ gene if it is spread over an extended region of genomic DNA, especially if the gene conversion hypothesis turns out to be correct and only

part of the gene, possibly not even including coding sequence, is contained within the cosmid clone which restored respiratory competence in Gal 73 cells.

To isolate the entire $gal^+$ gene, the easiest approach would be to check by Northern analysis and /or reverse transcription PCR whether C6-A, B1 or E5 contain DNA which is expressed in Gal 73 cells. If so, these clones could be used to isolate a putative $gal^+$ cDNA. However, when the M13 subclones were screened using radioactive probes prepared from 1st strand cDNA synthesis reactions from V79, Gal 73 and $Gal^+$73B mRNA, the results suggested that the genomic sequences in C6-A, B1 and E5 were not highly expressed. As mentioned above, genomic DNA in this region of the genome can be assayed over a larger scale for characteristics of expression (e.g. CpG islands in any cosmid clone in pool 2 which hybridises to C6-A, B1 or E5).

If the $gal^+$ gene is not contained entirely on one cosmid isolated from pool 2 using C6-A, B1 or E5 as probes, sequences at the pWE15/V79 junction of any C6-A, B1 or E5-hybridising cosmid clones can be used to design primers for ligation-mediated PCR, in order to initiate a genomic walk.

As a result of this work low copy-number clones are now available representing integrated DNA, and, indirectly, integration site (breakpoint) DNA. Most of the groundwork for the eventual characterisation of the $Gal^+$73 gene and of the precise molecular lesion in Gal 73 cell DNA has been performed. The data reported here strongly suggest that a homologous recombination event was required to restore respiratory competence, hence the low frequency of complementation of the $gal^-$ phenotype of Gal 73 cells. In retrospect, therefore, it would have been better to have used a cDNA library, instead of the cosmid genomic library, to complement Gal 73 cells. One possible problem with such an approach would arise if the $gal^+$ gene is not highly expressed. This would lead to a low representation of the gene within the cDNA library, and result once again in a low frequency of complementation of the $gal^-$ phenotype.

Given that a DNA conversion event has occurred in Gal$^+$73B to bring about the restoration of respiratory competence, one would predict that the same region of V79 genomic DNA, would have restored the gal$^+$ phenotype in Gal$^+$73A, as well as in Gal$^+$73B. When Gal$^+$73A genomic DNA was probed with pSK0.7 or pSK1.1 there was no difference in the pattern of hybridising fragments when compared to wildtype, suggesting that no extra copy of the V79 genomic DNA hybridising to C6-A had been integrated in Gal$^+$73A DNA. This result is consistant with Gal$^+$73A also having been generated by homologous integration. This could be confirmed by a southern blot of Gal$^+$73A DNA probed with C6 or C6-B showing C6-B hybridising to integrated sequences in Gal$^+$73A DNA as well as the breakpoint in Gal 73. However, the pattern of integration of the pWE15 recombinant into Gal$^+$73A is complicated and could confuse the interpretation of southern blots screened with C6 or C6-B. It would therefore seem more sensible to concentrate on the characterisation of the integrated DNA in Gal$^+$73B DNA using C6-A, B1 and E5, and of the breakpoint in Gal 73 DNA detected by subclone C6-B.

In conclusion, the necessary probes (B1, E5 and C6-A) are available to isolate the integrated recombinant cosmid in the Gal$^+$73B genome which will hopefully contain gal$^+$ sequences that can be characterised for function in mitochondrial Complex III biogenesis.

# CHAPTER 6

# CONCLUDING REMARKS

The aim of this project was to isolate and characterise mammalian nuclear-coded mitochondrial genes involved in mitochondrial biogenesis, in an attempt to analyse the mechanism by which the nuclear and mitochondrial genomes are co-expressed. In this study three mammalian cell-lines derived from Chinese hamster lung tissue were used, each of which has a nuclear mutation which affects mitochondrial respiration.

## 6.1 THE NUCLEAR MUTATION IN GAL 13 CELLS AFFECTS MITOCHONDRIAL PROTEIN SYNTHESIS.

Pulse-labelling of mitochondrial proteins of Gal 13 cells with $^{35}S$ methionine in the presence of the antibiotics emetine and chloramphenicol has shown that the synthesis of a subset of mitochondrially encoded polypeptides was significantly altered by the nuclear mutation in these cells. Good candidates for the polypeptides whose synthesis is affected are the mitochondrially encoded subunits of Complex I, (NADH dehydrogenase), at least four of which were lowered in relative abundance when compared to wildtype (Fig. 3.4). This would fit with the biochemical evidence that the nuclear mutation primarily affects Complex I activity in Gal 13 cells (Whitfield et al, 1981) lowering its activity to 5% of the wildtype level. Similar experiments in two other mutant cell-lines Gal 50 and Gal 73 show no such affect of their mutant genes on mitochondrial protein synthesis.

## 6.2 THE GAL 13 NUCLEAR MUTATION AFFECTS MITOCHONDRIAL PROTEIN SYNTHESIS BEYOND THE LEVEL OF TRANSCRIPTION.

Northern blot analysis using a mitochondrial DNA fragment containing sequences of at least 3 ND genes has shown that the abundance of the transcripts of ND4/4L, which are translated from the same mRNA, and ND3 are unaffected by the nuclear mutation in Gal 13 cells, suggesting that the mutation is not in a nuclear-coded transcription factor imported into mitochondria to regulate the transcription of the mitochondrially encoded subunits of Complex I.

The autoradiographs from the northern blot analysis also

imply that the processing of precursor ND transcripts to mature ND transcripts is unaffected by the nuclear mutation. The nuclear gene is therefore involved either in mitochondrial translation of the ND mRNAs or in post-translational processing of the resulting polypeptides. However, the abundance of not all the ND transcripts has been assessed by northern analysis, therefore the nuclear mutation could, conceivably, affect the transcription of one of those not detected by the mitochondrial DNA fragment used as a probe. Nevertheless, it would seem more likely, especially based on the precedent of the large number of nuclear mutations which affect mitochondrial translation in yeast, that the mutation in Gal 13 cells affects mRNA-selective mitochondrial translation.

In yeast many of the nuclear-coded genes isolated have been shown to be involved in the translation of specific mRNAs, e.g. PET494, PET54 and PET55 are all required for the translation of cytochrome c oxidase III mRNA (Fox, 1986). However, in Gal 13 cells the mutation affects the translation of at least four of the ND subunits, unlike the yeast nuclear gene products which only interact with a single mitochondrial mRNA. In yeast mitochondrial DNA, genes coding for subunits of NADH dehydrogenase have not been identified therefore the existence of a nuclear-coded polypeptide which regulates the translation of more than one mRNA, may be unique to this complex.

Another possibility is that the Gal 13 phenotype is similar to the yeast COX10 phenotype. COX10 is required for the assembly of cytochrome c oxidase (Fox and Costanzo, 1990). The $Gal^{+}13$ gene could be specifically required for the assembly of NADH dehydrogenase which when absent results in the degradation of some if not all the ND subunits.

### 6.3 THE MUTANT NUCLEAR GENE IN GAL 73 CELLS DOES NOT CODE FOR A STRUCTURAL SUBUNIT OF COMPLEX III.

Biochemical experiments have shown that the activity of Complex III in Gal 73 cells is 17% of the wildtype level. The

protein synthesis analysis, performed on this cell-line as for Gal 13 cells, showed that the mutation did not alter mitochondrial protein synthesis. Immunoprecipitation analysis, using an antibody against Complex III of the electron transfer chain, showed that for those Complex III subunits precipitated by the antibody, their relative abundance and rate of accumulation was not changed compared to wildtype cells. The immunoprecipitation analysis however, can not assess whether the function of any of the subunits of Complex III is affected by the mutation. The easiest way to determine whether any subunits of Complex III are functionally impaired by the nuclear mutation would be to isolate the mutated gene and assess directly its function in Complex III biosynthesis in Gal 73 cells.

In contrast to Gal 13, from the results obtained in this study it is difficult to relate the Gal 73 phenotype to any yeast PET mutants which affect Complex III of which there have been three types identified: 1) CBP1, required for the 5' processing of cytochrome b pre-mRNA to stabilise the mRNA; 2) CBP2, required for the splicing of the terminal intron of cyt b and 3) CBS1 and CBS2, required for the translation of the cyt b mRNA.

In relation to the complementation map devided by Chu (1974) and others from the results of cell fusion experiments between the $gal^-$ cell-lines (see section 1.8; Fig.1.3), Gal 13 and Gal 50 do not complement one another, but are in different complementation groups because Gal 50 complements Gal 73 but Gal 13 does not. This suggests that although Gal 13 and Gal 50 have a defect in Complex I the phenotype is due to different nuclear-coded mutations. This is confirmed by the protein synthesis analysis performed in this project. However, this does not explain why Gal 50 and Gal 13 do not complement one another. If in Gal 13 cells the $gal^+$ gene is a translation initiation factor specifically required for the translation of the mitochondrially-encoded ND mRNAs then this gene should be wildtype in Gal 50 cells and therefore capable of rescuing the Gal 13 phenotype, which in turn should rescue the Gal 50 phenotype. Why this does not happen is not yet clear.

This becomes even more difficult to explain for Gal 13 and Gal 73 cells which have defects in different redox complexes but still do not complement each other. The protein synthesis analysis performed here, shows that the nuclear-coded mutation in Gal 73 cells is different to that present in Gal 13 cells, but does not make it any clearer as to why they don't complement each other. However, it would be interesting to see if $Gal^+73B$ cells could restore respiratory competence in Gal 13 cells in cell fusion experiments. Although for a definitive insight into the overlapping pattern of complementation by the three $gal^-$ cell-lines used here, the isolation and characterisation of the $gal^-$ genes is required.

## 6.4 GAL 73 CELLS CAN BE COMPLEMENTED BY TRANSFECTION OF CLONED WILDTYPE GENOMIC DNA.

Wildtype genomic DNA from pools of cosmid recombinants, was used to restore respiratory activity in Gal 73 cells, giving two $gal^+$ cell-lines $Gal^+73A$ and $Gal^+73B$. Subsequent isolation of the integrated wildtype genomic DNA sequences by a marker rescue technique from $Gal^+73B$ cells has enabled a preliminary analysis on the molecular lesion in Gal 73 cells and the mechanism by which complementation of the mutation occurred, and has yielded molecular clones for the region containing the mutation. A similar strategy was employed to clone the $Gal^+13$ gene by complementation. This proved unsuccessful, although it was carried to a stage where this can be regarded as a definitive result. Analysis of further cosmids by this method might yet yield one or more recombinants capable of complementing the Gal 13 mutation.

### 6.4.1 COMPLEMENTATION OF THE MUTATION IN GAL 73 CELLS REQUIRED/INVOLVED HOMOLOGOUS RECOMBINATION.

Three lines of evidence support the view that the complementation of the mutation in Gal 73 cells occurred by homologous recombination, most important being the results obtained from southern blots of V79, Gal 73 and $Gal^+73B$ genomic DNA screened with the mixed M13 subclone C6. The putative subclone C6-B has been inferred to contain single-

copy genomic DNA sequences from the region of integration of the pWE15/V79 recombinant cosmid clone in the genome of $Gal^+$73B cells. A 3.5 kb EcoRI fragment was detected in Gal 73 genomic DNA by this subclone that was not seen in the genomic DNA of V79, the parental cell-line. This fragment disappeared again from $Gal^+$73B DNA. This evidence alone strongly suggests that the restoration of wildtype phenotype occurred by gene conversion via the homologous integration of wildtype genomic sequences from the cosmid recombinant, replacing mutant sequences in Gal 73 genomic DNA.

The other evidence comes from the low frequencey by which the complementation of the mutation in Gal 73 cells was achieved. This low frequency suggests that homologous recombination and not random integration was required to give the restoration of respiratory competence. Three paramenters which could affect the frequency of complementation are the size of the cosmid genomic library i.e. the frequency by which the $gal^+$ gene occurs in the library, the size of the gene and the requirement for specific integration events for complementation to occur. Obviously the size of the $gal^+$ gene would have a direct and compelling effect on the need for integration by homologous recombination.

When titred the wildtype genomic cosmid library was found to consist of $5 \times 10^5$ recombinants. This is more than the number required to be 99% certain that the whole Chinese hamster genome is represented by the library. Therefore, there is no reason to suppose that the $gal^+$ gene is underrepresented, although under representation remains possible, which could account for the low frequency of complementation. This low frequency however, most likely is due to the requirement for the conversion of the mutated region of the Gal 73 genome to $gal^+$ by homologous recombination.

The third line of evidence consistent with homologous recombination being required to restore respiratory competence in Gal 73 cells comes from the low frequency by which the $gal^+$ phenotype was passaged, when Gal 73 cells were transfected with DNA from the four Lorist 6 clones, L73B1-4, containing

DNA from the region of the Gal$^+$73B genome with the integrated pWE15/V79 recombinant. Southern blot analysis of the three gal$^+$ clones obtained from this transfection, Gal$^+$1, Gal$^+$2, and Gal$^+$3, screened with pSK1.1 containing DNA sequences close to but not covering the site of the molecular lesion in Gal 73 cells, detected no new fragments. This is again consistent with the mutant phenotype having been corrected by a gene conversion rather than a random integration event.

The strategy adopted here, had some success, but it appears that the gal$^+$ gene in Gal 73 cells could be very large hence the requirement for homologous integration. Other workers have used complementation to isolate mammalian nuclear genes eg. the chicken thymidine kinase gene (Perucho et al, 1980). However, this gene was found to be a small gene (2.2 kb), therefore homologous integration was not required to transfer the tk$^+$ phenotype to Ltk$^-$ cells. Two human bladder oncogenes have also been isolated using this method, both of which were also contained on small cloned inserts (<7 kb) of genomic DNA (Shih et al, 1982; Goldfarb et al, 1982).

## 6.5 FUTURE WORK

Two lines of research need now be followed in order to characterise the molecular lesion in Gal 73 cells and the isolation and characterisation of the corresponding 'Gal$^+$73' gene.

To characterise the molecular lesion within Gal 73 cells, the M13 subclone C6-B would have to be plaque-purified independently of C6-A. C6-B could then be sequenced and the sequence used to design primers for PCR analysis, to amplify this region of genomic DNA in V79 and Gal 73 cells. Comparisons of the PCR products from both wildtype DNA and mutant DNA should indicate whether the lesion is a point mutation in an EcoRI site, a deletion or an insertion which has altered the EcoRI restriction pattern of this region of the Gal 73 genome.
An analysis of the DNA which was integrated into Gal 73 cells to produce Gal$^+$73B can be performed by using the M13 subclones

B1, E5 and C6-A, as southern blot analysis has shown that they contain DNA sequences integrated into $Gal^+73B$ DNA as part of a pWE15/V79 recombinant clone. They could be used to screen the 20 000 original pWE15/V79 recombinant clones (pool 2) used in the transfection in which $Gal^+73A$ and $Gal^+73B$ were isolated. The inserts of any 'positive' clones resulting from thisd screen could then be subcloned and analysed for transcriptional start sites by looking for DNase I hypersensitive regions, CpG islands (Guo et al, 1993; Beland et al, 1993) or by subcloning and sequencing. If the clones do not contain the full $gal^+$ gene, as suggested by the requirement for homologous recombination to restore respiratory competence, exon sequences from the cosmid clone thus isolated could be used to screen a Chinese hamster cDNA library to isolate the entire $Gal^+73$ coding region.

## 6.6 RETROSPECTIVE REMARKS

The rate limiting step in the method used to isolate the mutated nuclear-coded mitochondrial genes in Gal 73 and Gal 13 cells has been the complementation of the mutation by transfection with wildtype V79 genomic DNA. This occurred at such a low frequency that for Gal 73 cells complementation only occurred twice in $2.6 \times 10^{10}$ cells transfected and for Gal 13 cells complementation was not achieved at all in the same number of cells trasnfected.

In retrospect, in order to increase the frequency of complementation, it probably would have been better to have used a Chinese hamster wildtype cDNA library as a source of the $gal^+$ genes for introduction into Gal 73 and Gal 13 cells. Although this would have overcome the requirement for homologous recombination for the restoration of respiratory competence, there are other disadvantages to using a cDNA library in this way. For example, it the transcripts of the $gal^+$ genes are only present at low abundance, then they might be under represented or even unrepresented in the library. Due to the lack of knowledge about the $gal^+$ genes there is no way of testing for the level of abundance of the transcripts prior to using the cDNA library. Therefore using a cDNA library may

not significantly increase the frequency of complementation of the mutations. More trivially, a suitable cDNA library was not available at the time the project was undertaken and the expertise in making cDNA libraries in the research department was also not available.

Ideally, an approach which enabled the isolation of nuclear-coded mitochondrial genes without first having to go through the process of complementing the mutation, would be beneficial to the aim of the project. One such approach would be to generate novel gal$^-$ cell-lines by an insertional mutagenesis procedure. This would entail randomly introducing plasmid DNa with a eukaryotic resistance marker into the Chinese hamster wildtype (V79) genome and then screening for the disruption of cellular respiration. This could be achieved in the same way as recorded by Chu et al, (1974), i.e. by growth in 5'-bromodeoxyuridine and treatment with light in nutritionally-deficient medium (galactose medium). The disrupted gene in any gal$^-$ cell-lines obtained as a consequence of this mutagenic process could be isolated by screening either lambda or cosmid genomic libraries prepared from these cell-lines, with a probe homologous to the integrated plasmid sequences or alternatively by marker rescue, as used in this project. The resulting genomic clones would contain the integrated plasmid sequences and also adjacent genomic DNA which could be characterised by the procedures discussed above, in respect of mitochondrial function.

If during mutagenesis and selection, the conditions permissive for maintenance of cell viability without aerobic respiration were used, this could enable the isolation of gal$^-$ cell-lines that may belong to complementation groups which would never have been isolated using earlier approaches, because the mutations would have been lethal.

A different approach could be taken to isolate the integrated DNA sequences, without having to screen libraries prepared from the genome of cells with the integrated DNA, exploiting PCR technology. Ligation-mediated PCR requires the sequence of only one of the primers to be known i.e. the integrated

plasmid sequences. The other primer sequence comes from the ligation of a linker sequence to the 3' overhang nucleotides produced by restriction digestion with certain endonucleases.

Another PCR approach would be to use "inverse PCR". For inverse PCR the genomic DNA is digested with a restriction enzyme which will not cut between the primer sites at either end of the plasmid DNA, and ligated under conditions to promote circularisation. Amplification should then proceed out into the genomic DNA adjacent to the plasmid sequences. Both of these PCR techniques would produce clones to use as probes to isolate the undisrupted wildtype $gal^+$ gene for characterisation.

However, the approaches which were adopted in this work were nevertheless successful in achieving the basic aim of the project, namely to isolate $gal^+$ conferring sequences from a mammalian Chinese hamster cell-line which can now be characterised and maybe explain why a mutation in this gene in Gal 73 cells does not allow the complementation of the Gal 13 phenotype.

## APPENDIX I

The sequence of the genomic DNA insert in the M13 subclone B1, referred to in this thesis.

GCTTGCATGC CTGCAGGTCG ACTCTAGAGG ATCATGCTAC
TCCTGGGCTT ACCATTATGT AGCCCGTAAG TTGCACCCAT
TGGTGAGTTC TAATAGGCTA TAGTTCTAGA TTTACAAATG
CCTCCCTCAA ATAATTTATG TCATAATGTT ATGCCCAAAT
TTATTCATTA ATATTTATTG ACTTAATATG TGGCCTCAAA
GAATTTATGG TTGTATGTAT AGGAAAGGCA GACAGATTGA
TGGAGAGCAA TACTCAGACT ACTCCTGACT GGCATCTCTC
AAGAATCTAT TCTACATCAT AC

## APPENDIX II

The sequence of the genomic DNA contained in the insert of the M13 subclone E5, referred to in this thesis.

GATCTAGCAC GGTAGGAAAC AACAGACTCA CTAATATCAT
AAGTGTGTCG ACTCACAGAG ATCTGCTAGA TGGTGTTACT
RATGATCGTT CCTTACCTGG AAGGTGTGAG ACAGGGCGTA
TACTAACATT

## APPENDIX III

The sequence of the genomic DNA contained in the insert of the M13 subclone C6-A, referred to in this thesis.

GATCTCAATA TGGCTTCTTT CCCCTTATGG TGAGTCTCAT
ATGCCTCCCT GATCCAGTTA GTAAGGATAG GAAAATTATA
TTTTATGTAA CAATAAGCAC AAGAATTAGT AACAATCTTA
ACTATAGAAG AGAGTCCATG AAAAGTCCGT GTGATGTAAA
TAGTAAAGCA CTTCTTCAGC ATCAAGGTTA GGTGTGTCCA
AGGTGAGTAC TGCTCCCATA GTCCAGGAGG CCTGGTCAAA
ATCAGGAGCT GCAAGTGTGC TTTATCTTGA TGTCAACTCT
TTCCCTCCTC CCTAGGTACG AAGATGCCCT GGTCCTTTGC
TCACTGAGGT GTTAAATCGA ATCCAGTTCA GATACAACCA
GGCCCAGCTG GAGGAGTTGG ATGATGA

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