JANNE TOIVONEN

A Model for Mitochondrial Deafness in Flies

Expression and Mutation Analysis of Mitoribosomal Protein S12

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ACADEMIC DISSERTATION

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To my parents

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LIST OF ORIGINAL PUBLICATIONS

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I **Toivonen JM**, Boocock MR and Jacobs HT (1999). Modelling in *Escherichia coli* of mutations in mitoribosomal protein S12: novel mutant phenotypes of *rpsL*. *Mol Microbiol* 31: 1735-1746.

II **Toivonen JM**, O'Dell KMC, Petit N, Irvine SC, Knight G, Lehtonen M, Longmuir M, Luoto K, Touraille S, Wang Z, Alziari S, Shah ZH and Jacobs HT (2001): *technical knockout*, a *Drosophila* model of mitochondrial deafness. *Genetics* 159: 241-254.

III **Toivonen JM**, Manjiry S, Touraille S, Wang Z, Alziari S, O'Dell KMC and Jacobs HT (2003): Gene dosage and selective expression modify phenotype in a *Drosophila* model of human mitochondrial disease. *Mitochondrion* 3, 83-96.

IV Mariottini P, Shah ZH, **Toivonen JM**, Bagni C, Spelbrink JN, Amaldi F and Jacobs HT (1999): Expression of the gene for mitoribosomal protein S12 is controlled in human cells at the levels of transcription, RNA splicing and translation. *J Biol Chem* 274, 31853-31862.

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine trisphosphate
ANT	adenine nucleotide translocase
APP	P^1,P^5-Di(adenosine-5´)pentaphosphate
bp	base pair
CHO	chordotonal organ
CS	Canton S
D-loop	displacement loop
Ef-Ts	elongation factor Ts
Ef-Tu	elongation factor Tu
ETC	electron transport chain
FAD	flavin adenine dinucleotide
GAL4	yeast transcriptional activator
GDP	guanosine diphosphate
GTP	guanosine trisphosphate
dB	decibel
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DmTTF	*Drosophila melanogaster* transcription termination factor
DNA	deoxyribonucleic acid
DOX	doxycyclin
DTNB	dithio-*bis*-nitrobenzoic acid
EDTA	ethylenediamine N,N,N',N' tetra-acetic acid
EF	elongation factor
ER	endoplasmic reticulum
EtBr	ethidium bromide
HCl	hydrochloric acid
HSP	heavy strand promoter
H-strand	heavy strand
IF	initiation factor
IM	inner membrane
IMS	intermembrane space
IPTG	isopropylthiogalactoside
KCN	potassium cyanide
L-strand	light strand
LSP	light strand promoter
LSU	ribosomal large subunit
mRNA	messenger RNA
MRPS12	human mitochondrial protein S12
mtDNA	mitochondrial DNA
mtSSB	mitochondrial single stranded binding protein
mRNP	messenger ribonucleoprotein
mt	mitochondrial
mtDNA	mitochondrial DNA
mTERF	mitochondrial transcription termination factor
MRP	mitochondrial RNA processing
MRPS12	human mitoribosomal protein S12

NAD	nicotinamide adenine dinucleotide
ND	NADH-dehydrogenase
nt	nucleotide
OD	optical density
oligo(Y)	oligopyrimidine
OM	outer membrane
ONP	ortho-nitrophenol
ONPG	ortho-Nitrophenyl-β-*D*-galactopyranoside
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
PM	paromomycin
Pol γ	mitochondrial DNA polymerase gamma
POLRMT	mitochondrial RNA polymerase
RI	restriction intermediate
RNA	ribonucleic acid
ROS	reactive oxygen species
rpS12	bacterial ribosomal protein S12
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
RRF	ribosome release factor
S12	ribosomal protein S12 (general name)
SD	Shine/Dalgarno
SM	streptomycin
SM^R	streptomycin resistant
SM^S	streptomycin sensitive
SSU	ribosomal small subunit
TCA	tricarboxylic acid cycle (Krebs cycle)
TFAM	mitochondrial transcription factor A
TFB1M	mitochondrial transcription factor B1
TFB2M	mitochondrial transcription factor B2
tko	*technical knockout* (gene for *Drosophila* mitoribosomal S12)
TMPD	N,N,N´,N´-tetramethyl-*p*-phenylenediamine
TOP	terminal oligopyrimidine
tRNA	transfer RNA
UAS	upstream activating sequence
UCP	uncoupling protein
uORF	upstream open reading frame
UTR	untranslated region
VDAC	voltage-dependent anoion channel (porin)
wt	wild-type
w/v	weight per volume

ABSTRACT

Mutations in the maternally inherited mitochondrial genome (mtDNA) or in nuclear genes involved in mitochondrial metabolism manifest with a wide range of clinical phenotypes, which frequently include sensorineural deafness in syndromic or non-syndromic form. The pathological mechanisms of such diseases are largely unknown, and their outcome is known to be affected by environmental and genetic modifiers. Relevant whole animal models are needed to explore the developmental and biochemical consequences of mitochondrial dysfunction, as well as mechanisms underlining the high tissue specificity of the diseases. The main purpose of my study is to elucidate the validity of *Drosophila melanogaster* (fruit fly) as a model system for human mitochondrial disorders, particularly those resulting from mitochondrial translational defects. To develop such models, I have manipulated the nuclear *Drosophila* gene *technical knockout* (*tko*), encoding mitoribosomal protein S12, a critical component of the ribosomal accuracy centre involved in the fidelity of protein synthesis both in bacterial and mitochondrial ribosomes. The prototypic mutation tko^{25t} results in conserved amino-acid substitution (L85H) and exhibits some close parallels with human mitochondrial disease, such as developmental delay, temporary paralysis in response to mechanical stress (followed by seizure-like episodes), hyporeactivity and defective auditory function. Based on bacterial modelling the mutation causes defective ribosome assembly and, in agreement with this, tko^{25t} shows decreased levels of mitochondrial small subunit ribosomal RNA, is hypersensitive to mitochondrial translational inhibitor doxycyclin, and shows greatly decreased mitochondrial oxidative phosphorylation capacity and diminished ATP synthesis. I infer that the tko^{25t} mutant provides a model of mitochondrial hearing impairment resulting from a quantitative deficiency of mitochondrial translational capacity. To extend our understanding of mitoribosomal function and to elucidate the critical times and cell types in development for manifestation of the disease-like phenotype, I have created novel transgenic flies and analysed their expression and phenotypes in various genetic backgrounds. Transgenic reversion of the tko^{25t} mutation results in complete rescue of the phenotype, whereas increased expression of the mutant allele shows partial attenuation of the biochemical defect. However, the latter is not associated with improved performance. In this sense, the model mimics biochemical and phenotypical threshold effects associated with heteroplasmic mtDNA mutations in humans (i.e. those in which mixture of wild-type and mutant mtDNA is present). Selective spatio-temporal expression of wild-type *tko*+ in

mutant flies results in a diverse range of phenotypes, and indicates tissues and developmental stages where mitochondrial translational capacity becomes limiting in the mutant. High level over-expression of *tko+ in vivo* leads to pre-adult lethality both in mutant and in wild-type genetic backgrounds, emphasizing the importance of correctly regulated expression of the components of the mitochondrial translation machinery. Differential regulation of expression may also be one important variable affecting the phenotypic outcome in human diseases. Using cultured human cells I have elucidated mechanisms involved in expression of the human homologue of *tko*, *MRPS12*, demonstrated that it is highly expressed in tissues dependent on oxidative metabolism, such as heart and skeletal muscle, and is subject to sophisticated regulatory mechanisms involving transcription, alternative splicing and cell growth-mediated translational control.

1. INTRODUCTION

Mitochondria are eukaryotic organelles responsible for cellular oxidative energy production. They are also involved in various physiological processes such as intermediary metabolism and cellular signalling events. Due to the endosymbiotic origin of this organelle, mitochondria have retained a circular (or in some organisms linear) chromosome (mtDNA) that encodes a small number of polypeptides essential for respiratory chain function. Additionally, mtDNA encodes RNA species required for expression of the mitochondrially encoded proteins. Mutations in mitochondrial transfer RNAs (tRNAs) or ribosomal RNAs (rRNAs) are frequently associated with maternally inherited diseases manifesting as either syndromic or non-syndromic disorders, often characterized by seizures, ataxia, muscle weakness and hearing impairment. Each cell contains a large number of mtDNA molecules, which complicates the genetics of these disorders. Frequently, the determining factor is the proportion of mutated and the wild-type molecules in cells or tissues (heteroplasmy). Mitochondrial disorders can also result from a mutation in nuclear genes involved in mitochondrial metabolism. Animal modelling of mitochondrial diseases has largely concentrated on nuclear genes, because mtDNA is intractable for conventional genetic manipulations.

In this thesis I will describe mitochondria, their various functions, gene expression, and common features behind mitochondrial biogenesis and disease. Particular emphasis will be laid upon description of the mitochondrial translation system because of its frequent involvement in human diseases, such as mitochondrial deafness. I will also discuss the use of the fruit fly, *Drosophila melanogaster*, as an experimental animal for studying human mitochondrial disease. The genetic power of *D. melanogaster* has long been recognised, but since it is a relatively ‘simple’ invertebrate, its biomedical relevance has been sometimes overlooked. Recent studies of insulin signalling in aging, and of multiple neurodegenerative disorders suggest, however, that this insect species might be physiologically more similar to humans than we have previously thought.

2. REVIEW OF LITERATURE

2.1 Mitochondria

This chapter describes mitochondria in general, their structure and diverse functions. The animal mitochondrial genome, its maintenance and expression at the RNA level will also be more specificly described. A more specific description of the mitochondrial protein synthesis machinery will be given in section 2.2.

2.1.1 Brief history

Mitochondria are eukaryotic cell organelles that contain their own genome and are responsible for cellular respiration and aerobic energy production. The name 'mitochondrion' was coined by C. Bender in 1898, and is derived from the Greek *mitos* meaning thread and *chondrion* meaning granule. Occasional descriptions of structures we now know to be mitochondria appeared here and there in the microscopic literature already 150 years ago. However, the first study characterizing these structures and showing them to be present in a wide variety of cell types was that of Richard Altmann in 1890, who considered these "bioblasts" to be independent entities, and suggested them to be tiny organisms forming colonies within eukaryotic cells. Among others he contributed to *The Serial Endosymbiotic Theory* for the origin of mitochondria, which states that in the early history of eukaryotes a protobacterium capable of aerobic respiration was engulfed by an anaerobic protoeukaryotic cell. By providing some metabolic advantage to the host (such as ATP, hydrogen or detoxification of oxygen) it became an endosymbiont currently known as the mitochondrion. Supportive evidence for this view was the discovery of deoxyribonucleic acid (DNA) inside mitochondria (Nass and Nass, 1963), and the theory was later popularised and received its best support and articulation by Lynn Margulis (Margulis, 1981).

2.1.2 Structure and function of mitochondria

The internal space of mitochondria (mitochondrial matrix) is surrounded by two membranes (Figure 2.1 a). The outer membrane (OM) is freely permeable to ions and most metabolites due to the presence of non-specific channels formed by porins (VDAC), for solutes of molecular weight less than 10 kDa (Scheffler, 1999). The inner membrane (IM) is impermeable to most such molecules and project to the mitochondrial matrix via

lamellar and/or tubular invaginations called cristae. Between the inner and outer membranes lies the intermembrane space (IMS), which was for a long time thought to be fully contiguous with the intracristal space. However, recent studies using three-dimensional electron tomography suggest some sort of compartmentalization between these structures (reviewed by Frey and Mannella, 2000). The cristal membranes seem to have only small tubular contacts (crista junctions) with the peripheral surface of the IM, and it is possible that this restriction is sufficient to limit exchanges of metabolites and proteins between the two compartments and the membranes enclosing them. The OM and the peripheral IM confront each other periodically by integral membrane protein-mediated contacts. This kind of contacts is required, for example, for protein import, and is achieved by interaction of the translocation complexes Tom and Tim of the outer and inner membrane, respectively (Pfanner and Meijer, 1997). The IM is extremely protein-rich due to the presence of all the complexes for oxidative phosphorylation (OXPHOS) and a large number of other proteins that provide import and export of polypeptides, metabolites and ions. It is also the membrane across which the membrane potential ($\Delta\psi$) is built up by the electron transport chain (ETC) to create proton motive force required for ATP synthesis. $\Delta\psi$ is also needed for protein import and for the transport of various substances into and out of mitochondria (Nicholls and Ferguson, 2002).

The morphology of mitochondria and cristae varies depending on environmental conditions and cell type, and is likely to reflect the energy demands of different tissues. Mitochondria are not merely floating around the cell but have non-random localization controlled by extramitochondrial cytoskeletal elements, such as microtubules (Heggeness *et al.,* 1978; Yaffe *et al.,* 1996; Yaffe, 1999), and their movements are affected by mutations in cytoskeletal motor proteins, such as kinesins (Pesavento *et al.,* 1994; Pereira *et al.,* 1997; Tanaka *et al.,* 1998). In some cells, mitochondria form a convoluted network (mitochondrial reticulum, figure 2.1 b) whereas in others they can appear as discrete filaments or ovoid structures (Bereiter-Hahn and Voth, 1994). The form of this dynamic reticulum is controlled by continuous fission and fusion events mediated by specific proteins that seem to be conserved among all eukaryotes (Shaw and Nunnari, 2002). Therefore, a traditional picture of mitochondrion as a static, individual, rod-shaped structure is at least in most cases misleading.

Mitochondria are often described as the power plants of the cell, which refers to their ability to synthesise energy in the form of adenosine trisphosphate (ATP) by OXPHOS. As postulated by Peter Mitchell (Mitchell, 1961), this is achieved by coupling of electron transport from reducing equivalents to proton pumping by a series of reduction-oxidation reactions that establish an electrochemical gradient across the IM. This gradient can subsequently be used to produce ATP, which functions as a major carrier of chemical energy and links the catabolic and anabolic networks of enzyme-catalysed reactions in all cells. The tricarboxylic acid cycle (TCA cycle) and fatty acid oxidation (β-oxidation) both take place in mitochondria in the matrix compartment. Besides their function to reduce freely diffusible coenzymes NAD+/NADH and FAD+/$FADH_2$ to provide substrates for OXPHOS, both of these pathways contain one membrane-associated enzyme-complex that is a component of the ETC.

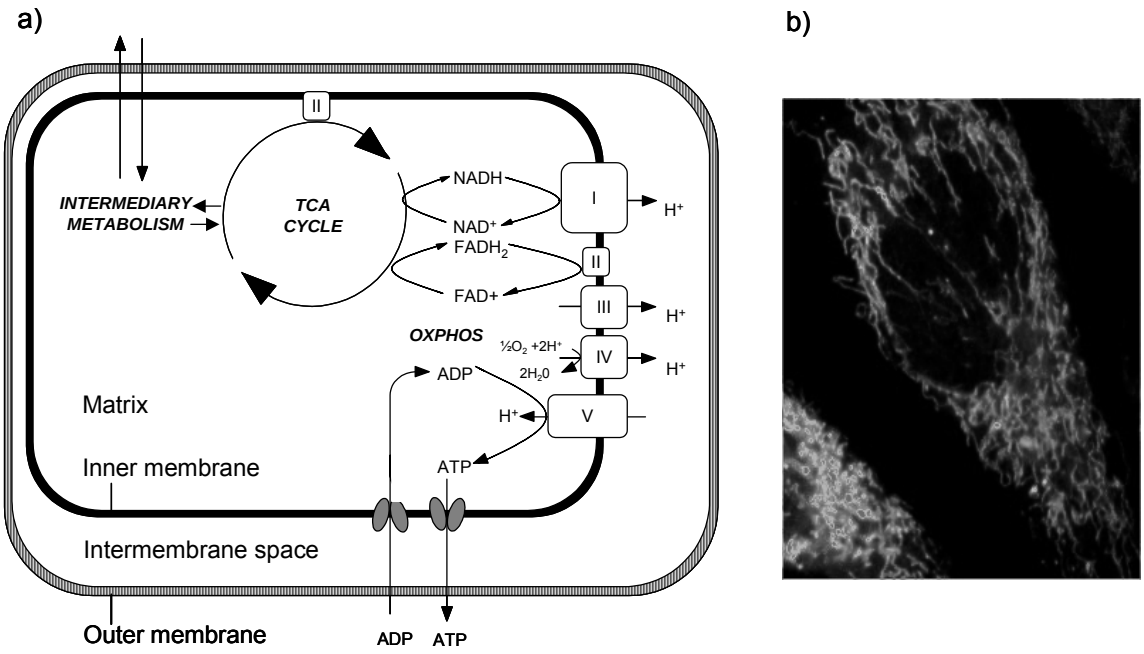


Figure 2.1. Mitochondrial metabolism and morphology. a) Schematic presentation of mitochondrion, with main metabolic activities presented (see text for details). For clarity, cristae are not shown. b) Confocal microscope picture of mitochondrial reticulum in cultured human cell-line stained with Mitotracker Red (kind gift from Dr. J.N. Spelbrink).

Mitochondria are involved in many other biochemical pathways, some of which are not directly linked to energy production. Intermediates produced by the TCA cycle provide precursors for amino acid and nucleotide biosynthesis. These intermediates are also important in the synthesis of the porphyrin ring of heme groups, which serve as oxygen carriers in the blood or electron carriers in dehydrogenase enzymes. Mitochondria are the

site for the synthesis of iron-sulphur (Fe/S) clusters involved in electron transport, substrate binding and biochemical catalysis, both inside and outside of mitochondria (Muhlenhoff and Lill, 2000). Intermediates derived from β-oxidation can be used as an alternative carbon source when glucose is unavailable, such as under starvation (Barger and Kelly, 2000). For detoxification of tissues, excess ammonia created by metabolism is transported to liver mitochondria where the nitrogen enters the urea cycle and is ultimately excreted. The first and rate-limiting step of steroid hormone synthesis from cholesterol occurs in mitochondria of steroidogenic tissues (Stocco, 2001, and references therein; Thomson, 2003). In some cases, such as in brown fat of newborn infants, cold adaptive rodents and hibernating animals, proton flow in mitochondrial membranes can be short-circuited by uncoupling proteins (UCPs), in order to produce heat instead of ATP (Kozak and Harper, 2000, and references therein). For a long time it has been known that mitochondria are also the primary site for the creation of reactive oxygen species (ROS, Boveris *et al.*, 1972), which might have impact on many pathological states in humans, as well as on ageing (reviewed by Droge, 2002; Mandavilli *et al.*, 2002). Milder uncoupling of mitochondria has been suggested to be involved in prevention of ROS formation and body weight control (Jezek, 2002).

A great number of studies have been published that confirm the importance of mitochondria in programmed cell death, *i.e.*, apoptosis (reviewed by Wang, 2001). Various apoptotic stimuli are transduced to mitochondria by BH3-only proteins (including Bad, Bid and its truncated form tBid), and their action can be either neutralised by anti-apoptotic proteins (such as Bcl-2 and Bcl-xL), or the signal can be further transduced to mitochondria by pro-apoptotic proteins (such as Bax or Bak). This signal causes appearance of the permeability transition pore, which uncouples the IM resulting in the collapse of the electrochemical gradient and swelling of mitochondria. Components of the permeability transition pore have been suggested to include VDAC and adenine nucleotide translocase (ANT), which are also responsible for transport of small metabolites and nucleotides, respectively, across the mitochondrial membranes. The rupture of the OM releases many pro-apoptotic mitochondrial proteins (including cytochrome *c*, Smac/Diablo, apoptosis inducing factor and endonuclease G) from the IMS to the cytosol and/or the nucleus, and results in subsequent activation of caspases that degrade various intracellular substrates. Simultaneously, this release causes caspase-independent nuclear chromatin condensation and large-scale DNA fragmentation. Other caspase-independent pathways

may be involved in programmed cell death, such as loss of mitochondrial function by uncoupling of electron transfer from OXPHOS, and alterations in calsium homeostasis, as shown by tBid treatment of isolated mitochondria. (Wang, 2001, and references therein). Another pro-apoptotic protein, Bad, has been recently shown to be a component of a multi-enzyme complex localised in the mitochondrial OM, and is required to nucleate the assembly of this complex (Danial *et al*., 2003). Other components of the complex are protein kinase A and protein phosphatase 1, responsible for Bad phosphorylation (inactivation) and dephosphorylation (activation), respectively, as well as the A kinase anchoring protein Wawe-1, and the glycolytic enzyme glucokinase. The work described by Danial *et al*. (2003) shows that Bad phosphorylation is required for maximal glucokinase activity, and suggests that in addition to being an integral participant in the apoptotic pathway, it also might ensure that glycolysis and apoptosis are coordinated.

There is also good evidence that mitochondria serve as temporary cellular calcium storages, or buffers preventing or delaying the spread of calcium signals in cells (for recent reviews, see Pozzan *et al.*, 2000, and Rizzuto *et al.*, 2000). Free cellular calcium exists normally at very low concentrations, but is increased in neuronal activation or by release from the endoplasmic reticulum (ER) in response to signalling events. The close proximity of mitochondria to the ER allows them to fine-tune the microenvironment of the calcium release sites and to rapidly respond in such release (Rizzuto *et al*., 1998). This can result in increased ATP production via stimulation of the dehydrogenases of the TCA cycle by intramitochondrial calcium (Denton *et al*., 1972). When these divergent tasks of mitochondria in cellular and physiological processes are taken into consideration it is perhaps too simplistic to think of them only as the ‘batteries’ of the cell. However, the bioenergetic functions performed by the OXPHOS machinery are clearly fundamental for multicellular life.

2.1.3 Mitochondrial genome organization and replication

The genetic information for mitochondrial biogenesis is mostly encoded by the nuclear DNA, and the proteins involved generally contain an amino-terminal targeting sequence that directs them to be imported into the organelle. These proteins include all those involved in transcription and translation, as well as the proteins of the mitochondrial DNA (mtDNA) replication and maintenance machinery. However, the mtDNA of animals codes

for a limited number of RNAs essential for intra-mitochondrial protein synthesis as well as some proteins needed for the function of ETC. This chapter describes a typical metazoan mitochondrial gene content and mainly uses human and the fruit fly *Drosophila melanogaster* as an example.

The mitochondrial genome in animals is a covalently closed circular molecule that is present in $10^3 – 10^4$ copies per cell (Lightowlers *et al.,* 1997). The mtDNA encodes 13 polypeptides (12 in those whose mtDNA do not encode A8), 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA) (Anderson *et al.,* 1981; Bibb *et al.,* 1981; Lewis *et al.,* 1995). Almost all the genes are contiguous and all lack introns; the genetic code used differs from the universal code in some respects (reviewed by Kurland, 1992). The open reading frames (ORFs) are generally punctuated by tRNA genes which serve as signals for precise nucleolytic cleavages to process long precursor RNAs to their mature forms (Ojala *et al.,* 1981), which involves both 5´ and 3´ processing of the tRNAs (Doersen *et al.,* 1985; Rossmanith *et al.,* 1995; Nagaike *et al.,* 2001; Puranam and Attardi, 2001). Released mRNAs and rRNAs are subsequently polyadenylated, a process that also completes the immature translation termination codon of some ORFs.

The mtDNA gene content is conserved from human to *Drosophila*, but variations in length, nucleotide content and organization of the genome exist (Figure 2.2). Mitochondrial ORFs encode subunits of complex I (NADH-ubiquinol oxidoreductase, EC1.6.5.3), complex III (ubiquinone-cytochrome-c oxidorteductase, or bc_1 complex, EC 1.10.2.2), and complex IV (cytochrome-c oxidase, 1.9.3.1) of the ETC, as well as two subunits of the complex V (ATP synthase, EC 3.6.1.3). Complex II (EC 1.3.99.11) of the ETC is the succinate dehydrogenase, a membrane bound enzyme of the TCA cycle, which has no mtDNA-encoded subunits.

Major variations between the mtDNAs of mammals and fruit flies are as follows. Firstly, the mtDNA of *Drosophila* contains a long, 96% A+T rich region in the equivalent position to the extensive non-coding region in mammals that contain the key signals for the initiation and regulation of transcription and DNA replication, as well as the displacement-loop (D-loop) whose function remains to be properly understood. The region contains the origin of replication in both species, but unlike in mammals, the length of the stretch varies substantially between *Drosophila* species (Goddard and Wolstenholme, 1978; Lewis *et al.,*

1994; Tsujino *et al.*, 2002) as a result of a variable number of tandemly repeated sequence elements. Secondly, genes encoded by mtDNA are more or less equally divided by the two strands in *Drosophila*, whereas in mammals most of the genes are encoded by the heavy strand (see below, Clary *et al.*, 1982; Garesse, 1988). This raises important questions about the regulation of mitochondrial gene expression in flies compared to mammals (see 2.1.4).

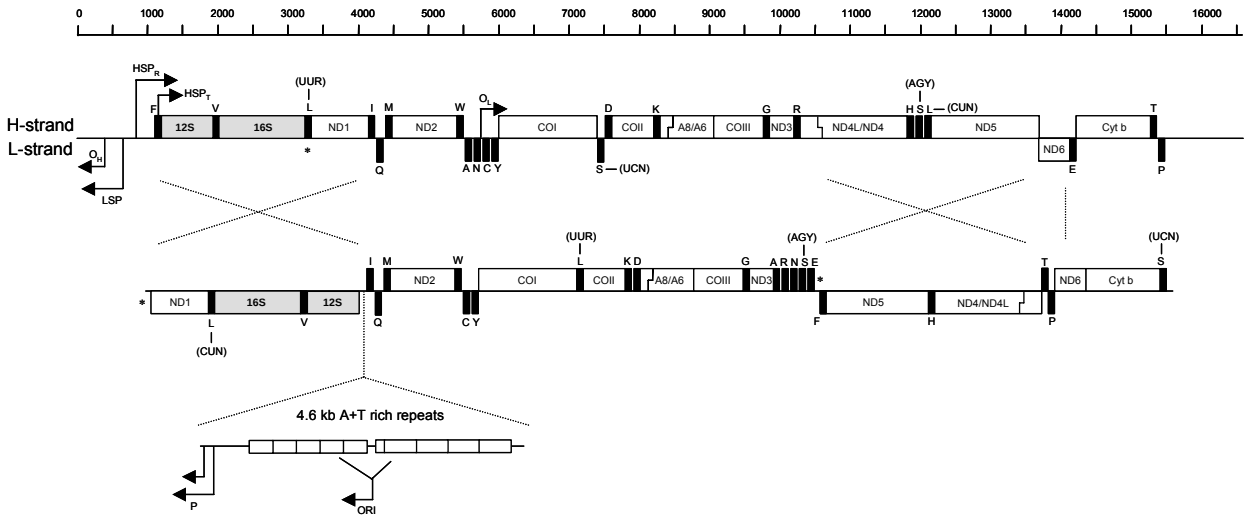


Figure 2.2. Linearised presentation of mitochondrial genomes of human (top) and *D. melanogaster* (bottom). Genes for rRNAs, tRNAs and for ORFs are shown as grey, black and open boxes, respectively. Heavy strand promoter (HSP) and light strand promoter (LSP) of the human mtDNA are shown by arrows that indicate the direction of transcription from each promoter. Dashed lines indicate the translocation and/or inversion events of the two blocks and ND6 gene between the two genomes. In *Drosophila*, an A+T-rich repeat sequence of variable length is located between 12S rRNA and tRNA for isoleucine. Approximate scale bar is shown above. Abbreviations for OXPHOS subunits and amino acids referring to corresponding tRNAs are as follows: ND1-ND6 (ND4L) = NADH-ubiquinol oxidoreductase 1-6 (4L), COI-COIII= cytochrome-c oxidase I – III, Cyt b= cytochrome *b*. tRNAs denoted by the one-letter amino-acid code as follows: A= alanine, C= cysteine, D= aspartate, E= glutamate, F= phenylalanine, G= glycine, H= histidine, I= isoleucine, K= lysine, L= leucine, M= methionine, N= asparagine, P= proline, Q= glutamine, R= arginine, S= serine, T= threonine, V= valine, W= tryptophan, Y= tyrosine. In the case of L and S, the cognate tRNA anticodons for the two-codon groups are indicated inside the brackets. Asterisks symbolise mTERF and DmTTF binding sites (see 2.1.4).

The two strands of mammalian mtDNA differ in their nucleotide composition, and are termed the heavy (H) and the light (L) strands. The replication of mtDNA in cultured HeLa cells was first described more than 20 years ago, when using electron microscopic techniques it was postulated that it replicates strand-asynchronously and continuously in both strands (Clayton, 1982). In this "orthodox" model, the replication starts from the H-strand origin (O_H) and proceeds approximately two thirds of the genome length until the L-strand origin (O_L) is exposed on the displaced H-strand. The replication of the L-strand can now start and proceed in the opposite direction. Recently, this model has been challenged by Holt and colleagues by interpretation of two-dimensional DNA electrophoresis gels,

which can be used to analyse mtDNA replication intermediates (RIs) in tissues and cell culture (Holt *et al.*, 2000; Yang *et al.*, 2002). The data suggested initially that two different mechanisms of mtDNA replication exist simultaneously: the orthodox mode, working mainly in the maintenance of a given copy number of the mitochondrial genome, and conventional, strand-coupled replication, with frequent lagging-strand initiation events predominating when efficient amplification of the mtDNA molecules is required (Holt *et al.*, 2000). Further analysis from highly purified mtDNA, however, has suggested that mammalian mtDNA is at least mainly replicated by the strand-coupled mechanism and previous data can be explained as an artefact of DNA preparation methods (Yang *et al.*, 2002). Very little is known about mtDNA replication in *Drosophila*, but the A+T rich region has been shown to contain an origin of replication by electron microscopic studies (Goddard and Wolstenholme, 1978). These studies suggested that in the mtDNA of *Drosophila* embryos most of the "leading" strand replication has been completed before the "lagging" strand replication starts, *i.e.* extreme strand asynchrony. How much this could be due to purification method artefacts as reported by Yang *et al.* (2002) is difficult to estimate, but the evident absence of a D-loop and the highly repetitive nature of the A+T rich control region suggest that some fundamental differences in the mechanisms are possible (Goddard and Wolstenholme, 1978; Lewis *et al.*, 1994).

Whatever the exact mechanism of mammalian mtDNA replication, it is putatively primed with extensive RNA transcripts derived from the light-strand promoter (LSP), which is located directly upstream of O_H (reviewed by Clayton, 1991; Shadel and Clayton, 1997; Lee and Clayton, 1998). RNase MRP recognizes and cleaves RNA-DNA hybrid (the R-loop) in a region that precisely matches the major observed RNA to DNA transition sites in the D-loop area, and is most likely providing the free 3´-end for the priming of replication (Tapper and Clayton, 1981; Lee and Clayton, 1998). Similarly, an RNA-primed mechanism has been suggested for the initiation of L-strand replication at a conserved stem loop structure at O_L (Hixson *et al.*, 1986).

The enzymatic machinery that replicates mtDNA has been characterized to some extent in human and mouse, and quite extensively in *Drosophila*. DNA polymerase gamma (pol γ) is a heterodimer consisting of a catalytic (pol γ-α) and accessory (pol γ-β) subunits and is believed to be the only DNA polymerase active in mitochondria (Bolden *et al.*, 1977; Wernette and Kaguni, 1986; Kaguni and Olson, 1989; Lewis *et al.*, 1996; Ropp and

Copeland, 1996; Wang *et al.,* 1997; Carrodeguas and Bogenhagen, 2000). The pol γ-α is related to Pol I of *E. coli* and to phage T7 DNA polymerase, and contains both polymerase and proofreading activities (Lewis *et al.,* 1996). In addition, pol γ-α has potential function in mtDNA repair, since it is active in base excision repair of abasic sites of DNA (Olson and Kaguni, 1992; Longley *et al.,* 1998; Pinz and Bogenhagen, 2000). pol γ-β is related to prokaryotic aminoacyl-tRNA synthetases, serves as a processivity factor increasing the catalytic efficiency of the pol γ-α *in vitro*, and might have an additional role in primer recognition during mtDNA replication (Olson *et al.,* 1995; Fan *et al.,* 1999; Wang and Kaguni, 1999).

Null mutations in the *Drosophila* gene encoding the catalytic subunit pol γ-α (*tamas*), result in a defect of late larval locomotory behaviour followed by prepupal death, and are associated with aberrations of the visual system and disruption of the mitochondrial distribution pattern in the central nervous system (Iyengar *et al.,* 1999). Over expression of pol γ-α in transgenic flies results in late pupal lethality, and is characterised by severely reduced mtDNA copy number and cuticular defects including altered numbers of macrochaetae (Lefai *et al.,* 2000a). However, over expression of the same gene to a more or less similar extent in *Drosophila* Schneider cells does not cause any obvious functional defects or decrease in mtDNA levels (Lefai *et al.,* 2000a). This serves as a good example of fundamental differences that may exist between cell-culture models and the whole organism with complex developmental requirements. Similarly, over expression of human wild type pol γ-α (*POLG*) in cultured human cells causes no alterations in mitochondrial function or mtDNA, although dominant negative polymerase deficient mutants cause mtDNA depletion (Spelbrink *et al.,* 2000; Jazayeri *et al.,* 2003). Null mutations and substitutions of conserved amino-acids in the *Drosophila* gene for the accessory subunit, pol γ-β, result in reduced cell proliferation in the central nervous system followed by lethality during early pupation. This is associated with aberrant mitochondrial morphology and loss of mtDNA (Iyengar *et al.,* 2002). In contrast to pol γ-α null mutants, pol γ-β mutants show only mariginal effects on locomotory behaviour as well as later lethality, which might be explained either by differential temporal expression patterns of the two genes during development (Lefai *et al.,* 2000b) or by differences in relative maternal contribution in tissues that are critical for viability, as discussed by Iyengar *et al.,* 2002.

Both polymerase and proofreading activities of pol γ, as well as the rate of initiation of DNA strands, are further stimulated by mitochondrial single-stranded DNA-binding protein (mtSSB) *in vitro* (Thommes *et al.,* 1995; Farr *et al.,* 1999). mtSSB is encoded in *Drosophila* by the gene *lopo* (*low power*, Maier *et al.*, 2001) and shows similar properties to *E. coli* SSB, which functions in bacterial DNA replication by stabilizing the displaced single-stranded segment of DNA during passage of the replication fork and enhancing the catalytic activity of the polymerase. Insertion of a transposable element into the mtSSB gene, producing essentially a null mutant, results in a drastic decrease in mtDNA copy number, loss of respiration capacity, late larval lethality and aberrations of the visual system similar to pol γ-α mutants. Unlike these, however, it shows no alterations in the number or structure of mitochondria (Maier *et al.,* 2001).

Other proteins required for mtDNA replication and maintenance include Twinkle (Spelbrink *et al.,* 2001), a helicase that shows similarity to the bacteriophage T7 primase/helicase gene 4, and is which associated with the human mitochondrial disorder adPEO (autosomal dominant progressive external ophthalmoplegia) characterised by multiple mtDNA deletions. Additionally, topoisomerases are likely to play roles in mtDNA transactions (Zhang *et al.,* 2001; Wang *et al.,* 2002).

mtDNA is organised in mitochondria as protein-DNA complexes called nucleoids which at least in yeast, and possibly in mammalian cells, seem to be attached to the mitochondrial inner membrane and are suggested to be the structural units of mtDNA inheritance (Berger and Yaffe, 2000; Jacobs *et al.,* 2000; Lehtinen *et al.,* 2000; MacAlpine *et al.,* 2000; Garrido *et al.,* 2003). Nucleoid structures, mostly identified in yeast, contain 2-4 copies of mtDNA and approximately 20 polypeptides and recently it has been shown that in mammalian cell lines at least Twinkle, mitochondrial transcription factor A (TFAM - see section 2.1.4 for more detailed discussion of its role in gene expression) and mtSSB colocalize with mtDNA in punctate structures (Miyakawa *et al.,* 1987; Spelbrink *et al.,* 2001; Garrido *et al.,* 2003). TFAM in mammalian cell lines and placenta is abundant enough to cover all of the mtDNA and, indeed, mtDNA is far from naked (Takamatsu *et al.,* 2002; Alam *et al.,* 2003). Since essentially all TFAM is associated with mtDNA it seems likely that the stability of both mtDNA and TFAM are dependent on each other (Alam *et al.,* 2003; Garrido *et al.,* 2003). Supporting this view, TFAM levels seem to be severely reduced in mtDNA-less (*rho*^{0}) cell lines whereas mtSSB is not, although its

punctate localization disappears (Garrido *et al.*, 2003). In addition, the yeast counterpart of TFAM (Abf2p) is required for mtDNA maintenance (Diffley and Stillman, 1992; Zelenaya-Troitskaya *et al.*, 1998) but probably not for gene expression (Lightowlers *et al.*, 1997), and can be complemented by TFAM (Parisi *et al.*, 1993) or by the bacterial, histone-like protein HU (Megraw and Chae, 1993).

2.1.4 Mitochondrial transcription

The mitochondrial genome in mammals is transcribed as long polycistronic pre-mRNAs from three different promoters (Montoya *et al.*, 1982; Montoya *et al.*, 1983). The H-strand contains two promoters (Figure 2.2), HSP_R for the transcription of the two rRNAs and the tRNAs for phenylalanine and valine, and HSP_T for the transcription of all genes in the H-strand except tRNA for phenylalanine. The L-strand contains only one promoter (LSP) from which all eight tRNAs and a single ORF (ND6) of the L-strand are transcribed (Figure 2.2). A transcript generated from the LSP is also proposed to prime mtDNA replication, functionally coupling mitochondrial gene expression with genome maintenance (Chang and Clayton, 1985; Chang *et al.*, 1985; Shadel and Clayton, 1997) (see also 2.1.3). In *Drosophila*, RNA end-mapping has suggested many putative transcription start sites, and it is possible that in this organism all the 'blocks' of genes are transcribed from their own promoters (Berthier *et al.*, 1986).

Mammalian mitochondrial genes are transcribed by mitochondrial RNA polymerase (POLRMT), which shows similarity to T3/T7 phage-like RNA polymerases (Tiranti *et al.*, 1997). Initiation of transcription requires also mitochondrial transcription factor A (TFAM, also known as mtTFA), and mitochondrial transcription factors B1 (TFB1M, also known as mtTFB) and/or B2 (TFB2M), all of which interact directly with POLRMT (Fisher and Clayton, 1985; Parisi and Clayton, 1991; Falkenberg *et al.*, 2002; McCulloch *et al.*, 2002). TFAM is a high mobility group (HMG) box protein that exhibits low sequence specificity (Parisi and Clayton, 1991), and is required both in transcription and mtDNA maintenance, and is essential for embryogenesis in mice (Parisi and Clayton, 1991; Larsson *et al.*, 1998). However, it binds with high affinity to upstream elements of both HSP and LSP *in vitro*, where it facilitates specific transcription initiation indicating a role in proper promoter recognition and recruitment of POLRMT (Fisher and Clayton, 1985). When over expressed in tissue-culture or imported into isolated mitochondria, TFAM is able to

enhance the expression of H-strand transcript levels (12S rRNA and COI) as well as stimulate the nascent H-strand (7S DNA) synthesis from the LSP (Montoya *et al.,* 1997; Gensler *et al.,* 2001). The *Drosophila* homologue of TFAM has been studied by RNA interference (RNAi) in cell culture, which showed that 95% reduction of TFAM protein levels deplete mtDNA to less than half of the controls with only mariginal effects on mitochondrial transcription (Goto *et al.,* 2001). This might not be conclusive for the *in vivo* situation, because of differences with respect to developmental requirements of the whole organism, as discussed earlier (Lefai *et al.,* 2000a). In cell culture models, it is possible that TFAM can be subjected to substantial down regulation without any obvious effect on mitochondrial transcription, since it seems to exist in excess for what is required for these processes (Takamatsu *et al.,* 2002; Alam *et al.,* 2003).

TFB1M and TFB2M further promote mitochondrial transcription *in vitro* from both HSP and LSP, TFB2M being at least ten times more active, which might partly account for flexible regulation of mtDNA expression (Falkenberg *et al.,* 2002). These proteins are homologous to bacterial rRNA dimethyltransferases, and at least TFB1M, showing higher homology, appears to be a dual-function protein which can methylate bacterial small subunit (SSU) rRNA in a conserved stem loop structure that seems to be also partially methylated in mitochondrial SSU rRNA (McCulloch *et al.,* 2002; Seidel-Rogol *et al.,* 2003). Therefore, these genes have been probably recruited to mitochondrial transcription during evolution, and since homologues for both TFB1M and TFB2M can be found in mouse and *Drosophila*, but only one in *C. elegans*, a gene duplication event during early metazoan evolution is the most plausible explanation (Rantanen *et al.,* 2003).

Reduced levels of TFAM are found in muscle fibers of patients with mtDNA depletion and in *rho*^{0}-cell lines lacking mtDNA (Larsson *et al.,* 1998). Ethidium bromide (EtBr) is a lipophilic cation that accumulates into mitochondria, intercalates into mtDNA and prevents its transcription and replication. It can be utilised in tissue culture media to produce stabile *rho*^{0}-cell lines, or in more temporary mtDNA depletion-repletion experiments. In HeLa cells subjected to and recovering from EtBr treatment, TFAM and POLRMT proteins exhibit similar depletion-repletion profiles suggesting that mitochondrial transcription machinery is co-ordinately regulated in response to changes in mtDNA copy number, and that this control is most likely post-transcriptional (Seidel-Rogol and Shadel, 2002). Currently it is not known if TFB1M and TFB2M expression follows similar patterns, but it

is clear that not all factors required for the maintenance of the mitochondrial genetic system are co-regulated, since transcription and translation of human pol-γ is unaffected by changes in level or even by the total loss of mtDNA (Davis *et al.,* 1996). As discussed before (see 2.1.3), in addition to its importance in transcription and transcription-mediated replication TFAM, but not pol-γ, is also proposed to function as an mtDNA packaging protein (Alam *et al.,* 2003; Garrido *et al.,* 2003).

Premature termination of the pre-rRNA transcript starting from HSP_R is believed to be brought about in mammals by mitochondrial transcription termination factor mTERF (Kruse *et al.,* 1989; Daga *et al.,* 1993), which binds mammalian mtDNA at the site within the gene for $tRNA^{Leu(UUR)}$ (Christianson and Clayton, 1988). In *Drosophila*, a putative counterpart of mTERF (DmTTF) has recently been characterised (Roberti *et al.,* 2003), but the conserved sequence corresponding to the human mTERF binding site in *Drosophila* is not occupied by DmTTF. Instead, the latter binds specifically homologous, non-coding sequences at the ends of the convergent gene units of each strand, indicated as asterisks in Figure 2.2 (Roberti *et al.,* 2003). These sites coincide with regions previously suggested to be transcription termination sites by RNA mapping (Berthier *et al.,* 1986). Only one transcription termination site in each strand would imply that, unlike in mammals, the rRNA transcript would have to be produced by post-transcriptional processing in *Drosophila*. The experiments carried out thus far do not exclude the possibility that mtDNA transcription in *Drosophila* is initiated and/or attenuated in several positions, but points out possible differences in the regulation of rRNA versus mRNA genes in this organism compared to mammals (Berthier *et al.,* 1986).

In mammals, the steady-state levels of the rRNAs compared to H-strand mRNAs are increased 50-100 fold (Gelfand and Attardi, 1981). At the level of initiation, the two promoters of the H-strand seem to play a role in regulation of the relative abundance of mRNAs and rRNAs, the ratio of which has been shown to be modulated directly by thyroid hormone (Enriquez *et al.,* 1999). Also, changes in ATP levels might affect the preferential use of the two promoters, as observed *in vitro*, although the nature of the ATP-requiring step is unknown (Gaines *et al.,* 1987). Processing efficiency, differential transcript stability and changes at the level of termination frequency at the mTERF-dependent termination site have been proposed to have a role in controlling this process.

2.2 Mitochondrial translation system

Mitochondria have their own separate protein synthesis machinery for translation of the messages encoded by mtDNA. The RNA components of this apparatus are mtDNA derived, but all ribosomal proteins, translation factors, and aminoacyl-tRNA-synthetases must be imported into mitochondria, at least in animals.

2.2.1 Components of mitochondrial translation machinery

To get insight into mitochondrial protein synthesis it is helpful to first consider the analogous process in prokaryotes (reviewed by Brock *et al.,* 1998. See also Figure 2.3). The bacterial translation system contains three translation initiation factors, named IF1-IF3. IF3 works essentially as a recycling factor by binding to the free ribosomal small subunit (SSU) and by inhibiting its association with the large subunit (LSU) after translation termination. Prior to subsequent ribosomal re-association at a new initiation site, IF3 promotes the correct positioning of mRNA on the SSU by facilitating the codon-anticodon interactions between the start codon and the aminoacylated initiator tRNA (fMet-tRNA^{fmet}) at the peptidyl-site (P-site) of the ribosome. IF2 is a GTPase that binds the fMet-tRNA^{fmet} and brings it to the initiation complex. Its affinity for the ribosome is increased by IF1, which also occludes the A-site during the initiation, thus preventing other tRNAs to bind this position. In the classical *E. coli* model of the elongation cycle, the active GTP bound form of elongation factor (EF) -Tu interacts with aminoacylated tRNA (aa-tRNA) forming a ternary complex which promotes binding of the aa-tRNA to the aminoacyl site (A-site) of the mRNA-programmed ribosome. Once locked into the A-site by cognate codon-anticodon interactions (and other rRNA/protein -mediated contacts), the GTP in the ternary complex is hydrolysed, and EF-Tu is released from the ribosome as an EF-Tu·GDP complex which is recycled to the active form in a reaction catalysed by EF-Ts. Peptidyl transfer is catalysed by a reaction centre located in the LSU and a GTPase EF-G is required for translocation of the peptidyl-tRNA from the ribosomal A-site to the P-site, during the elongation cycle. (Brock *et al.,* 1998). Four factors are involved in termination of protein synthesis in bacteria, which are called release factors (RFs). RF1 and/or RF2 are responsible for recognition of stop codons, a process that is stimulated by RF3, which is a GTPase (RF1/2-RF3·GTP complex). Ribosome recycling factor (RRF), in combination with EF-G, is essential for the release of the ribosome from the mRNA at the stop codon.

The precise mechanism of this process is not currently understood, although it might mimic the translocation process (Kim *et al.*, 2000).

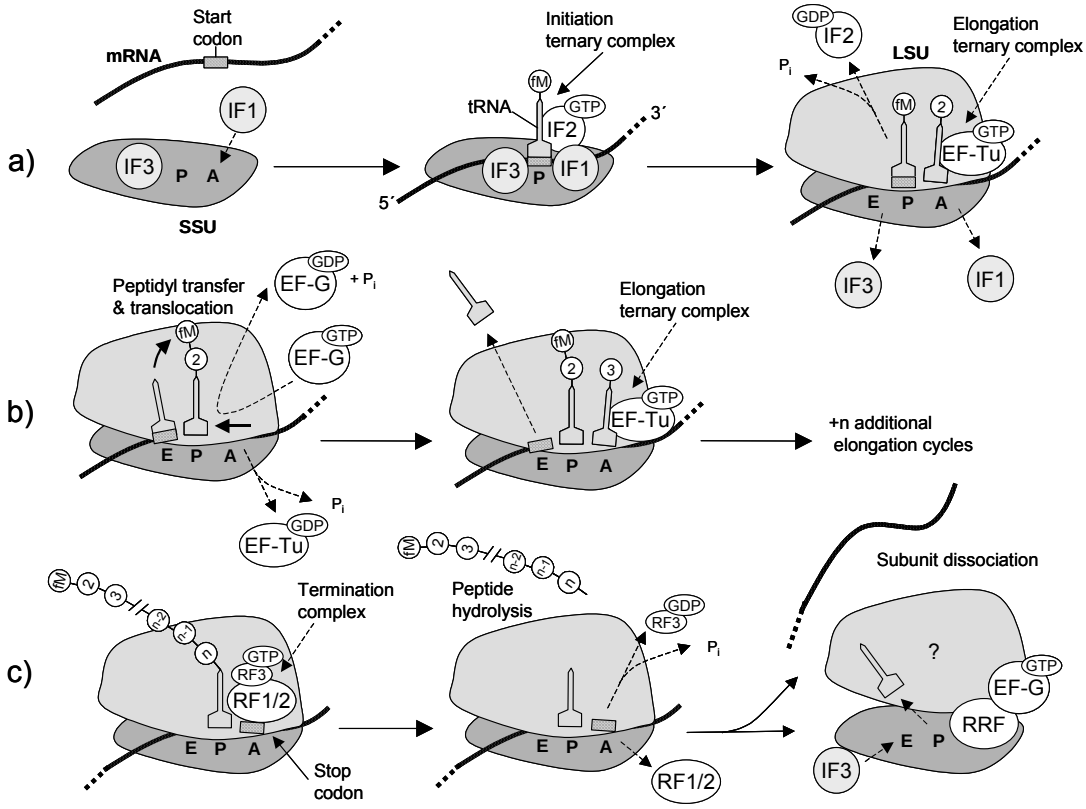


Figure 2.3. Schematic presentation of bacterial translation. a) Initiation. The mRNA and the initiation ternary complex bind to the IF3-bound ribosomal small subunit in random order, and IF1 occupies the A-site. The large ribosomal subunit can now bind the 30S initiation complex to form the 70S initiation complex. Concomitantly, IF1 and IF3 are ejected, and IF2-bound GTP is hydrolysed. The unoccupied A-site can now bind the first elongation ternary complex. b) Elongation. The EF-Tu-GTP is hydrolysed and its dissociation causes conformational change that leads the initiator tRNA in the P-site to form a peptide bond with an A-site aa-tRNA in a reaction catalysed by the peptidyl transferase center. The A-site is concomitantly occupied by EF-G-GTP, hydrolysis of which promotes translocation of the A-site peptidyl-tRNA to the P-site. Initiator tRNA is ejected via the E-site, and another elongation cycle can start. EF-Tu is reactivated in a reaction catalysed by EF-Ts, and EF-G is reactivated by autocatalysis (not shown). c) Termination. When a termination codon is exposed in the A-site, the RF1/2-RF3 complex recognises it. Hydrolysis of the RF3-bound GTP causes this complex to dissociate and the translated polypeptide to be released. Subsequent binding of ribosome release factor causes mRNA and tRNA to be ejected and results in dissociation of the ribosomal subunits. The exact mechanism of this process is not properly understood, but it seems to be EF-G assisted. The small ribosomal subunit is subsequently bound by IF3, which prevents premature re-association with the large subunit. See text for more details.

Mitochondrial orthologues of both IF2 and IF3, but not IF1, have been studied in mammals (Liao and Spremulli, 1991; Ma and Spremulli, 1996; Koc and Spremulli, 2002), as well as mitochondrial elongation factors EF-Tu and EF-Ts, and EF-G (Schwartzbach *et al.*, 1996; Cai *et al.*, 2000; Karring *et al.*, 2002). RF1 have been identified in rat and human mitochondria, as well as RRF in humans (Lee *et al.*, 1987; Zhang and Spremulli, 1998). Despite the differences with respect to kinetic properties or apparent lack of homologues of

some factors operating in prokaryotes, the mitochondrial translation system seems to be mechanistically closely related to the bacterial one.

The 13 polypeptides of the mtDNA are translated from nine monocistronic and two dicistronic mRNAs. None of these mRNAs possesses significant 5´- or 3´-untranslated regions (UTRs) (Montoya *et al.,* 1981; Ojala *et al.,* 1981). How then, are mitochondrial mRNAs recognised by the translation initiation complex? Eukaryotic cytoplasmic ribosomes use a 5´-cap binding and scanning mechanism, and usually contain a conserved Kozak sequence in the translation start site (Kozak, 1987; Kozak, 1999). In prokaryotes, ribosomes recognise the start codon using the Shine/Dalgarno (SD) interaction between the mRNA and the SSU rRNA. The SD region of the mRNA (upstream of the initiation codon) base pairs with the 3´ end of the 16S rRNA, helping to position the small ribosomal subunit in relation to the initiation codon (Shine and Dalgarno, 1975). Since mitochondrial mRNAs are not 5´-capped and do not contain SD-like sequences, it is unlikely that sequence-directed base pairing between SSU rRNA and mRNA is involved in the initiation process in the organelle. It is possible, however, that functional homologues of the SD sequence are located internally to mitochondrial mRNAs and have been therefore overlooked. Mitochondrial ribosomal SSUs have an intrinsic ability to bind mRNAs *in vitro* even in the absence of start codon and initiation factors, and this interaction is affected by the length of the mRNA but does not require a free 5´-end, as evidenced by efficient binding of circularised mRNA (Liao and Spremulli, 1989; Liao and Spremulli, 1990; Farwell *et al.,* 1996). It has been suggested that sequence-independent binding is an early step of translation initiation. IFs may facilitate the re-localisation of the bound SSU on the mRNA, and the initiation site could be stabilized by recognition of the start codon by initiator tRNA using codon-anticodon interaction (Farwell *et al.,* 1996). This is supported by the fact that the addition of mitochondrial extract stimulates the 5´-specific protection of mRNAs (Denslow *et al.,* 1989). In fact, this mode of initiation would not be dedicated to mitochondria only, since recent data in *E. coli* suggest that translation of leaderless mRNAs is dependent on IF2 and IF3, and that recognition of the initiation codon is independently achieved by the ribosome-IF2-fmet-tRNA^{fmet} complex (Moll *et al.,* 2002). A problem in initiation has been suggested as a possible pathological mechanism resulting from the aminoacylation defect in A3243G-mutated tRNA$^{Leu\ (UUR)}$, since cell lines carrying this heteroplasmic mutation show decreased association of mRNA with mitochondrial ribosomes (Chomyn *et al.,* 2000).

Mitochondrial translation initiation might also be affected by direct interaction with the inner membrane, since mitoribosomes are largely associated with IM both in yeast and bovine (Spithill *et al.*, 1978; Liu and Spremulli, 2000). This might relate to the fact that all mtDNA-encoded polypeptides are hydrophobic membrane proteins, which need to be assembled into IM to form ETC complexes. It is not known if insertion into the membrane in animals is a post-translational or co-translational process, but in yeast, examples of both have been described (Fox, 1996). In yeast mRNAs are activated for translation by mRNA-specific protein complexes that bind the 5´-UTRs and direct the message to the vicinity of the IM. Although yeast is an organism in which most significant progress have been made in understanding translational activation (Fox, 1996), the mechanism of initiation is likely to be different in mammals due to the lack of mRNA leader sequences.

2.2.2 Mitochondrial ribosomes

Ribosomes are ubiquitous ribonucleoprotein particles that are responsible for the fundamental process of protein synthesis. Combinations of X-ray crystallography and cryo-electron microscopy have recently produced detailed structures of both of the ribosomal subunits of *Thermus thermophilus* and *E. coli*, sometimes associated with tRNAs, mRNA, translation factors or antibiotics (reviewed by Ramakrishnan, 2002; Gao *et al.*, 2003). The composition and properties of mitochondrial 55-60S ribosomes (mitoribosomes) have mostly been studied using bovine and rat tissues (O'Brien and Kalf, 1967; Denslow and O'Brien, 1984; Koc *et al.*, 2000; Koc *et al.*, 2001a; Koc *et al.*, 2001b; Patel *et al.*, 2001; Suzuki *et al.*, 2001a; O'Brien, 2002). In contrast to cytoplasmic (80S) ribosomes, mitoribosomes seem to be more “bacterial (70S)-like” with respect to sensitivity to some antibiotics, which can be rationalized in the light of their endosymbiotic origin. However, the size of the rRNAs compared to those of bacteria has been reduced in mitochondria, and this reduction has apparently been compensated by replacing the lost RNA segments with additional mitoribosomal proteins, or by enlarging the proteins in order to maintain the structural and/or functional integrity of the ribosome (Suzuki *et al.*, 2001b; O'Brien, 2002). More than 80 mitoribosomal proteins have been found using a proteomic approach from humans, but only a few of them have been characterised in detail. Like many mitochondrial transcription and replication factors, some of these extra proteins appear to have been recruited to mitochondrial translation from some other

cellular duties in the course of evolution, and might still be bifunctional (Spirina *et al.*, 2000; O'Brien, 2002). A good example of this is MRP-S29, also known as death associated protein 3 (DAP-3), which is a pro-apoptotic protein and mediates interferon- and tumor necrosis factor mediated cell death (Kissil *et al*., 1999). Structural analysis suggests that it is a GTP-binding protein, and it possesses a putative phosphorylation site (Koc *et al*., 2001c). Interestingly, mammalian mitochondrial ribosomes bind GDP and GTP, which makes this protein a likely candidate for this action. Mutants deficient for the yeast orthologue of DAP-3 are defective in the maintenance of mtDNA (Berger *et al*., 2000), suggesting loss of mitochondrial translational capacity (Fox, 1996). It is therefore possible, that mitochondrial translation machinery serves as a component of cellular apoptotic signalling pathways.

The rRNAs from bacteria and mitochondria show little primary sequence conservation but major secondary structures have been preserved between bacterial and mitochondrial ribosomes, as well as the structures of functionally important proteins in the ribosomal accuracy centre, involved in the maintenance of faithful protein synthesis (Brakier-Gingras and Phoenix, 1984; Alksne *et al.,* 1993).

2.2.3 Mitoribosomal protein S12

Ribosomal protein S12 (S12) in *E. coli* is encoded by the *str* operon gene *rpsL* (Funatsu *et al.,* 1977; Post and Nomura, 1980). Various organellar S12 proteins are closely related to *E. coli* S12 and to their homologues in other organelles, but only distantly related to their isologues in cytosolic ribosomes (Shah *et al.,* 1997; Johnson *et al.,* 1998). The organellar S12 orthologues in plants and protists are usually mitochondrial or plastid-encoded, and an amino-terminal targeting sequence is required for the delivery of the (nuclear-coded) metazoan S12 orthologues to mitochondria (Shah *et al.,* 1997). There is substantial variation in the size of the relevant mRNAs in different animal species studied, mainly because of differences in the length of the 5´-UTR. In humans (MRPS12) and flies (*tko*) this region is long (>300 bp), and in humans it contains an upstream open reading frame (the uORF) indicative of translational regulation (see section 2.2.4, Shah *et al.*, 1997). In addition, the promoter region of the human MRPS12 gene seems to contain binding sites for transcriptional regulatory factors NRF-1 and NRF-2 (Johnson *et al.,* 1998). None of these putative regulatory elements have been functionally elucidated.

2.2.3.1 S12 in bacteria and its role in translational fidelity

Bacterial S12 is an important component of the ribosomal decoding centre, which seems to be structurally and functionally conserved over 2 billion years of evolution (Alksne *et al.,* 1993). The decoding centre consists of conserved domains of SSU rRNA, such as helix 44, helix 27 and the 530 loop (*E. coli* nomenclature), plus proteins S4, S5 and most notably S12. From a structural point of view, S12 belongs to the class of OB-fold proteins (Murzin, 1993) containing a five stranded β-barrel, a motif that is shared with many functionally unrelated single stranded RNA and DNA binding proteins (Brodersen *et al.,* 2002). The core C-terminus of S12 is located at the interface of the small and large ribosomal subunits, which is almost devoid of other proteins. Conserved residues of S12 interact with and stabilise bases of rRNA (G530, A1492 and A1493 in the *E. coli* numbering) involved in recognition of codon-anticodon base pairing when aa-tRNA binds to the ribosomal A-site (Ogle *et al.,* 2001; Ogle *et al.,* 2003). S12 also interacts directly with EF-Tu bound aa-tRNA in the A-site, which has been suggested to stabilize the binding of the ternary complex (Stark *et al.,* 2002). This contact is most likely broken as the aa-tRNA moves into the peptidyl transferase center in the 50S subunit. S12 can be also crosslinked to IF1 of *E. coli* and may therefore interact with it in the initiation complex (Boileau *et al.,* 1983). The unusually long N-terminal tail of S12 traverses the body of the SSU, contacting proteins S8 and S17 on the other side. This extension is sandwiched between two domains of the mature SSU, which implies that these domains cannot be packed against each other before S12 interaction. Therefore, S12 might have an additional role in assembly and stabilisation of the SSU structure (Brodersen *et al.,* 2002), although S12 mutations affecting assembly have not been previously described in any organism.

Mutations in the rRNA or protein components of decoding centre can result in increased or decreased translational fidelity, and hence alter the ribosomal susceptibility to agents that cause translational misreading, such as streptomycin (SM) (Brakier-Gingras and Phoenix, 1984; Kurland and Ehrenberg, 1984). Mutations found either from clinical samples or created intentionally under SM selection have been reported in S12 that result in SM resistance (SM^R) or dependence (SM^D) (Funatsu and Wittmann, 1972; van Acken, 1975; Timms *et al.,* 1992; Finken *et al.,* 1993; Timms and Bridges, 1993; Meier *et al.,* 1994; Timms *et al.,* 1995; Sreevatsan *et al.,* 1996; Inaoka *et al.,* 2001). Generally these mutants show increased accuracy of translation and are sometimes associated with decreased

ribosomal elongation rate (restrictive mutations, Bilgin *et al.*, 1992). Two conserved regions of S12 that are affected by mutations locate close to the A-site, namely amino acids 41-42 and 85-91 in *E. coli* numbering. SM^R can also result from conserved SSU rRNA mutations in bacteria or chloroplasts (Montandon *et al.,* 1985; Honore *et al.,* 1995; Sreevatsan *et al.,* 1996; Springer *et al.,* 2001). Animals are not normally particularly sensitive to streptomycin, and all animal and fungal species studied this far carry glutamine in mitochondrial S12 at the residue corresponding to conserved lysine 87 (K87Q) in *E. coli* (Shah *et al.*, 1997). The analogous mutation has been reported in SM^R *Mycobacteria*, although it occurs together with a SSU rRNA mutation (Meier *et al.,* 1994). This raises the question as to whether K87Q can confer SM^R in its own right, and if so, could it play a part in the natural SM resistance of mitochondria (Shah *et al.,* 1997)?

Mutations in other residues of the SSU rRNA and in proteins S4 and S5 of the ribosomal accuracy centre can cause ambiguity in ribosomal decoding (*ram*). These mutants show relaxed accuracy of translation and are characterised by suppression of nonsense codons both in bacteria and yeast (Andersson *et al.,* 1986; Shen and Fox, 1989; Alksne and Warner, 1993; Anthony and Liebman, 1995; O'Connor *et al.,* 1997). *ram* strains show increased sensitivity to SM and can be counteracted by restrictive mutations in S12. Recent structural data (Ogle *et al.,* 2003) implies that, although located in the opposite side of the subunit, both *ram* mutations and SM facilitate the rotation of the ribosomal "shoulder" domain that takes place during the decoding process. This "domain closure" is a major rearrangement necessary for tRNA selection, and by reducing the energetic cost of the process *ram* and SM will facilitate the acceptance of near-cognate (*i.e.* wrong) tRNAs by the ribosome. In contrast, restrictive S12 mutations increase the activation barrier of the rearrangement, hence favouring the selection for cognate tRNAs (Ogle *et al*., 2003). This interpretation is supported by the fact that S12 mutations decrease GTP hydrolysis by Ef-Tu and increase accuracy during initial selection and proofreading (Bilgin *et al.,* 1992).

The *str* operon in *E. coli* also encodes three other proteins of the translational machinery, namely ribosomal protein S7, EF-G and EF-Tu. Normally *E. coli* maintains roughly equal amounts of rRNA and ribosomal proteins, and ribosomal operons show feedback inhibition at the translational level to prevent pools of free ribosomal proteins from accumulating, as reported for example in the case of S4, S7 and S8 (Nomura *et al.,* 1980). This control is based on structural similarities between the autoregulated mRNA and the binding site of

the corresponding ribosomal protein on the small subunit rRNA. Although there is no evidence of binding of S12 to its own mRNA, it has been reported that several ribosomal proteins, most prominently S12, facilitate *in vitro* and *in vivo* splicing of the T4 phage *td* intron and enhance the activity of the hammerhead ribozyme (Coetzee *et al.*, 1994; Semrad and Schroeder, 1998). As an RNA-binding factor, it is conceivable that prolonged, high-level over-expression of S12 could be deleterious to the cell via non-specific binding to undefined RNA species. Alternatively, this could interfere with ribosome assembly by changing the stoichiometry of the interplayers.

2.2.3.2 technical knockout (tko)

The orthologue of S12 in *Drosophila* is encoded by the X-linked gene *tko* (*technical knockout*). The gene name is based on the phenotype found in screens for behaviour after induced mutagenesis (Judd *et al.*, 1972; Shannon *et al.*, 1972; Royden *et al.*, 1987). The only viable allele described thus far is tko^{25t} (Judd *et al.*, 1972; Royden *et al.*, 1987; Shah *et al.*, 1997), which falls into the category of "bang sensitive" (BS) mutants (listed in Table 2.1, see also 2.4.1) (Ganetzky and Wu 1982; Engel and Wu 1994; Pavlidis and Tanoye 1995; Kuebler *et al.*, 2001; Lee and Wu, 2002). tko^{25t} is recessive, and shows lethality in female hemizygotes, *i.e.* in heterozygous condition with deficiencies of (large deletions affecting) the locus (Judd *et al.*, 1972). Homozygous flies are described as semi-lethal, and show slender bristle phenotype associated with altered mechanosensory function in response to bristle deflection (Judd *et al.*, 1972; Shannon *et al.*, 1972; Engel and Wu, 1994). Royden *et al.*, (1987) have shown by transgenesis that tko^{25t} can be rescued by a genomic fragment containing the gene for mitoribosomal protein S12. However, the nature of the mutation was not identified by this study. Shah and co-workers showed later that the tko^{25t} allele contains a mutation converting leucine at position 85 (56 in *E. coli* numbering) to histidine (L85H). High conservation of this residue suggests that it is responsible for the phenotype, but formal proof is still lacking because an effect of the mutations, *e.g.* outside the coding regions, could not be excluded. In any case, no difference was observed in the mutant strain in the expression of the *tko* gene at the mRNA level or in the gross arrangement of the genomic region, when mutant and wild-type flies were compared (Shah *et al.*, 1997).

When BS flies are observed after physical concussion they exhibit a stereotyped sequence of behavioural abnomaly, initially muscle spasm (uncoordinated movements), followed by paralysis (lack of motion and responsiveness), delayed seizures during recovery (often combined with high frequency of wing flapping, leg shaking and abdominal muscle contraction), and finally full recovery (normal posture, Engel and Wu, 1994). After recovery there is a refractory period during which sensitivity to further such shocks is reduced. Wild-type flies are not significantly responsive to even the harshest physical jolt, such as vigorous vortexing in a vial. BS mutations occur in genes encoding a heterogeneous group of proteins (Table 2.1), only one of which in, addition to tko^{25t}, has been confirmed to encode a mitochondrial gene product. This gene, *sesB*, encodes one isoform of the mitochondrial adenine nucleotide translocase (ANT), which is located in the mitochondrial inner membrane and responsible for the import of ADP and export of ATP (Zhang *et al.,* 1999). Mutation $sesB^{1}$ results in an amino-acid change in the putative sixth transmembrane domain of ANT. It causes a temporary paralytic phenotype similar to tko^{25t}, associated with hypoactivity, conditional temperature sensitivity, developmental delay and reduced homozygous female viability (Zhang *et al.,* 1999).

GENE NAME	**PRODUCT**	**REFERENCE**
tko - technical knockout	Mitoribosomal protein S12	Royden *et al.,* 1987
sesA - stress sensitive A	unknown	Homyk *et al.*, 1980
sesB - stress sensitive B	Adenine nucleotide translocase	Zhang *et al*., 1999
sesD - stress sensitive D	unknown	Homyk *et al.*, 1980
bss - bang senseless	unknown	Pavlidis and Tanouye, 1995
eas - easily shocked	Ethanolamine kinase	Pavlidis *et al*., 1994
bas - bang sensitive	unknown	Pavlidis and Tanouye, 1995
Atpalpha	Na+ / K+ ATPase α-subunit	Palladino *et al.*, 2003
sda - slamdance	Aminopeptidase N	Zhang *et al*., 2002
jbug - jitterbug	Filamin	Ren and Tanouye, 2001
kdn - knockdown	unknown	Pavlidis and Tanouye, 1995
rex – rapid exhaustion	unknown, allelic to *kdn*?	Homyk and Pye, 1989

Table 2.1. Bang-sensitive mutants. Names of the BS genes, their products (if known) and most relevant (or recent) reference are shown. More detailed description of some of the BS mutants is given in text (see 2.4.1).

2.2.4 Co- regulation of nuclear and mitochondrial gene expression

Control of gene expression is achieved at various levels, such as transcription, post-transcriptional processing, mRNA stability, translation, post-translational modifications and protein stability. Little is known about physiological and developmental processes that regulate co-ordinated gene expression of the mitochondrial and the nuclear genomes. Because mitochondria are involved in important cellular and physiological processes, it is plausible to expect that at least some mitochondrially located nuclear genes need to be controlled in an orchestrated fashion. Many transcriptional regulatory elements have been shown, or suggested, to play roles in nucleo-mitochondrial communication, most notably nuclear respiratory factors NRF-1 and NRF2 (reviewed by Scarpulla, 2002), the OXBOX/REBOX system (Chung *et al.,* 1992) and Mt3/4 elements (Suzuki *et al.*, 1995). Genes possessing NRF-1/2 binding sites include those encoding many subunits of the ETC complexes, components of the mitochondrial transcription/replication machinery and genes involved in protein import and assembly pathways and intermediary metabolism (Scarpulla, 2002). As already mentioned, human mitoribosomal protein S12 has also putative NRF-1 and NRF-2 binding sites in its promoter (Johnson *et al.,* 1998). In addition, DNA replication-related elements have been shown to be important in the regulation of both nuclear and mitochondrial replication machineries in *Drosophila*, where they probably have a role in the regulation of at least Pol γ-β, mtSSB and TFAM (Wang *et al.,* 1997; Lefai *et al.,* 2000b; Ruiz De Mena *et al.,* 2000; Takata *et al.,* 2001; Takata *et al.,* 2003).

Alternative splicing and usage of alternative transcription start sites has been shown to be a major regulator of gene expression and generator of diversity from the relatively small number of genes in metazoan genomes, and they might have a role also as mitochondrial disease modifiers (Caceres and Kornblihtt, 2002; Ladd and Cooper, 2002; Nissim-Rafinia and Kerem, 2002). For example, many enzymes responsible for mtDNA repair are encoded by the same genes as their nuclear counterparts, and mitochondrial targeting sequences are generated through alternative splicing of mRNAs, alternative use of transcription initiation sites, or alternative use of translation initiation sites (Kang and Hamasaki, 2002). Similarly, human cytosolic and mitochondrial lysyl-tRNA synthetases are encoded by the same gene, and the mitochondrial version is produced by alternative splicing that results in exon inclusion and insertion of the mitochondrial targeting peptide (Tolkunova *et al.,* 2000).

Well-defined elements that regulate the translational 'potential' of many key mRNAs are 5´-terminal oligopyrimidine tracts (TOPs) and upstream open reading frames (uORFs). TOPs are present in mRNAs for many components of cytosolic translation machinery, most notably ribosomal proteins, and in addition to mammals they are found in many mRNAs in other vertebrates, and even in insects (reviewed by Meyuhas, 2000). Growth arrest in response to a wide variety of signals, such as contact inhibition or amino-acid/serum starvation, leads to selective repression of the translation of TOP mRNAs and is characterised by their shift from polysomes (complex of ribosomes bound to a single mRNA) into the subpolysomal fraction (mRNP particles). Conversely, growth stimuli cause efficient accumulation of these mRNAs to the actively translated polysomal fraction. A particularly complex example of a TOP-regulated protein in vertebrates is poly(A)-binding protein (PABP), that itself has been implicated in translational regulation, where it assists initiation of translation and controls mRNA stability. Furthermore, PABP is subject to autoregulation through an A-rich sequence in its 5´-UTR (Meyuhas, 2000, and references therein).

Probably close to 40 % of vertebrate mRNAs contain at least one uORF in their 5´-UTR, and large proportion of these messengers are rare and poorly translated at least in normal growth conditions (reviewed by Meijer and Thomas, 2002). uORFs generally encode putative peptides of 5 to 25 amino-acids and are recognised by the scanning initiation complex with varying efficiencies, depending on properties of the uORF itself, the regions surrounding it and the intercistronic length between the uORF and major ORF (Lovett and Rogers, 1999; Meijer and Thomas, 2002). In some cases, the uORF-encoded short peptide might directly interact in *cis* with translating ribosome and prevent further translation, for example by inhibiting the peptidyl transferase centre (Lovett and Rogers, 1999). However, this mechanism is probably specific to certain metabolic pathways, since uORF-encoded peptides are not generally conserved in their amino-acid sequence. Leaky scanning (ribosomes do not recognise uORF), or possibly re-initiation after translation of the peptide encoded by the uORF might result in translation of the downstream ORF (Morris and Geballe, 2000; Meijer and Thomas, 2002). A good example of these mechanisms is yeast transcription factor GCN4, which is involved in upregulation of a large group of amino-acid biosynthetic genes (Ruiz-Echevarria and Peltz, 2000; Dever, 2002; Meijer and Thomas, 2002). GCN4 contains a total of four uORFs in its 5'-leader, the first (uORF1)

and the last (uORF4) of which are functionally most important. During amino-acid starvation eukaryotic initiation factor eIF2a is phosphorylated, a mechanism that leads to reduced efficiency of ternary complex formation. Even in these conditions most of the ribosomes can translate the uORF1, then resume and continue scanning towards uORF4. In normal conditions ternary complex formation is efficient, and when scanning reaches uORF4 ribosomes will recognise and translate it, which results in disengagement of the ribosome from the GCN4 message without producing Gcn4p. Under starvation, however, the low levels of ternary complexes enable scanning to continue for longer distances without re-initiation of translation. Ribosomes then scan past the uORF4 and subsequently acquire the ternary complex in time to initiate at the GCN4 start codon, which results in production of Gcn4p and transcriptional upregulation of amino-acid biosynthetic genes. (Meijer and Thomas, 2002). Interestingly, one of these target genes in yeast is *ILV5*, the product of which can improve the mtDNA stability in lines lacking Abf2p, the yeast counterpart of TFAM (Zelenaya-Troitskaya *et al.*, 1995). It turns out that Ilv5p controls the organization of mtDNA in nucleoids (Kaufman *et al.*, 2000; MacAlpine *et al.*, 2000), and hence it can be said that regulation of mtDNA organization and transmission in yeast is indirectly under translational regulation by uORFs. In mice, uncoupling protein 2 (UCP2), which is expressed in a tissue-specific manner, is under both transcriptional and translational control, the latter being due to uORFs. Under oxidative stress conditions increase in UCP2 protein levels occurs without any change in UCP2 mRNA levels, which allows strong and rapid induction in tissues containing high, basal expression at mRNA level (Pecqueur *et al.*, 2001).

Through nonsense or frameshift mutations, up to 30% of mutant alleles contributing to human genetic disease harbour a premature termination signal (Mendell and Dietz, 2001). In many cases, premature termination codons in the coding sequence or nonsense codons in the 5´-UTR lead to nonsense-mediated mRNA decay, or mRNA surveillance, an mRNA quality control mechanism that is ubiquitous in eukaryotes (reviewed by Mendell and Dietz, 2001). The exact nature and location of this process are still under investigation, but both nuclear and cytosolic compartments have been suggested (Wilkinson and Shyu, 2002). In principle, uORF-containing mRNAs can be sensitive to this pathway, which could lead to mRNA degradation. Certain uORFs in yeast are followed by *cis*-acting stabilizer element which prevent the transcript from undergoing nonsense-mediated mRNA decay, such as in the case of GCN4 (Ruiz-Echevarria and Peltz, 2000). The *cis*-acting

stabilizer element is bound by the RNA-binding protein Pub1, mammalian homologues of which have been shown to play important roles in differentiation and proliferation, and which has been proposed to prevent rapid decapping and destruction of the bound mRNAs by the surveillance complex (Ruiz-Echevarria and Peltz, 2000).

2.3 Mitochondria and disease

Mitochondrial disease can be caused by mutations in nuclear DNA or in mtDNA, most of which target subunits of OXPHOS complexes directly or else indirectly, via genes involved in mitochondrial translation (reviewed by Smeitink *et al.,* 2001). Mitochondrial disorders can show Mendelian or matrilineal inheritance, are usually progressive, and have no known treatment. Nonsyndromic mitochondrial disorders affect only single organs, such as the eye or cochlea, whereas syndromic disorders involve multiple organ systems. Generally, mitochondrial disorders affect highly energy-dependent, post-mitotic tissues, such as the central nervous system (CNS) and skeletal and cardiac muscle, and are often characterised by exercise intolerance, seizures and sensorineural deafness. Typical examples of syndromic manifestations (reviewed by Schmiedel *et al.,* 2003) include MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes), MERRF (myoclonic epilepsy with ragged red fibers), LHON (Leber hereditary optic neuropathy), Leigh syndrome (infantile subacute necrotizing encephalopathy) and NARP (neuropathy, ataxia and pigmentary retinopathy). However, there is considerable clinical variability and many patients do not fit into one particular category. Over 100 different mtDNA point mutations and even more mtDNA deletions have been described since the first mutations associated with disease were described 15 years ago (Holt *et al.,* 1988; Wallace *et al.,* 1988a; Wallace *et al.,* 1988b). A search for genes causing "nuclear-mitochondrial disorders" is ongoing, and has revealed already many genes involved in OXPHOS, mtDNA maintenance, ETC assembly and protein import (Koehler *et al.,* 1999; reviewed by Zeviani, 2003). Based upon the available data, an estimate for the prevalence of all mitochondrial diseases is at least 1 in 8000 of the population, making them among the most common genetically determined diseases (reviewed by Chinnery and Turnbull, 2001). Additionally, mtDNA mutations and impairment of mitochondrial function has been implied also in cancer (reviewed by Copeland *et al.,* 2002; Tomlinson *et al*., 2002), diabetes mellitus (reviewed by Berdanier, 2001), many age-related neurodegenerative disorders, such as Parkinson's disease (reviewed by Sherer *et al.,* 2002), Alzheimer's

disease (reviewed by Castellani *et al.*, 2002), Huntignton's disease (reviewed by Ross, 2002), and the pathogenesis of Down's syndrome (reviewed by Arbuzova *et al.*, 2002).

2.3.1 Inheritance, heteroplasmy and segregation of mtDNA

The human mitochondrial genome is thought to be transmitted strictly from the mother to the offspring (Birky, 1995; Birky, 2001), although a single case of muscle-specific, paternal transmission has been recently reported (Schwartz and Vissing, 2002). Because mtDNA lacks sexual recombination and has approximately 10 times higher mutation frequency compared to nuclear genes it is in principle susceptible to the accumulation of deleterious mutations over generations, the so-called Muller's ratchet (Brown *et al.*, 1979; Bergstrom and Pritchard, 1998). Mutations in mtDNA can either be inherited or generated sporadically, and the presence of two or more kinds of mtDNA molecules in the individual, tissue or single-cell is called heteroplasmy. At the population level mtDNA is highly polymorphic, but this is not the case at the individual level. During early embryogenesis only a small proportion of the available mtDNA molecules repopulate the embryonic tissues. It is thought that this genetic "bottleneck" causes a heteroplasmic pool of mtDNAs to segregate rapidly. Genotype frequencies in shift greatly in a single transmission from mother to offspring (reviewed by Chinnery *et al.*, 2000). As a result most individuals are homoplasmic for a single species of mtDNA. Since mtDNA exists in thousands of copies per cell, the proportion of pathogenic mtDNA molecules needs to be high enough in order to cause dysfunction. This can result by unequal partitioning of mtDNA (vegetative segregation) in dividing cells, or by clonal expansion in non-dividing cells due to cell cycle-independent replication of mtDNA. Although homoplasmic pathogenic mutations exist, most disease causing mutations occur in the heteroplasmic state, implying that energy deficiency can result from absence of complementation by wild-type mtDNA. It is possible that oocytes containing mutated mtDNA are subjected to negative selection during oogenesis to eliminate cells with defective OXPHOS capacity (quality control), but this is not currently supported by available data (Chinnery *et al.*, 2000).

The segregating unit of mtDNA inheritance have been suggested to be a nucleoid (Jacobs *et al.*, 2000). In mouse models, evidence for both random genetic drift and highly tissue dependent directional selection for different, seemingly neutral (non-pathogenic) mtDNA genotypes in the same animal have been observed (Jenuth *et al.*, 1996; Jenuth *et al.*, 1997). Since genetic background has an effect on these processes, mtDNA segregation seems to

be under complex nuclear control (Battersby *et al.,* 2003). It is still a mystery how pathogenic mtDNAs are selected (or counter-selected) in the female human germ-line, or if the predominant mechanism is random genetic drift (Chinnery *et al.,* 2000).

2.3.2 Threshold effects

Clinical diagnosis and genetic counselling of mitochondrial disorders is difficult because the same mutation can result in different symptoms. Conversely, similar pathological states can be caused by different mutations. Both the nature of the mutation and the level of heteroplasmy affect the severity of the disease, but the relationship with severity is not necessarily linear (Morgan-Hughes *et al.,* 1995). Heteroplasmy levels can alter in various tissues of the same individual or even in different cells of the same tissue. Furthermore, the same level of heteroplasmy can result as dysfunction of some tissues but not in others. These differential phenotypic manifestations are explained by varying, tissue-specific tolerance levels for a particular mutation, *i.e*. threshold effects (reviewed by Rossignol *et al.,* 2003). Even homoplasmic (or nearly homoplasmic) mutations do not necessarily cause disease in all individuals carrying them, but might require involvement of environmental factors or alterations in nuclear, possibly tissue-specific genetic modifiers (Carelli *et al.,* 2003). Good examples of homoplasmic mtDNA mutations with variable penetrance are aminoglycoside induced deafness associated with the mitochondrial SSU rRNA mutation A1555G (Prezant *et al.,* 1993; Guan *et al.,* 2001) and Leber's hereditary optic neuropathy (LHON) most often associated with mtDNA-encoded genes for complex I subunits (reviewed by Man *et al.,* 2002), both of which are tissue-specific, affecting mainly hearing and vision, respectively.

Threshold effects have been analysed in more detail by studying consequences of heteroplasmy for OXPHOS capacity at the cellular level. For example, individual muscle fibers from patients exhibiting different clinical manifestations (*e.g.* MELAS, PEO, DM or Leigh syndrome) but the same mtDNA mutation (A3243G in $tRNA^{Leu(UUR)}$) have been studied by combined quantification of heteroplasmy levels and histochemical detection of cytochrome c oxidase (COX) activity. In these studies high mutant load was associated with COX negative fibers, and the differences in the somatic distribution of the mutation among tissues were suggested to be responsible for the differential phenotypic expression of the disease (Petruzzella *et al.,* 1994; Koga *et al.,* 2000). Secondly, cytoplasmic hybrid

(cybrid) technology (King and Attardi, 1989) has been used to transfer patient-derived mitochondria to cell-lines lacking mtDNA (*rho*^{0}) in order to produce cybrids with varying heteroplasmy levels. In combination, transmitochondrial studies indicate that both heteroplasmy level and nuclear background affect biochemical capacity of the cells (Attardi *et al.*, 1995; Koga *et al.*, 1995; Raha *et al.*, 1999; Vergani *et al.*, 1999). Finally, influences of heteroplasmy have been modelled by inhibiting OXPHOS complexes by titration with specific inhibitors. The studies carried out mainly in rat tissues have revealed biochemical threshold effects, *i.e.* the activity of ETC complexes can be decreased substantially without affecting respiration or ATP synthesis (Rossignol *et al.*, 1999). Comparable observations have been made in a *Drosophila subobscura* strain carrying partially deleted mtDNA, which manifests decreased activities of some ETC complexes (particularly complex I), but shows no effect on net ATP synthesis, nor obvious phenotype (Beziat *et al.*, 1997). However, the affected ETC complexes of these flies are more susceptible to inhibition, and the threshold level for complex I inhibition, where a large drop in respiration and ATP synthesis is observed is only 20% in the deletion mutant strain compared to 70% in wild-type flies (Farge *et al.*, 2002a). Whether or not the mutation directly affects on ETC subunit, or has a more general impact on production of mitochondrially encoded polypeptides, migh influence the levels of heteroplasmy that can be tolerated, since threshold effects can operate at the transcriptional or translational level or affect enzyme activity via kinetic or assembly defects (Rossignol *et al.*, 2003).

2.3.3 Animal models of mitochondrial disease

Whole animal models are crucial in order to understand complex pathophysiology, tissue specificity and developmental aspects of mitochondrial disorders, as well as segregation of mtDNA. Although cybrid technology has been useful in defining pathogenic effects of mtDNA mutations in cellular level, the research has been hindered by the lack of animal models with mtDNA mutations due to inaccessibility of mtDNA for targeted mutagenesis (reviewed by Jacobs, 2001). For this reason, animal modelling of mitochondrial disease has concentrated on manipulations of nuclearly encoded mitochondrial genes, some of which have been studied in knock-out mice, or alternatively in *Drosophila*. In *Drosophila* the studies have historically used conventional or “forward” genetics, *i.e.* from phenotype to genotype, whereas in mice, known mitochondrial genes have been subjected to (conditional) targeted disruption. Manipulation of mouse embryonic stem cells has

produced knockout lines of many nuclear encoded mitochondrial proteins, such as TFAM, adenine nucleotide translocase 1 (ANT1), uncoupling proteins, Mn superoxide dismutase and many genes involved in apoptotic pathways (reviewed by Wallace, 2002). Recently the very first transmitochondrial lines carrying either a partial deletion (Inoue *et al.*, 2000) or pathogenic point mutation (Sligh *et al.*, 2000) in mtDNA were described. These have proved that heteroplasmic point mutation producing symptoms of mitochondrial disease in mouse can be transferred through the female germline, and this approach has given a great promise for future research (Hirano, 2001).

2.3.4 Drosophila as a model organism

The question arises as to whether relatively simple invertebrates such as fruit-flies or worms (*Caenorhabditis elegans*) can serve as reasonable models for human disease. Animal experimentation in general is a trade-off between genetic power and biomedical relevance (Dow and Davies, 2003). To maximise biomedical relevance studies should be carried out using human patients or patient-derived tissues. Even if this is possible in some cases, genetic manipulation of humans is impossible for ethical reasons, and studies in most closely related vertebrates are hindered by technical problems. Major vertebrate models offering at least partial genomic sequence data, and in which classical genetics is possible, are rat (*Rattus rattus*), mouse (*Mus musculus*) and zebrafish (*Danio rerio*). The mouse, which is regarded generally as the most suitable model organism for human disorders, shares reasonably conserved physiology with humans and can be subjected to targeted gene disruption by homologous recombination. However, this is both slow and very expensive, and might therefore create restraints that could be avoided by using non-vertebrate models. Additionally, the life cycle of the mouse is far too long to perform genetic screens routinely (Dow and Davies, 2003).

Drosophila melanogaster has been under intensive investigation since its "domestication" by Thomas Hunt Morgan and his students in the early 20th century. This work started genetics as we know it today, and *Drosophila* was the first organism in which chromosomes were identified as the carriers of the hereditary material, with the genes arranged in linear order (Sturtevant, 1913; Bridges, 1914). It was also the first experimental animal in which ionizing radiation was shown to cause genetic damage, mutations (Muller, 1927). Today, the *Drosophila* genome is completely sequenced (Adams

et al., 2000) and the recent update (Release 3) has closed most remaining gaps in the euchromatic portion of the genome, as well as producing improved sequence quality and validating the assembly (Celniker *et al.*, 2002). Its small size, short life cycle (Figure 2.4) and high reproductive rate make the fruit fly a convenient experimental animal. Furthermore, the genetics of the organism is well described and modified, natural transposable elements and two-component systems can be utilised in transgenesis, and to bring about tissue specific expression of genes of interest (Rubin and Spradling, 1982; Spradling and Rubin, 1982; Brand and Perrimon, 1993; Brand *et al.*, 1994). Large collections of mutations, chromosomal deficiencies and P-element insertions are available, which provide valuable tools for molecular genetics (FlyBase, 2003). Recently, a method has been described that allows targeted disruption in *Drosophila* and this technique has been used successfully to target several loci (Rong and Golic, 2000; Rong and Golic, 2001; Rong, 2002; Rong *et al.*, 2002). In addition, functional knock-outs can be created by RNA-mediated interference (RNAi) techniques *in vivo* (Kennerdell and Carthew, 1998), even at the resolution of a single tissue, cell-type or a single transcript isoform (Lam and Thummel, 2000; Roignant *et al.*, 2003).

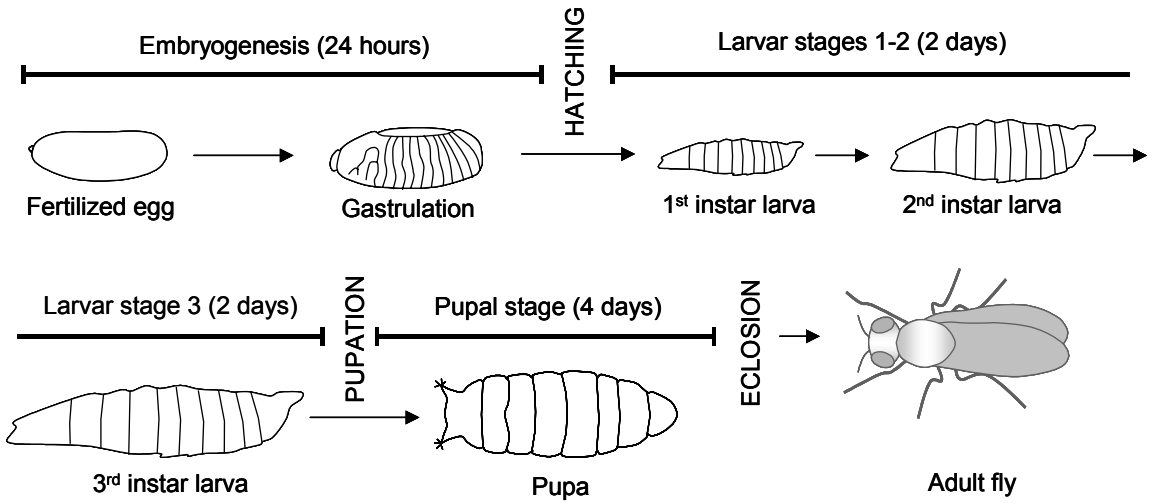


Figure 2.4. Life cycle and developmental stages of *Drosophila melanogaster* at 25° C. After fertilization, the eggs are deposited by females on the surface of the culture medium. A female can lay up to 100 eggs per day at her peak. Embryogenesis takes approximately one day, and is followed by emergence of the larva (hatching). The larva feeds on food substrate for approximately four days and passes through two molts of larval cuticulum during this time (larval instars 1-3). The first instar larva feeds on the surface of the medium, the second and third instar larvae burrow into the medium. At the end of third instar, the larva leaves the food substrate (wandering stage), finds an appropriate site to pupate, and develops an immobile pupal case. During the pupal stage, metamorphosis to the adult stage occurs, and approximately 9 days after egg laying, the adult imago escapes the pupal case (eclosion). Newly emerged flies are normally ready to mate and produce offspring within 12 hours of eclosion. For genetic crosses, segregation of males and females within the first 8 hours of adult life ensures that females are virgin. All developmental stages can be used for purposes of molecular biology and biochemistry. When larval stages are used, third instar larvae are commonly selected for their higher biomass and easy collection at the wandering stage. (Sullivan *et al*., 2000).

The *Drosophila melanogaster* genome is ~180 Mb in length (a third of which is centric heterochromatin) and encodes approximately 13 500 genes. Compared to humans (~ 3.2 Gb, approximately 30 000 genes) it is markedly smaller in size, but contains about half the number of genes and transcripts. Importantly, according to the Homophila database (Chien *et al.*, 2002), approximately 75% of the human disease gene associated sequences found in the OMIM database (Online Mendelian Inheritance in Man, (Hamosh *et al.*, 2002) have strong matches to one or more sequences in the *Drosophila* genome database (FlyBase, 2003). It is clear that at least insights to the basic mechanisms of human disease can be gained from studies in flies. Good examples of these are neurodegenerative diseases, such as Huntington's disease (HD) which is caused by expansions of a polyglutamine repeat found in the protein huntingtin, leading to neuronal loss and characterized by insoluble protein aggregates in brains (Ross, 2002; Rubinsztein, 2002). Even if mouse models have provided valuable data about molecular mechanisms underlying HD, the pathogenesis of the disease is not currently understood. A powerful approach to identifying pathways involved in HD pathology and possible protective strategies, is to perform suppressor screens to identify genes that alleviate or modify the disease, for instance by using P-element insertions in a *Drosophila* model (Kazemi-Esfarjani and Benzer, 2000).

Especially where a pathway shares a common evolutionary origin between different eukaryotic lineages, genetic dissection of its components, combined with comparative structural studies, can produce valuable data for understanding disease processes. For example, the insulin signalling pathways in humans, mice, flies and worms shows remarkable conservation between the organisms (Leevers, 2001; Claeys *et al.*, 2002): reduction in the function of this pathway seems to increase longevity in all these organisms (reviewed by Partridge and Gems, 2002). Similarly, developmental and comparative studies suggest that the organs of mechanoreception, from worms to humans most probably share a common evolutionary origin (Garcia-Anoveros and Corey, 1997; Caldwell and Eberl, 2002). Alterations in the human cochlea are difficult to study, a case in which *Drosophila* can provide a valuable tool for experimental manipulations (Jarman, 2002), as discussed in the next chapter.

2.4 Genetics of deafness

2.4.1 Principles of mechanosensation

Mechanoreceptors can take various forms depending on the mechanical input to which they have adapted. In humans mechanoreceptors can be found, for example, in skin (touch, sharp pain), joints and muscles (limb proprioception), the vestibular system (balance) and cochlea (audition). Hearing is based on the conversion of mechanical vibration (movement) to electrical signals, which are subsequently transmitted further through synaptic connections with afferent sensory neurons. In vertebrates sound detection is mediated by inner-ear hair cells (Hudspeth and Jacobs, 1979). The hair cell (Figure 2.5) is a sensory receptor that responds to mechanical stimulation of its sensory hair bundle, which usually consists of numerous large microvilli (stereocilia) and a single true cilium (the kinocilium). In *Drosophila* mechanoreceptive sensory cells are also found all over the body, including the thorax, abdomen, legs, wings and antennae. The most noticeable of these are the sensory bristles inserted in the cuticle (external sensory organs, Figure 2.5). Another type of mechanosensors, chordotonal organs (CHOs, Figure 2.5), do not have an external bristle and are located within the cuticulum. Developmental and comparative studies suggest that external sense organs and CHOs in *Drosophila* have a common evolutionary origin (Merritt, 1997). The most famous assembly of CHOs, Johnston's organ, is localised in the antennae, and serves as the principal auditory organ in insects (Eberl *et al*., 2000). Fruit-flies use Johnston's organ to discriminate species-specific courtship songs at close range, which can be used in genetic screens for mutations that disrupt auditory function (Eberl *et al.,* 1997). Indeed, many mutations have been found that disrupt the CHO structure, and hence show defective courtship behaviour, such as *atonal* (*ato*), *beethoven* (*btv*) and *touch-insensitive-larva-B* (*til*B, Eberl *et al*., 1997; Eberl *et al*., 2000).

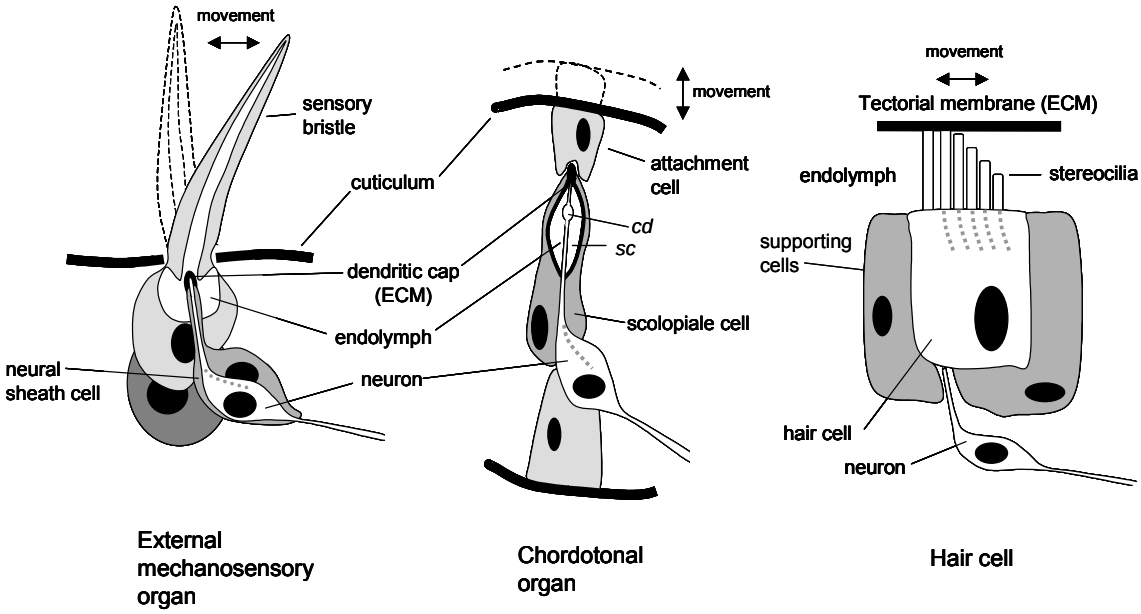


Figure 2.5. Spatial arrangement of mechanosensory cells in flies and of human hair cells. From left to right: *Drosophila* external mechanosensory organ, *Drosophila* chordotonal organ, and human inner-ear hair cell. Ciliated neurons or hair cell stereocilia are attached to the extracellular matrix (dendritic cap and tectorial membrane, respectively). In flies, the neural sheath cell and scolopiale cell secrete a dendritic cap, which connects the tip of the cilium to the cuticulum directly or via a attachment cell. Deflection of the bristle or stretching of the CHO impinge on the ciliated dendrite of the sensory neuron. Similarly, sound-induced relative movements of the hair cells with respect to the tectorial membrane deflect stereocilia, which causes depolarization of the hair cell and activation of the synapsing neuron. Abbreviations: *cd*, ciliary dilation, *sc*, scolopidium (see text for details).

It has been suggested that each mechanoreceptor type arose from exaggerations or reductions of different structural features found in a more generic, primordial sense organ (Eberl *et al.*, 2000; Jarman, 2002). There is genetic evidence that essential components of insect sensory organs and vertebrate hair cells may have evolved before arthropods and chordates diverted. A good example is the *Drosophila* gene *no mechanoreceptor potential C* (*nompC*) that encodes an ion channel of the transient receptor potential superfamily, and is expressed in external sensory organs and CHOs (Walker *et al.*, 2000). *nompC* mutants lack receptor potentials in external mechanosensory bristles, are defective in sound-evoked potentials of antennal CHOs, and are generally uncoordinated (Kernan *et al.*, 1994). Zebrafish orthologue of *nompC* is expressed selectively in sensory hair cells, and loss of its function causes larval deafness (lack of stimulus-evoked potentials in lateral-line hair cells) and an imbalanced swimming pattern (Sidi *et al.*, 2003). Also from a structural or biomechanical point of view the structures are analogous, since both the invertebrate and the vertebrate transduction apparatus are bathed in K^+-rich endolymph secreted by the supporting cells (Jarman, 2002, Figure 2.5), show directional sensitivity, and are capable

to mechanically tune themselves to varying stimulus intensities (Walker *et al*., 2000; Gopfert and Robert, 2003).

Deafness can result from developmental, structural or functional defects of the mechanoreceptors or associated cells. In flies mutant for the proneural gene *atonal*, chordotonal organs, photoreceptors, and a subclass of olfactory receptors fail to develop because their precursors are not specified (Jarman *et al.,* 1993; Jarman *et al.,* 1995; Gupta and Rodrigues, 1997). *atonal* flies are devoid of scolopidia, a specific structure in the CHO (Figure 2.5), although the antennal nerve is present (Jhaveri *et al.,* 2000). Evidence underlying developmental homology between CHOs and mammalian hair cells is provided by the mouse homologue of *atonal* (MATH1) which is expressed in the auditory sensory epithelium at the time hair cell precursors are specified and is required for hair cell generation (Bermingham *et al.,* 1999). Furthermore, the *atonal* mutation in *Drosophila* is rescued by MATH1 expression (Ben-Arie *et al.,* 2000). In btv^{5P1} mutants the chordotonal cilium of auditory mechanosensory neurons displays an anatomically aberrant ciliary dilation (Figure 2.5), in the contact zone between the ciliary axoneme and membrane (Eberl *et al.*, 2000; Gopfert and Robert, 2003). btv^{5P1} as well as $tilB^{2}$ affects only the CHOs, which is shown by complete loss of sound-evoked potentials measured from the antennal nerve but normal touch-evoked potential due to deflection of thoracic bristles (Kernan *et al.,* 1994; Eberl *et al.,* 2000). In addition to being deaf, $tilB^{2}$ males are sterile due to a motility defect caused by malformation of sperm tail axonemes, the other of the two ciliary structures in the fly (Eberl *et al.,* 2000). Screens for defects in the larval response to touch have revealed a set of mutations that affect mechanotransduction both in external sensory organs and CHOs, such as *uncoordinated* (*unc*) (Kernan *et al.,* 1994). This mutant seems to exhibit more "syndromic" defects of mechanotransduction, since in addition to affecting both CHOs and external sense organs, it also shows uncoordination of movement and a sperm axoneme defect similar to that seen in $tilB^{2}$ (Baker and Kernan, 2003). Despite this, *unc* shows a spontaneous response to light and to mechanical stimuli indicating no defects in neuromuscular excitability (Kernan *et al.,* 1994). These variable phenotypes underlie a wide range of organ systems responsible for mechanotransduction, touch, balance, and hearing.

Sensitivity to mechanical stress (bang-sensitivity, BS, see Table 2.1, section 2.2.3.2) has become a classic tool for dissecting neural function in *Drosophila*. It should be noted that

mutations affecting development and structure of sense organs (discussed above) do not generally show a BS phenotype. However, a decreased frequency of action potentials in response to external sensory bristle displacement is a common defect in many BS mutants studied, *i.e. bang-sensitive* (*bas*^{1}), *bang-senseless* (*bss*^{MW1}), *easily-shocked* (*eas*^{2}) and *tko*^{25t} (Engel and Wu, 1994).

In addition, BS flies show seizures and paralysis when subjected to direct electrical stimulation of the brain (Pavlidis and Tanouye, 1995; Lee and Wu, 2002). The giant fibers (GFs) are a pair of interneurons that project from the fly brain to the thoracic ganglion, and have several outputs in the thorax (Figure 2.6). The GFs synapse with a peripherally synapsing interneuron (PSI), which drives flight motor neurons (DLMmns) that synapse with dorsal longitudinal muscle (DLM) fibers. When an electrical stimulus is applied to the brain of a fly, the GF pathway is activated, which results in synchronous activation of all DLM fibers. When neuronal activity was (indirectly) measured from DLM fibers, BS mutants (including *tko*^{25t}) showed seizures of ~0.5-3 sec, which are likely to correspond to the preparalytic seizures after mechanical disturbance (Pavlidis and Tanouye, 1995; Lee and Wu, 2002). During the paralysis following the seizures, subsequent GF stimulation could not evoke DLM fiber potentials. However, since muscle fibers could be stimulated directly from their motor neurons, it seems that this stimulation failure is not due to defects in flight muscles, motor neurons, or connections between them, but occurs at the level of PSI-DLMmn synapses (Pavlidis and Tanouye, 1995). After recovery, a refractory period, during which a stimulus was less effective at producing seizures and paralysis, was observed with different BS mutants (Pavlidis and Tanouye, 1995; Lee and Wu, 2002). These events closely follow the characteristics and time-course of mechanically induced BS behaviour. It has been suggested that BS is due to a similar physiological defect in all the mutants studied, but there are probably additional processes affected in some mutants but not others (Pavlidis and Tanouye, 1995). It is also likely that other neural circuits in addition to GF pathway are involved, which makes the interpretation of the results more difficult. Mutations in at least two of the most studied BS genes, *bas* and *bss*, can also be suppressed genetically by mutations affecting Na^{+}-channels, K^{+}-channels and electrical synapses (Kuebler *et al*., 2001).

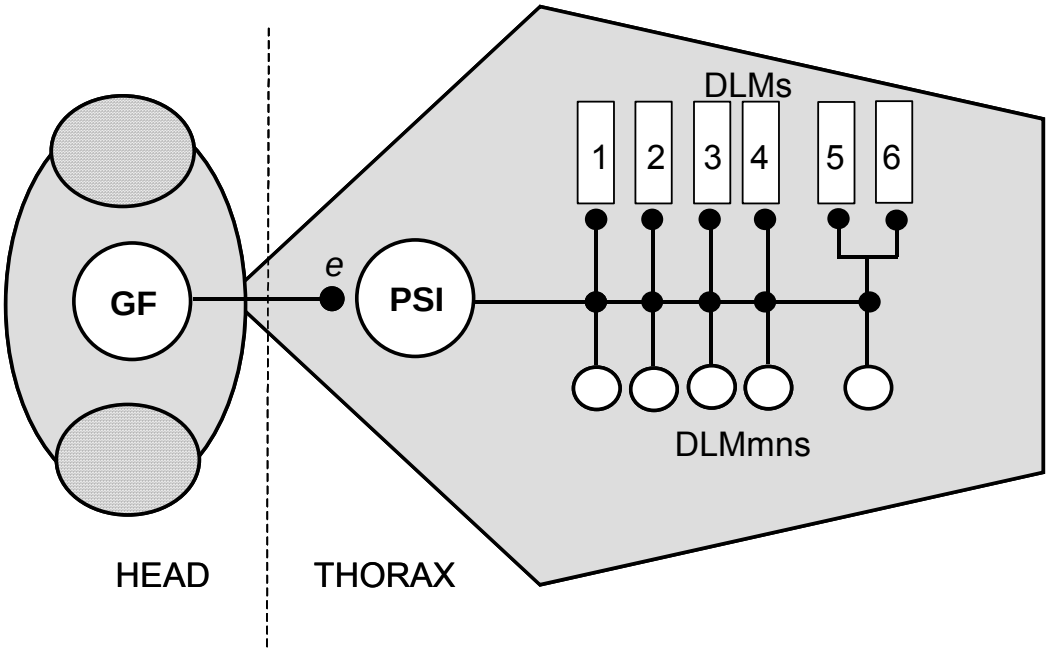


Figure 2.6. The giant fiber pathway of *Drosophila*. One of the two bilateral pathways is shown. Neurons are presented as circles and muscles as boxes. Small black spots are chemical synapses (except the one marked *e*, which is electrical). The Giant fiber (GF) projects from brain to thorax, where it has several outputs. One of these outputs, the peripherally synapsing interneuron (PSI) is shown. The PSI drives dorsal longitudinal flight motor neurons (DLMmns) via chemical synapses. When GF is stimulated, all six DLM fibers are activated synchronously.

From BS mutants, in addition to *tko*, only *eas* has thus far been characterised at the gene level. It encodes ethanolamine kinase, which controls the first step in phosphatidylethanolamine synthesis (Pavlidis *et al.,* 1994). This protein is highly expressed in sensory organs, peripheral neurons, glia and the brain, and as an enzyme affecting membrane phospholipid composition. It could be involved in ion channel function, synaptic transmission or energy metabolism in the cell (Nyako *et al.,* 2001). A mutant that is more clearly involved in energy metabolism, *sesB*, encoding one of the two isoforms of ANT in *Drosophila* (see 2.2.3.2), has been reported to display profound structural overgrowth at neuromuscular synapses (Trotta *et al.,* 2003). In response to high frequency stimulation, *sesB* mutants display pronounced synaptic fatigue and very slow synaptic recovery. This suggests an inability to sustain mitochondrial function sufficient to maintain rapid synaptic vesicle cycling under conditions of high demand (Trotta *et al.,* 2003).

2.4.2 Mitochondrial deafness

At least 58 genes have been identified in humans that are associated with auditory defects, either in syndromic or non-syndromic form. Six of them are encoded by mtDNA (Morton,

2002). Mutations in these genes frequently target components of the mitochondrial translation machinery, such as A1555G mutation in mitochondrial SSU rRNA (for discussion, see 2.3.2 and 2.4.3). Mutations in mitochondrial tRNAs for serine, leucine and lysine have been implied in sensorineural hearing impairment and in syndromes including sensorineural hearing impairment (reviewed by Jacobs, 1997). The tRNA mutations have been suggested to act at the molecular level in diverse ways, such as impaired rate of tRNA synthesis, decreased aminoacylation efficiency, increased turnover rate or defects in processing and nucleotide modifications (Schon *et al.,* 1992; El Meziane *et al.,* 1998; Borner *et al.,* 2000; Levinger *et al.,* 2001; Toompuu *et al.,* 2002). Generally, mutations that exist in the homoplasmic or nearly homoplasmic state show only a mild phenotype in cell culture, such as decreased growth on selective (glucose-free) medium or modest hypersensitivity to the antibiotic doxycyclin (Reid *et al.,* 1997; Toompuu *et al.,* 1999). Such mutations may only be pathogenic in certain cell types or tissues, such as in the cochlea, when the biochemical threshold for the mitochondrial dysfunction is reached. In heteroplasmic cases associated with more severe mutations, a sharp threshold in the capacity to support protein synthesis is observed, such as in the case of A3243G in $tRNA^{Leu(UUR)}$ or T7445C in $tRNA^{Ser(UCN)}$ (Dunbar *et al.,* 1996; Guan *et al.,* 1998).

Knowing the importance of mtDNA-encoded RNAs in deafness, the protein components of the mitochondrial translation machinery are strong candidates for SHI in their own right, as well as modifiers of expression of the mitochondrial tRNA/rRNA mutations (Shah *et al.,* 1997). Chromosome 19q13.1, to which the autosomal deafness locus *DFNA4* has been mapped, contains two genes encoding components of this machinery: ribosomal protein S12 (MRPS12) (Shah *et al.,* 1997; Johnson *et al.,* 1998) and mitochondrial seryl-tRNA synthetase (SARSM) (Yokogawa *et al.,* 2000). These genes have been screened for possible mutations in two *DFNA4*-linked deafness families, but no plausible disease mutations were found (Shah *et al.,* 2001). Therefore, at least in *DFNA4*-families identified thus far, these genes can be excluded unless distant regulatory mutations are considered. However, as putative modifiers of mitochondrial disorders these genes are still of interest.

2.4.3 Aminoglycoside antibiotics and deafness

The most common mutation associated with maternally inherited non-syndromic deafness is A1555G in the mitochondrial SSU rRNA (Prezant *et al.,* 1993). The mutation is largely

homoplasmic, exists in numerous families with different ethnic origins, and sometimes the affected members have a history of aminoglycoside antibiotic treatment (Estivill *et al.*, 1998; Hutchin and Cortopassi, 2000). As mentioned earlier, not all the carriers of the mutation suffer from SHI, and genetic modifiers of the phenotype have been suggested to play a role in the pathogenesis (Bu *et al.*, 1993; Guan *et al.*, 1996; Guan *et al.*, 2001). However, no nuclear modifiers of the disease have thus far been identified in humans. Moreover, the pathogenic mechanism of the mutation is still largely unknown, although many studies using patient-derived primary cell-lines and cybrid cell-lines have been studied.

Aminoglycoside antibiotics have a general capacity to bind to nucleic acids that have internal 'bubbles', bulges and other non-Watson-Crick duplex elements (Ryu and Rando, 2001) and show high affinity for the decoding centre of prokaryotic ribosomes (Cundliffe, 1987). Streptomycin (SM) and paromomycin (PM), for example, cause mistranslation of mRNAs in bacteria (Ruusala and Kurland, 1984) and this is due to an altered conformational state favouring acceptance of near-cognate tRNAs, as discussed in section 2.2.3. The mitochondrial A1555G mutation introduces a new base pair into the SSU rRNA and thereby changes the region involved in decoding to a more bacterial-like conformation (position 1491 in *E. coli*). This can bind certain aminoglycosides, such as PM, efficiently *in vitro* (Hutchin *et al.*, 1993; Hamasaki and Rando, 1997). Therefore, it has been suggested that a decreased rate (or mistranslation) of mitochondrially encoded polypeptides due to interaction with the drug could be the basis for aminoglycoside-induced deafness (Hutchin *et al.*, 1993). Indeed, cell-culture models have shown an approximately 30% decrease in the rate of mitochondrial protein synthesis in the presence of PM in A1555G harbouring lymphoblastoid cell-lines compared to controls (Guan *et al.*, 2000). More recently, similar experiments were carried out on patient-derived fibroblasts, transmitochondrial cybrids with uniform nuclear background, and with transmitochondrial fibroblasts in patient or control nuclear background simultaneously (Giordano *et al.*, 2002). These studies did not reveal a translational defect in any combination of mtDNA and nuclear background without antibiotics, but confirmed the PM sensitivity reported previously by Guan *et al.* (2000). Surprisingly, SM did not cause any defect in the same experiments, which contrasted with the previous results suggesting moderate reduction in mitochondrial translation products in the HeLa cell background (Inoue *et al.*, 1996). These experiments have further supported the idea that nuclear, possibly tissue-specific modifiers

have an important impact on manifestation of the disease, and that potential selective uptake of the drugs by the cochlea could also be involved in the specificity of the disease. The role of SM as an environmental inducer of deafness still remains to be elucidated, because in contrast to PM, this specific mutation does *not* increase SM binding in vitro (Hamasaki and Rando, 1997). It is notable that not all effects of this group of antibiotics are attributable to decoding *per se*, since aminoglycosides have been shown to inhibit RNase P RNA cleavage and aminoacylation of yeast $tRNA^{Asp}$ *in vitro* (Mikkelsen *et al.,* 1999; Walter *et al.,* 2002).

Modifiers of the paromomycin resistance mutation P^{R}_{454} in yeast mitochondrial SSU rRNA, corresponding to human A1555G have been described (Weiss-Brummer and Huttenhofer, 1989; Li and Guan, 2002; Li *et al.,* 2002). Mto1p and Mss1p are mitochondrially located tRNA modification enzymes homologous to bacterial *gidA* and *mnmE*, respectively, and mutant alleles of the genes only manifest as respiration deficiency when combined with the P^{R}_{454} mutation (Decoster *et al.,* 1993; Colby *et al.,* 1998; Bregeon *et al.,* 2001). Human homologues of both MTO1 and MSS1 can complement this deficiency in yeast, and at least MTO1 is expressed highly in the cochlea (Li and Guan, 2002; Li *et al.,* 2002). P^{R}_{454} has been suggested to result in stringent (restrictive) proofreading during mitochondrial translation (Weiss-Brummer and Huttenhofer, 1989), and since many mitochondrial tRNAs contain modifications it is plausible that hypomodified tRNAs in *mto1* and *mss1* mutant strains are not functional in the highly accurate ribosomal environment (Bregeon *et al.,* 2001). Therefore, human homologues of these proteins could be potentially involved in adjustment of codon-anticodon interactions that might lead to disease in the context of the A1555G mutation (Li and Guan, 2002; Li *et al.,* 2002). A possibility that the tRNA modification deficiency could lead to additional direct susceptibility of tRNAs to aminoglycosides can also not be excluded, since aminoglycosides are implicated in the inhibition of binding of fMet-$tRNA^{fmet}$ to IF2 (Evans *et al.,* 2003).

3. AIMS OF THE STUDY

The main purpose of this study is to elucidate the validity of *D. melanogaster* as a model system for human mitochondrial disorders, particularly those resulting from translational defects. Two different mutations in the *Drosophila* mitoribosomal protein S12 have been studied for effects on translation, on mitochondrial OXPHOS function, and on the development, physiology and behaviour of the fly. Additional experiments to understand the molecular effects of these mutations on ribosome function were carried out in a bacterial model system. Genetic and environmental factors affecting the fly phenotype were investigated, showing that the model exhibits some close parallels with human mitochondrial disease. One important variable affecting the phenotypic outcome may be differential regulation of expression of the S12 gene. To investigate this at the molecular level, the human homologue was studied using cultured human cells. More specifically, the aims of the study were:

1) to model in *E. coli* the molecular consequences of the L85H mutation found in tko^{25t} flies, as well as the potential involvement of a conserved glutamine residue (Q116) in the natural streptomycin resistance of mitochondria (I)
2) to characterise the mutant phenotype in prototypic tko^{25t} mutants and in various transgenic variant lines with respect to molecular, biochemical, developmental and behavioural alterations (II, III)
3) to determine whether the L85H mutation is the sole cause of the tko^{25t} phenotype by a transgenic reversion test (II)
4) to create and characterise tko^{Q116K} transgenic lines for streptomycin resistance and other possible phenotypes (II)
5) to create GAL4-responsive UAS-*tko*+ transgenic lines and characterize the effects of *tko*+ over-expression in both wild-type and *tko* mutant flies (III)
6) to elucidate the mechanisms of regulation of human mitochondrial S12, especially with respect to post-transcriptional control (IV)

4. MATERIALS AND METHODS

This chapter briefly describes the main methods used. For more detailed descriptions of general methods, such as PCR, various blotting techniques, transformation, transfection and molecular cloning, please refer to the original publications I-IV.

4.1 Bacterial strains

Streptomycin sensitive (SM^S) *E. coli* strain XL-1 Blue was obtained from Stratagene (La Jolla, CA). Streptomycin resistant (SM^R) strain DS941 was supplied by Dr. Martin R. Boocock, University of Glasgow, and strains UD1D2 and UD5B6 used in nonsense suppression analysis, and strain UD8C1 used in elongation rate assays were provided by Dr. Diarmaid Hughes and Dr. Charles Kurland, University of Uppsala, Sweden. Strains were stored as 50% v/v glycerol stocks at -70°C. Cultures were grown on L-agar plates or in L-broth (Sambrook *et al*., 1989) supplemented with appropriate antibiotics, except for the experiments carried out with UD1D2, UD5B6 and UD8C1, which were done using M9 minimal medium (Sambrook *et al*., 1989). To produce competent cells for transformations, cells were grown to mid-logarithmic phase in a medium supplemented with 10 mM $MgCl_2$, cooled on ice and washed with TB (10mM Pipes, 55mM $MnCl_2$, 15 mM $Cacl_2$, 250 mM KCl). Cells were resuspended in TB containing 7% DMSO, aliquots were frozen in liquid nitrogen, and stored at -70°C.

4.2. Mammalian cell culture

HEK293-EBNA cells were maintained in DMEM, 10% FCS, supplemented with 50µg/ml uridine, 1 mM glutamine, 1 mM sodium pyruvate plus 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were routinely passaged weekly. For serum starvation conditions, please check original article (IV).

4.3 *Drosophila* strains and P-element mediated transgenesis

The tko^{25t} mutant strain was obtained from Dr. Chun-Fang Wu and the tko^3/FM7 stock was obtained from the Bloomington stock center (Bloomington, IN). The strains Canton S (wild-type) and w^{1118} were obtained from Dr. Kevin O'Dell, University of Glasgow, Scotland, and the inbred control strain 0713 was supplied by Dr. Serge Alziari, Université

Blaise-Pascal, Clermont II, France. The stocks were maintained at 25 °C on a 12 hr:12 hr light:dark cycle in yeast sugar medium (1% agar, 1.5% sucrose, 3% glucose, 3.5% active dried yeast, 1.5% maize meal, 1% wheat germ, 1% soya flour, 3% treacle, all w/v) supplemented with 0.5% propionic acid and 0.1% methyl paraben (Nipagin M), with w/v. The tko^{25t} stock was maintained both as a homozygous mutant (inbred) and tko^{25t}/FM7 balanced stock (for explanation of balancers, see below). P-element mediated transformation was carried out essentially as described by Spradling (Spradling, 1986). Various versions of *tko* (*i.e.* tko^{L85H}, tko^{H85L}, tko^{Q116K} and *tko*+) were cloned into pP{CaSpeR-4} (Thummel and Pirrotta, 1992) or pP{UAST} (Brand and Perrimon, 1993) vectors, and co-injected into recipient w^{1118} embryos with pUChsΔ2-3 helper plasmid. Survivors were mated to the recipient strain, and putative transgenic progeny were selected on the basis of the *w*+ eye colour marker. Siblings were crossed in order to produce homozygous stocks. After molecular characterization, homozygous viable strains were bred to various endogenous *tko* backgrounds (*e.g.* tko^{25t} and tko^{3}).

Insertions in the X, second and third chromosomes were maintained over balancers FM7, *CyO* and TM6*Sb*, respectively. In *D. melanogaster* recombination occurs in females, and it can be controlled by balancer chromosomes that contain dominant (and recessive) markers. Balancers are no longer capable of pairing or recombining with their normal homologue during meiotic prophase. Most recessive mutations are invisible in heterozygous conditions. Because of the markers, transmission of balancers to progeny can be tracked unambiguously and, therefore, also transmission of the homologous chromosome containing the desired mutation (*e.g.* tko^{25t}). If the progeny do not have the balancer, it must have the homologue. By this way, invisible genotypes can be scored in progeny with virtually 100% reliability.

4.4 *Drosophila* behavioural tests

4.4.1 Bang-sensitivity

Freshly eclosed flies were collected and separated into fresh food vials, with females and males separated. After three to four hours flies were briefly CO_2-anaesthetised Single flies were transferred to empty vials and allowed to recover for half an hour. Stress sensitivity was measured by vortexing single flies in vials for 15 s and by measuring the time taken

for the fly to stand on its feet again (recovery time). Stress-sensitivity of the delayed flies eclosing from the doxycyclin experiment (see 4.4.3) were measured similarly.

4.4.2 Reactivity

Locomotion in response to mild mechanical disturbance was measured by counting the distance travelled by a single fly in a 10 cm X 10 cm gridded open-field chamber. The distance travelled during ten minutes after stimulus (in consecutive one minute periods) was recorded. A total of 50 flies of each genotype were analysed.

4.4.3 Developmental time and antibiotic sensitivity

The stage of the developmental delay was determined by following the growth of larvae on food plates containing an inert dye (neutral red) at 25 °C. The embryos were synchronised by letting fertilised females lay eggs, and then replacing the plates at three-hour intervals. The onset and length of the first and second larval molts, pupariation and eclosion of imago were followed daily. Two antibiotics known to affect bacterial translation were tested for potential additional effects on developmental phenotype on yeast-sugar medium vials containing varying concentrations (0, 1, 3, 10, 30, 100, 300 or 1000 μg / ml food) of doxycyclin (DOX) or streptomycin (SM). The timing and number of females and males eclosing under each condition were recorded.

4.4.4 Courtship analysis

Courtship was analysed as in O'Dell *et al.* (1989). Briefly, virgin flies were collected within six hours of eclosion and aged for 4 days in vials containing 10 females or males. Flies were briefly CO_2-anaesthetised and the lack of any obvious defects in appearance (e.g. wing morphology) was confirmed. Males and females were briefly CO_2-anaesthetised and transferred to the opposite sides of round mating chambers (∅ diameter 2.2 cm) divided in half by a removable separator. After one hour of recovery at 25°C, the separator was removed and the status of the flies was observed once every minute for one hour at 25°C. The duration of latency (the period preceding courtship), of courtship and of copulation was measured (see also Figure 5.6 a). Ten pairs of each combination of sex and genotype were analysed simultaneously in order to minimize the effect of possible environmental factors (e.g. air pressure).

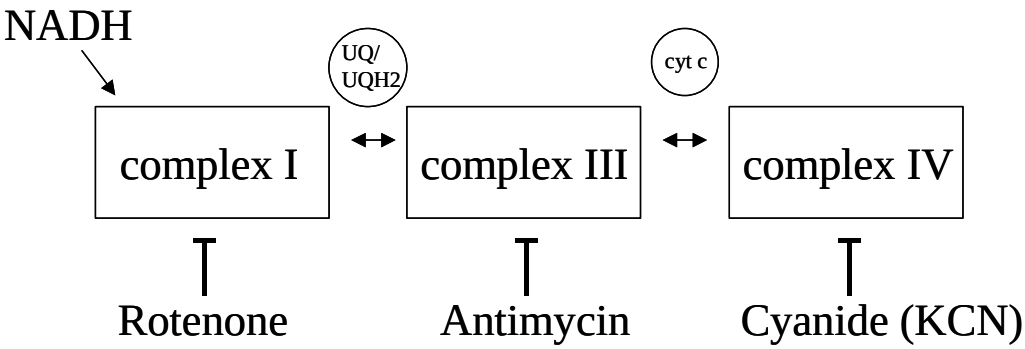
4.4.5 Deafness assay

Hearing was measured (by K. Luoto and K.M.C. O'Dell) by a modification of the assay used by Von Schilcher (1976b) and Eberl *et al*., (1997). Canton S and *tko*^{25t} males were collected in batches of ten within six hours of eclosion. After one day, flies were briefly anaesthetised and the wings surgically removed. At the age of 5-7 days six males from each batch were transferred to a mating chamber and exposed to 5 minutes of silence, followed by 10 minutes of computer-generated pulse song (Ritchie *et al.*, 1998) of varying intensities (60, 70, 80, 100 or 110 dB). The number of males exhibiting courting behaviour (max = 6) was scored before exposure to song (4.40-4.50 min), and at early (6.00-6.10 min) and late (14.30-14.40) timepoints thereafter. The short-term effect was calculated by subtracting the scores of "before" from "early", and long term effect by subtracting the scores of "before" from "late", in order to eliminate any possible differences caused by spontaneous (*i.e.* not song-induced) homosexual mating behaviour between the strains.

4.5 Respiratory enzyme activities and ATP synthesis

The principles of respiratory complex activity measurements are shown in Figure 4.1. Complex I activity was monitored by NADH oxidation at 340 nm in pH 7.2 buffer containing 35 mM NaH_2PO_4, 5 mM $MgCl_2$, 2.5 mg/ml BSA, in the presence of 2 µg/ml antimycin (complex III inhibitor), 2 mM KCN (complex IV inhibitor), 97.5 µM ubiquinone, 0.13 mM NADH and 40 µg mitochondrial protein. Only the rotenone (complex I inhibitor) sensitive activity was recorded. For measurements of complex III, cytochrome *c* reduction was monitored at 550 nm in pH 7.2 in the same buffer, but in the presence of 2 mM KCN, 2 µg/ml rotenone, 15 µM ubiquinol, 15 µM cytochrome *c* and 5 µg mitochondrial protein. The non-enzymatic reduction of cytochrome *c* with ubiquinol (in the absence of mitochondria) was subtracted from this measurement. Complex IV activity was determined by oxidation of partially reduced cytochrome *c*, monitored at 550 nm in pH 7.4 buffer containing 30 mM KH_2PO4, 1 mM EDTA, 56 µM cytochrome *c* and 5 µg mitochondrial protein. Citrate synthase activity was assayed by the appearance of thionitrobenzoic acid (yellow) derived from the reduction of dithio-bis-nitrobenzoic acid (DTNB-colourless) with coenzyme A, monitored at 412 nm in 100 mM Tris-HCl pH 8 buffer, 2.5 mM EDTA, 37 µM acetyl-CoA, 75 µM DTNB, 300 µM oxaloacetate and 5 µg mitochondrial protein. All activities were measured at 28 ºC.

Mitochondrial ATP synthesis was measured using a technique based on the luminescence of luciferin in the presence of luciferase, which is proportional to the ATP concentration in the test medium (Wibom *et al.*, 1991). Kinetics were recorded at 25 ºC, pH 7.5, with 50 µl of the incubation medium containing 0.15 mM ADP, 54 µM APP and 1 µg mitochondrial protein. After 2 min of incubation, 5 µl of substrate and 100 µl of reagent were added simultaneously and fluorescence was monitored for one minute. The final concentrations of substrates were 10 mM pyruvate + 10 mM malate, 20 mM α-glycerophosphate or 3.3 mM ascorbate + 80 µM TMPD. Calibration was performed at the end of each measurement by the addition of 200 pmol of ATP.



cI: NADH oxidation (+UQ, antimycin, KCN), only rotenone sensitive activity noted
cIII: cyt c reduction (+UQH2, rotenone, KCN), non-enzymatic reduction deduced
cIV: oxidation of partially reduced cyt c
Citrate synthase: Ac-CoA + OAA → citrate + CoASH
CoASH + DNTB → colour

Figure 4.1. Principles of mitochondrial respiratory complex activity measurements. Electron donors and acceptors are shown, as well as specific inhibitors of the three complexes. The boxed text indicates the measured parameters and inhibitors used in each measurement. For more details, please see the text.

4.6 Translational kinetics in bacteria

E. coli strains UD8C1, UD1D2, and UD5B6 were grown on M9 medium (Sambrook *et al.*, 1989) containing 0.2% glucose and supplemented with 0.04 mM arginine, and 0.2 mM thiamine. To obtain 'mutation specific' values for the translational elongation rate and translational nonsense suppression of the wt-*rpsL*, K87Q-*rpsL*, and L56H-*rpsL* -expressing transformants, the various host strains were transformed with the various pBR322-*rpsL* constructs (I) and were grown in the presence of 100 µg/ml ampicillin (to select for the

presence of the plasmid), as described below. Both elongation rate and nonsense suppression assays are based on the appearance of yellow ortho-nitrophenol (ONP) from chromogenic (colourless) ortho-nitrophenyl-β-*D*-galactopyranoside (ONPG), which serves as a quantitative estimate of β-galactosidase expression from the *lacZ* gene (Figure 4.2).

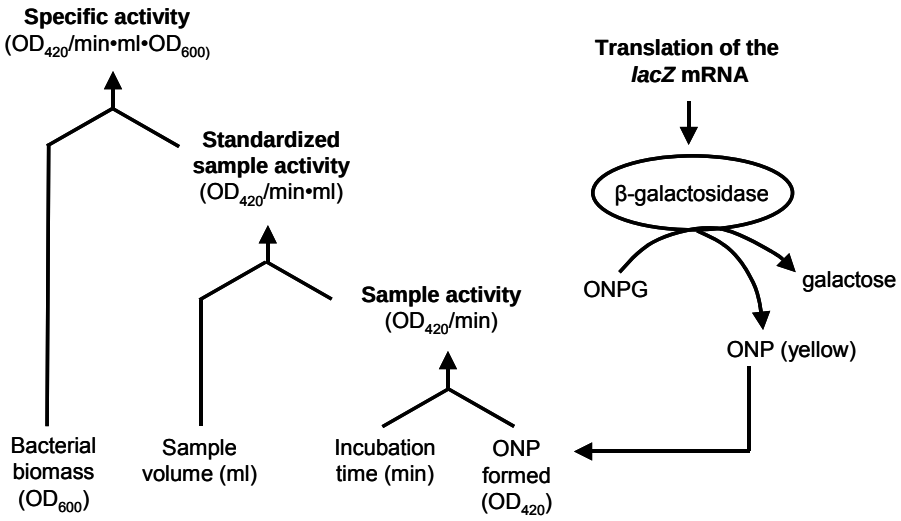


Figure 4.2. Principles of β-galactosidase expression measurements. The *lacZ* mRNA is translated by ribosomes containing various *rpsL*-mutated proteins. The amount of β-galactosidase enzyme produced is proportional to the yellow colour in the buffer, and it is normalized against bacterial biomass and incubation time to get specific β-galactosidase activity.

Elongation rate measurements were carried out using strain UD8C1 that carries an inducible β-galactosidase gene, essentially as described previously (Miller, 1972), but using ampicillin selection. Cells were grown on a shaker at 37°C until OD_{600} was approximately 0.5, and a 6 ml sample was then quickly transferred to a sterile 30 ml Corex tube placed in a 37 °C water-bath. Stirring of the culture was by means of a small magnetic stirrer bar inside each Corex tube. Immediately before induction, a sample from each tube was taken and kept on ice for cell density measurements (OD_{600}). At time zero, 50 µl of 0.1 M IPTG was added to induce β-galactosidase production and a 0.2 ml "zero" sample was taken at 15 s and immediately mixed with 300 µl of ice-cold chloramphenicol (CM) solution (0.5 mg/ml in H_2O) on ice. Starting from 1 min, 0.2 ml samples (n = zero +12) were mixed with cold CM solution every 15 sec. Two parallel 0.2 ml samples from each cold bacterial / CM solution were then transferred to 2 ml eppendorf tubes containing 0.8 ml Z-buffer (Miller, 1972), 40 µl 0.1% SDS, and 80 µl chloroform. The tubes were shaken vigorously for 20 s and transported to the dark. Subsequently, 0.2 ml of ONPG solution (4 mg/ml in ONPG buffer) was added and samples were shaken in a 28 °C water-bath in the dark for 13-15 h. The reactions were stopped by adding 1 ml of 1M Na_2CO_3 and the tubes

were centrifuged at 16 000 g_{max} in a microcentrifuge at 4°C for 15 min. The average rate of nitrophenol formation (OD_{420}) of these was measured. The data was transformed by subtracting the average value of parallel zero-time samples, and by normalizing cell densities at the start of the assay to OD_{600} = 1.0 and ONPG incubation times to 1 hour, and values were plotted as square roots of corrected OD_{420} values. The time required to produce the first active β-galactosidase monomer was estimated from the point of intersection of the trend-line and the x-axis (time). Eighteen different clones expressing wt protein, 12 clones expressing the K87Q mutated protein, and 5 clones expressing the L56H mutated protein were analysed. Freshly transformed clones were used in order to avoid additional mutations in plasmid constructs, and each strain used was verified by sequencing the single colony PCR product of the cell pellet from the sample taken at the actual time of the β-galactosidase measurements. All results obtained from clones containing additional mutations in the reading frame or in the upstream sequence, were discarded.

E. coli strains UD1D2 and UD5B6 were used to measure nonsense suppression *in vivo*. UD1D2 carries an F´ plasmid that includes a constitutively expressed, wild-type β-galactosidase fusion protein (*lacIZ*Δ14), and UD5B6 carries a nonsense mutation in this same gene (*lacIZ*Δ14, UGA at 189) in an otherwise isogenic background. Because the chromosomal copy of the *lacZ* gene has been deleted in these strains, all β-galactosidase message will be transcribed from the F´ fusion gene, and functional β-galactosidase produced from strain UD5B6 can be explained only by read-through of a stop codon by ribosomes. Freshly transformed clones were grown to mid-log phase, growth was stopped on ice and parallel samples (0.1 ml from UD1D2 clones and 1.0 ml from UD5B6 clones) were pelleted, and resuspended in 1 ml of ice cold Z buffer (Miller, 1972). From this point on, β-galactosidase activity was measured as in the case of elongation rate measurements, but with typical incubation times of 2 h and 15-16 h for UD1D2 and UD5B6 clones, respectively. Average OD_{420} values of parallel samples were normalized to the amount of cells and time of incubation. Values for each UD5B6 clone expressing a given *rpsL* mutation were then divided by the values of corresponding UD1D2 clones to get a suppression value specific for the given mutation in the *rpsL*-gene. The results were calculated using the following formulae:

STEP 1:

$$\frac{OD_{420} - (OD_{550} \times 1.75)}{Vol\ (ml) \times OD_{600} \times time\ (min)} \times 1000 \rightarrow \text{Miller unit (} \beta\text{-gal x } 10^3\text{) values}$$

STEP 2:

$$\frac{\text{Value of } \Delta 14 \text{ UGA at 189 (UD5 B6)}}{\text{Value of } \Delta 14 \text{ (UD1 D2)}} \times 10{,}000 \rightarrow \text{suppression by } 10^4$$

Standard deviations for the suppression values of the clones carrying different *rpsL* mutant plasmids were obtained using following equation

$$SD = \frac{Var(x) + Var(y) \times \rho^2}{mean(y)^2}$$

where Var(x) = variance of UD5B6 suppression values, Var(y) = variance of UD1D2 suppression values, and ρ = mean(x) / mean(y).

4.7 Construction of uORF -mutagenised *MRPS12* expression plasmids

For the 5´-UTR characterization (unpublished), uORF constructs were made using the same Invitrogen expression vector as in the other constructs (pcDNA3.1(-)/Myc-His B) described in article IV. Splice variant *b* was created using combinations of primers rps-d1 (GGTTAGACCTATAGAGGGACGGCCCAGGTG), the two bases surrounding the splice site underlined) and rps-d2 (CACCTGGGCCGTCCCTCTATAGGTCTAACC) with commercial primers BGH and T7, respectively, using MRPS12-Myc/B construct as a template. The two first round PCR products were used as a template for second round PCR with primers BGH and T7, and the product was then cut with *Bam*HI and *Hind*III and ligated to the vector. To mutate the uORF, the following primers were designed: rps-uORF1 (TCTGTTAGCAGCCTGAGGGCCTGTGG, mutated base underlined) and its reverse complement rps-uORF2 (CCACAGGCCCTCAGGCTGCTAACAGA) to create construct b2, rps-uORF3 (CAGCATGGGGCCTGTGGTTTAGACCTA, bases surrounding the deleted A-residue underlined) and its reverse complement rps-uORF4 (TAGGTCTAAACCACAGGCCCCATGCTG) to create construct b3, and rps-uORF5 (GCAGCATGCGTGCATGCGGGTGAACCT, altered bases underlined) and its reverse complement rps-uORF6 (TAGGTTCACCCGCATGCACGCATGCTGC) to create

construct b4. Primers rps-uORF1, -3 and -5 were used in combination with primer BGH, and primers rps-uORF2, -4 and -6 were used with primer T7, using cloned construct b as a template. Second round PCR, digestion, and cloning was performed as above.

5. RESULTS

The main results are summarized in this section. Additional details of each finding will be found in the original communications. Amino-acids affected by the mutations are named here corresponding to their bacterial or fly gene products, i.e. L56H in *E. coli* corresponds to L85H in *D. melanogaster*, and position K87 in *E. coli* corresponds to Q116 in *Drosophila* (Figure 5.1).

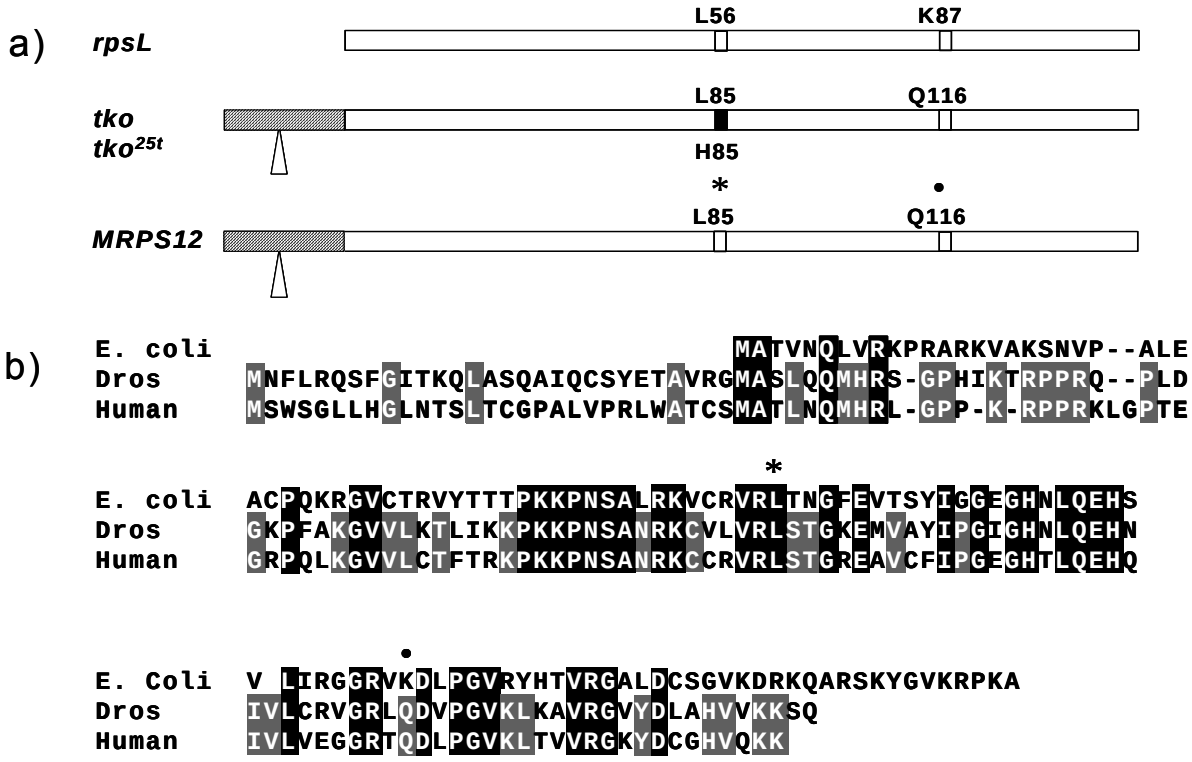


Figure 5.1 Coding regions of the ribosomal protein S12 homologues. a) Schematic picture of *E. coli rpsL, D. melanogaster tko*, and human *MRPS12* coding regions. Corresponding amino-acid positions in bacteria and animals are indicated, and amino-terminal mitochondrial targeting sequences are shown as diagonally striped boxes. Conserved intron positions in *tko* and *MRPS12* are indicated as arrowheads. a) Amino-acid alignment of the same genes. The first 29 amino-acids of *tko* and *MRPS12* are mitochondrial targeting sequences. The residues conserved in all variants are highlighted as black boxes, and the residues identical between human and flies are highlighted as grey boxes. Amino-acid identity between human and fly genes is 63.7%, and bacterial S12 identity to flies and humans is 38.7% and 41.9%, respectively (mitochondrial targeting sequences omitted).

5.1 Mutational analysis of *rpsL* (I)

Unless otherwise stated, bacterial overexpression studies were carried out in streptomycin resistant (SM^R) *E. coli* strain DS941. It should be kept in mind that, because streptomycin

sensitivity (SM^S) is a dominant trait in bacteria, overexpression of wild-type rpsL (wt-*rpsL*) will result in loss of SM resistance in DS941.

5.1.1 L56H-rpsL

The analogous mutation to *tko*^{25t} has not been previously reported in *rpsL*. The possibility that this mutation could be involved in SM resistance led us to test the ability of bacteria overexpressing L56H- *rpsL* to grow in media supplemented with various concentrations of SM. I found that L56H-*rpsL* could be expressed stably, both under normal growth conditions and in medium containing high levels of SM (25 µg/ml), confirming that the mutation does not affect the steady-state level of the protein *per se* (Figure 5.2). Overexpression of L56H- *rpsL* did not alter the phenotype of the SM^R host strain to SM^S, which is clearly the case when the wt-*rpsL* was expressed (Figure 5.2). Conversely, L56H-*rpsL* expression did not cause SM resistance when expressed in an SM^S host strain. The loss of dominant SM sensitivity due to the L56H replacement indicates that the mutant protein, although expressed at a much higher level than endogenous S12, cannot efficiently contribute to ribosomal assembly. Most substitutions of residue L56 obtained from saturation mutagenesis gave a similar phenotype, *i.e.* loss of dominance. No effects on translational fidelity (nonsense suppression) or ribosomal elongation rate were observed (Table 5.1).

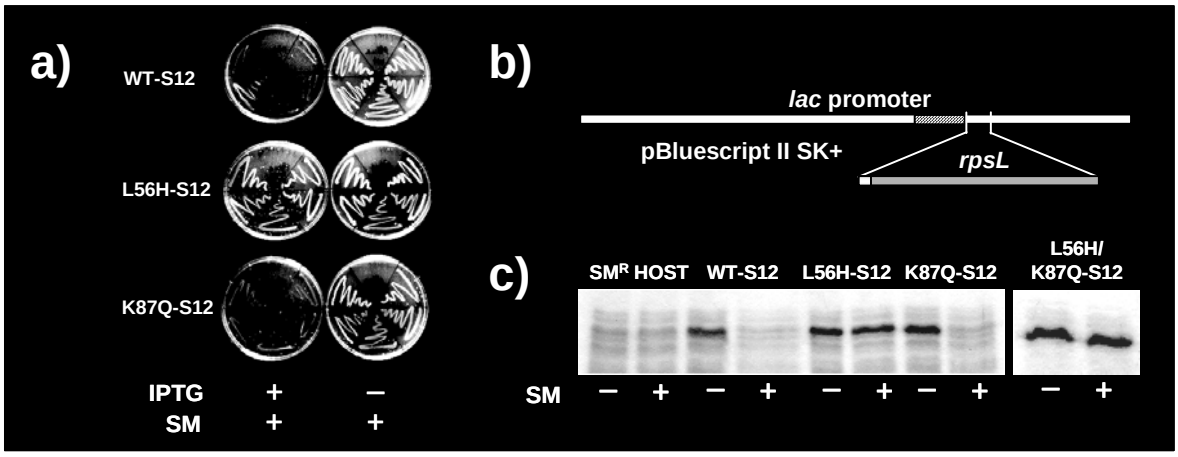


Figure 5.2. Streptomycin phenotype of the *rpsL* variants. a) Growth of the three *rpsL* variants on plates containing 25 µg/ml SM. b) Gross structure of the expression construct used. Plasmid borne *rpsL* variants were induced with IPTG. c) SDS-PAGE showing stability of the proteins in over-night cultures, in the presence and absence of SM. Product of doubly mutated L56H/K87Q-*rpsL* is also shown. Contents of figure 5.2 c) are reprinted from original article I, Copyright (1999), with permission of Blackwell Publishing.

5.1.2 K87Q-rpsL

Similar expression constructs carrying K87Q- substituted *rpsL* can be expressed stably at the protein level and in comparable amounts to L56H-*rpsL* or to wt-*rpsL*, in the absence of SM. In the presence of high levels (25 µg/ml) of SM, K87Q-*rpsL* behaved like wt-*rpsL*, i.e. confers dominant SM sensitivity (Figure 5.2). When colonies on plates or turbidity in over-night liquid cultures were observed, this was always associated with loss of protein production due to mutations in the expression construct (Figure 5.2). Expression of the K87Q-*rpsL* in lower concentrations (1, 2, 5, and 10 µg/ml) of SM resulted in a growth phenotype intermediate between that of the host alone and the wt-*rpsL*, i.e. the amino-acid substitution conferred a low-level resistance to SM. This was confirmed by expression of the K87Q-*rpsL* in the SM^S host. However, the level of resistance was lower in the SM^S background, indicating incomplete dominance compared to wt-*rpsL*.

Because the K87Q substitution is involved in SM resistance, I then measured the effects of the mutation on translational fidelity and ribosomal elongation rate. Expression of K87Q-*rpsL* resulted in decreased elongation rate (~15%) compared to wt-*rpsL*. However, the mutation did not cause significant differences in nonsense suppression (Table 5.1).

	Nonsense suppression (X 10^4)	Elongation rate*
WT-*rpsL*	99 ± 16	76.6 ± 3.3
K87Q-*rpsL*	94 ± 5	88.9 ± 4.8
L56H-*rpsL*	114 ± 9	77.9 ± 3.1

Table 5.1 Translational parameters for *rpsL* variants. * Expressed as ribosomal transit time for lacIZΔ14 mRNAs. For details, see chapter 4.6. Contents of the table are reprinted from original article I, Copyright (1999), with permission of Blackwell Publishing.

5.2 Molecular and biochemical characterization of *tko*^{25t} (II, III)

Because many mutations affecting ribosome assembly in bacteria and yeast result in degradation of rRNA, we investigated the abundance of mitochondrial rRNAs in *tko*^{25t} mutants in larvae. In addition, OXPHOS capacity and ATP synthesis were measured to

investigate potential mitochondrial translational dysfunction. Third instar larvae were used because biomass of the larvae at this stage is highest, and the developmental delay caused by *tko*^{25} mutation occurs at this stage (see 5.3.5).

5.2.1 Decreased mitochondrial 12S:16S rRNAs ratio

Northern Blot analysis of mitochondrial 12S (SSU) and 16S (LSU) rRNAs suggested initially a decrease of approximately 70% in the relative level of SSU rRNA (compared with LSU rRNA) in *tko*^{25t} mutant larvae (Figure 5.3a). Re-analysis by real-time RT-PCR indicated that this drop is brought about partially by an increase in the LSU rRNA level (Figure 5.3b). The standardized steady state levels of the 12S rRNA and 16S rRNA were determined as 0.67 ± 0.02 and 1.53 ± 0.05, respectively, in the mutant, compared with levels in Canton S (Figure 5.3b).

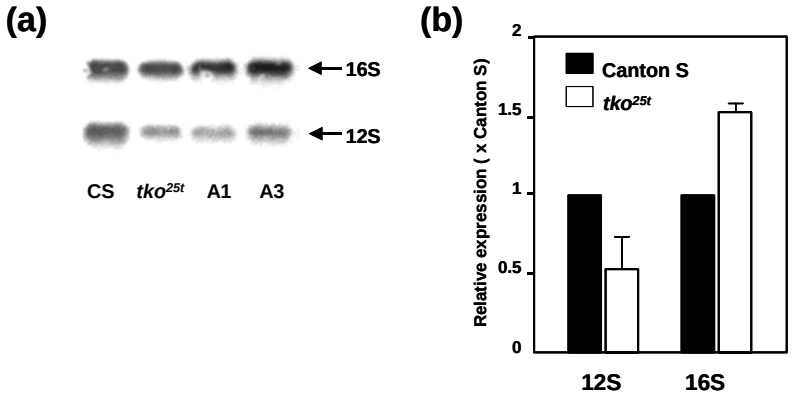


Figure 5.3. Decreased 12S:16S rRNA ratio in *tko*^{25t} mutants. a) Northern blot from third instar larvae showing decrease in relative abundance of the 12S rRNA compared with 16S rRNA in *tko*^{25t}. Transgenic line A3 shows improvement in the ratio compared to the prototypic *tko*^{25t} or line A1. b) Relative expression levels of 12S and 16S rRNAs in *tko*^{25t} mutant (white bars) compared to Canton S (black bars). Values were corrected against cytosolic rp49 mRNA, used as an internal standard in all reactions. Means and SDs are for three independent measurements. Figure 5.3 a) is reprinted from original article III, Copyright (2003), with permission from Elsevier.

5.2.2 Decreased OXPHOS capacity and rate of ATP synthesis

Activities of the three respiratory chain enzymes containing mitochondrially encoded subunits, i.e. complexes I, III and IV were analysed by spectrophotometry from mitochondrial extracts of inbred *tko*^{25t} and Canton S (wild type) flies, as well as from another (inbred) control line (0713). Nuclearly encoded citrate synthase (CS) was used as a control to rule out general effect on metabolic pathways, and its activity showed no significant differences between the strains. Large decreases in activities of complexes I, III and IV are evident in *tko*^{25t} larvae, compared with either reference strain (Figure 5.4a). Citrate synthase activity showed no significant differences (not shown). ATP synthesis

capacity was less affected, but the effects were still significant, for at least two of the substrates used (Figure 5.4b).

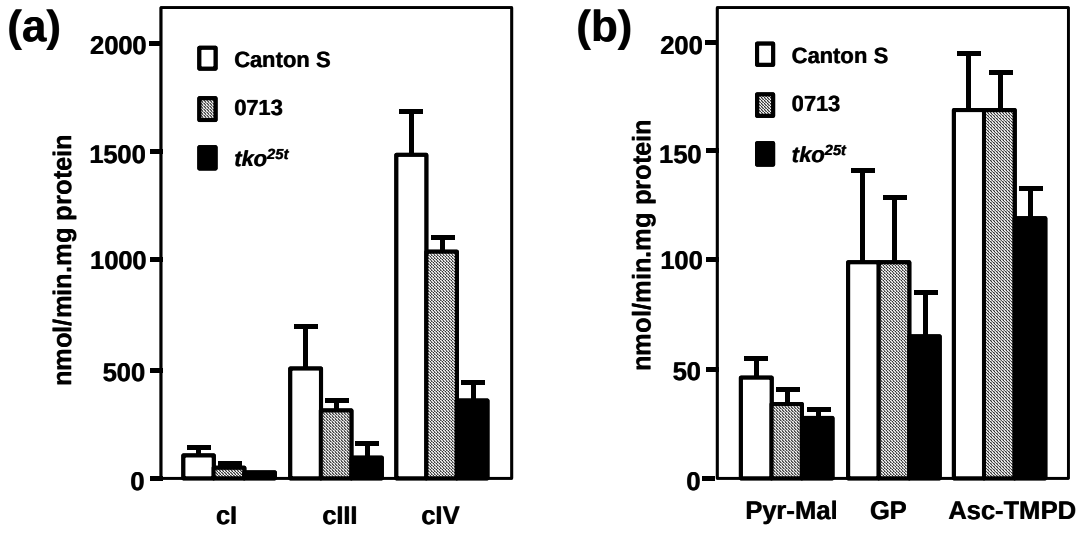


Figure 5.4. Metabolic analysis of *tko*^{25t} mutant third instar larvae. a) Mitochondrial enzymatic activities of Canton S, inbred wild-type 0713, and *tko*^{25t} . cI, Complex I; cIII, complex III; cIV, complex III. b) ATP synthesis capacities from the same mitochondrial extracts. Substrates used were: Pyr-Mal, pyruvate and malate; GP, α-glycerophosphate; Asc-TMPD, Ascorbate plus TMPD. Figures are reprinted from original article II, Copyright (2001), with permission from the Genetics Society of America.

5.3 Behavioural and developmental alterations (II)

Various tests were carried out to elucidate the effects of the *tko*^{25t} mutation on behaviour of the fly. I confirmed the previous finding of stress sensitivity, and tested the effects of the mutation on courtship behaviour and sound perception (the latter experiments were performed by K. Luoto and K.M.C. O'Dell). In addition, the developmental consequences of rearing flies on doxycyclin were investigated.

5.3.1 Bang-sensitivity

Sensitivity for mechanical vibration was confirmed by brief vortexing of three to four hour-old flies individually in an empty food vial (for details, see section 4.4.1). The inbred *tko*^{25t} mutants showed paralysis with a mean recovery time of more than 30 seconds, whereas Canton S controls were virtually unaffected (Figure 5.5a). Flies outbred for several generations showed increased bang-sensitivity, suggesting selection against this phenotype during long term culture. The sensitivity to mechanical concussion also altered

during the adult life-span of individual flies, since 4 day-old flies were less bang-sensitive (data not shown).

5.3.2 Hyporeactivity

The mutant flies showed normal circadian rhythm, i.e. their spontaneous activity seemed not to be affected, at least in the case of inbred *tko*^{25t} stock (data not shown). However, initial reactivity to gentle mechanical disturbance was affected, as shown by decreased distance travelled by the mutants after the stimuli (Figure 5.5b).

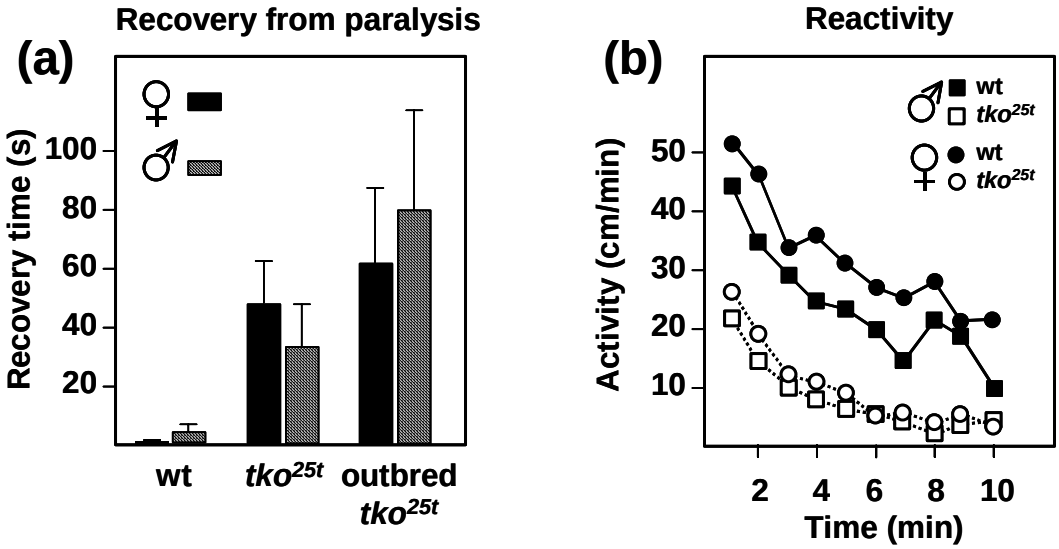


Figure 5.5. Bang-sensitivity and activity of *tko*^{25t} flies. a) Recovery times from paralysis resulting from 10 sec vortexing in a vial. Male (black bars) and female (striped bars) recovery times are shown from wild-type (wt), plus inbred (in the middle) and outbred *tko*^{25t} mutants. b) Distance travelled after stimulus by wild-type and inbred *tko*^{25t} flies. Standard error bars are not shown in the figure, but were generally in the the range of 1-4 cm. Figures are reprinted from original article II, Copyright (2001), with permission from the Genetics Society of America.

5.3.3 Impaired courtship behaviour

The courtship of flies is a complicated ritual that includes many sensory modalities (Figure 5.6 a). A crucial step is a complex wing-beating behaviour of the males that produces the species-specific courtship song (the 'love song') that is a primary determinant of female acceptance or rejection (Ritchie *et al.*, 1998). Courtship success was tested with *tko*^{25t} and wild-type (Canton S) flies in all possible combinations of sex and genotype (Figure 5.6). The *tko*^{25t} males were rejected by wild-type females, but were moderately successful in courting mutant females. The mutant males also showed prolonged time in courting and reduced mating time. Wild-type males, on the other hand, were equally successful in courting wild-type and mutant females (Figure 5.6).

5.3.4 Impaired sound responsiveness

The response of wild-type and *tko*^{25t} mutant males to computer generated 'love song' was assayed as described in Materials and Methods section 4.4.5 (see also Figure 5.6.b). In each batch tested, wings of six males were surgically removed and their behaviour was recorded during the pulse song playback. In the case of normal auditory capacity, the pulse song triggers groups of males to court one another. Wild-type males showed a typical response at 70-90 dB, but *tko*^{25t} mutant males showed only a very slight response at 100 dB (Figure 5.6 b). Similar results were obtained when response was recorded at 1 min or 10 min timepoints. The deafness assay was performed by K. Luoto and K.M.C. O'Dell.

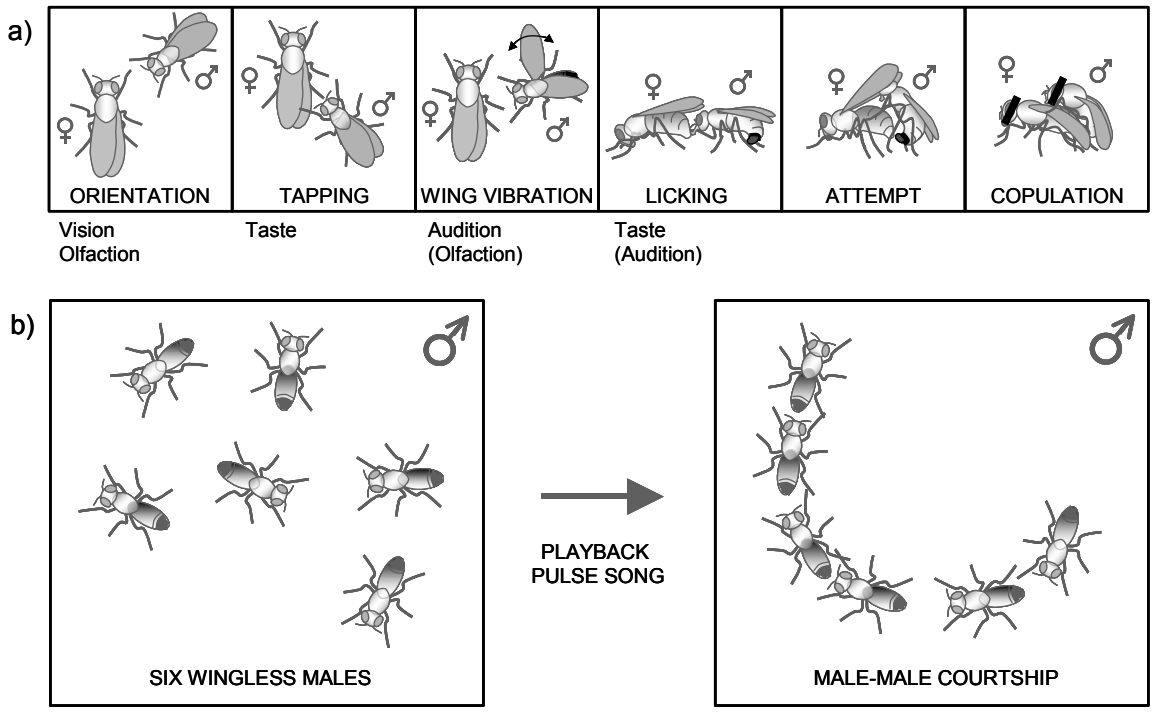


Figure 5.6. Courtship behaviour and pulse song induced male-male courtship. a) Courtship behaviour in D. melanogaster requires many sensory modalities such as vision, olfaction, taste and audition. One of the primary determinants of the species-specificity is the love song played by the male. b) Typical behaviour of wild type males in response to computer generated pulse song. In the experiments reported in Fig. 5.6 the wings were surgically moved to prevent sound-production by wing-beating of the responding flies.

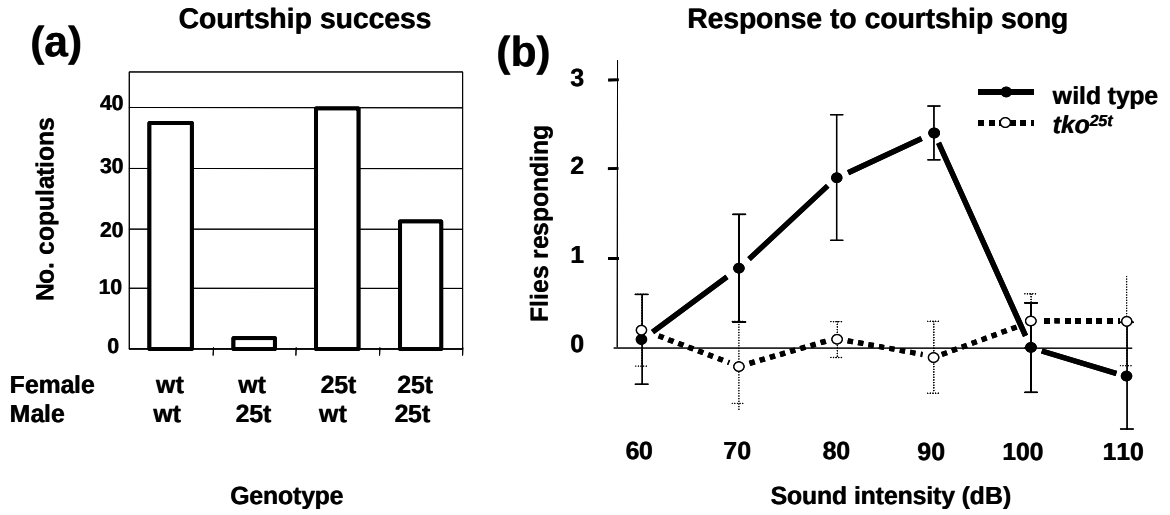


Figure 5.7. Courtship analysis and auditory behavioural assay. a) A graphical presentation of the data shown in original article (II), which shows poor courtship success of the mutant males with wild-type females, but moderate success with mutant females. A total of 50 pairs of each combination of gender and genotype were analysed. b) Response to computer generated pulse song by wild type and *tko*^{25t} mutant males. The average number or flies responding at each sound intensity is shown. For details see (4.4.5). Figure 5.7 b) is reprinted from original article II, Copyright (2001), with permission from the Genetics Society of America.

5.3.5 Developmental delay and doxycyclin hypersensitivity

The *tko*^{25t} mutant flies showed a developmental delay of approximately 2-3 days compared with wild-type. Based on the approximate timing of larval molts, this delay was inferred to be entirely attributable to the second and third larval instars, *i.e*. embryogenesis and the pupal stage are not prominently affected. Doxycyclin (DOX) is a translational inhibitor that selectively inhibits mitochondrial ribosomes. The addition of various amounts of DOX to *Drosophila* culture medium increased systematically the delay of the mutant flies (Figure 5.8a) starting from a concentration of 30 µg/ml DOX. The wild-type flies did not respond by exhibiting any developmental delay before the concentration of the DOX was ten times higher. At 1000 µg/ml DOX, *tko*^{25t} flies exhibited an additional delay of 7 days, whereas wild-type flies showed a delay of only 3 days. Interestingly, wild-type flies grown at the highest concentration of DOX (1000 µg/ml, arrowed in Figure 5.8a), and which exhibited a developmental retardation typical of the *tko*^{25t} mutants in normal culture medium, were also significantly bang-sensitive (mean recovery time of 50 flies tested was 12.6 ± 1.6 sec). In bacteria, DOX acts by blocking the binding of aminoacyl tRNA to the A site on the ribosome. The synergistic effect of the mutation and DOX, as well as the partial BS phenocopy in wild-type flies (resulting from drug inhibition only) strongly support the

idea that the *tko*^{25t} mutation impairs mitochondrial translation in a quantitative manner, either by decreasing the rate of successful initiation or elongation.

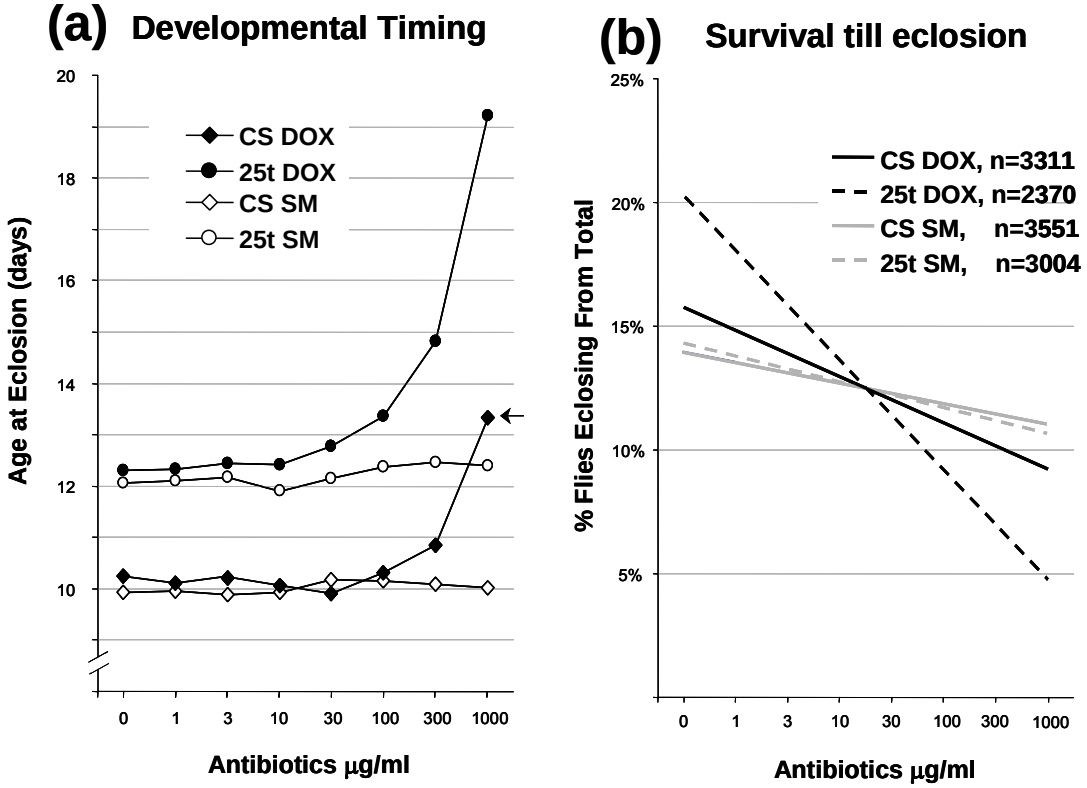


Figure 5.8. Developmental delay and doxycyclin hypersensitivity of *tko*^{25t} mutants. a) Age at eclosion of *tko*^{25t} and wild-type Canton S (CS) flies in various concentrations of DOX or SM in the food. The arrow indicates wild-type flies that exhibited a partial BS phenocopy of the mutant. b) Trendlines of the amount of eclosing flies from the same experiments, presented as percentage of the total number of flies eclosing from each genotype / drug combination (numbers for each are shown, n).

The increasing concentration of DOX also decreased the proportion of flies surviving till adulthood (Figure 5.8 b). Again, the effect was seen much more prominently in *tko*^{25t} mutants. Similar experiments in the presence of SM instead of DOX did not reveal any extra developmental delay or decrease in survival, even at the highest concentrations of the drug (Figure 5.8).

5.4 Transgenic analysis of *tko* (II, III)

Transgenic lines were created to confirm that the L85H mutation is the sole cause of the *tko*^{25t} phenotype, and to characterize further the effects of increased or decreased *tko*^{25t} expression. Additionally, GAL4-responsive UAS-*tko*+ lines were created and initially characterized, as well as lines *tko*^{Q116K}, which carry a mutation in the residue corresponding to *E. coli* K87 (see 5.1.2).

5.4.1 Phenotypic rescue of the tko^{H85L} revertant strains (II)

Transgenic lines A, carrying additional an transgenic copy (or, in the case of line A3, three copies) of the *tko^{25t}* allele, as well as lines B, carrying an extra transgenic copy of the reverted *tko^{H85L}* allele, were created as described in Materials and Methods, section 4.3. In the lines studied in detail, a hemizygous, autosomal dose of the reverted transgenic insertions in lines B (but not additional mutant alleles in lines A) was sufficient to rescue fully both the developmental delay and BS (Figure 5.9) in the *tko^{25t}* background, as well as hemizygous lethality in *tko^{25t}*/*tko^{3}* females. Similarly, the courtship behaviour of line B5 (the only representative of lines B studied for this parameter) was identical to that of the wild-type, but that of line A1 was comparable to the original *tko^{25t}* mutant. This confirms that the L85H mutation in the *tko^{25t}* allele can alone account for the mutant phenotype. In other words, any mutations outside of the coding region or on a closely linked portion of the X-chromosome are not relevant to the phenotypes studied.

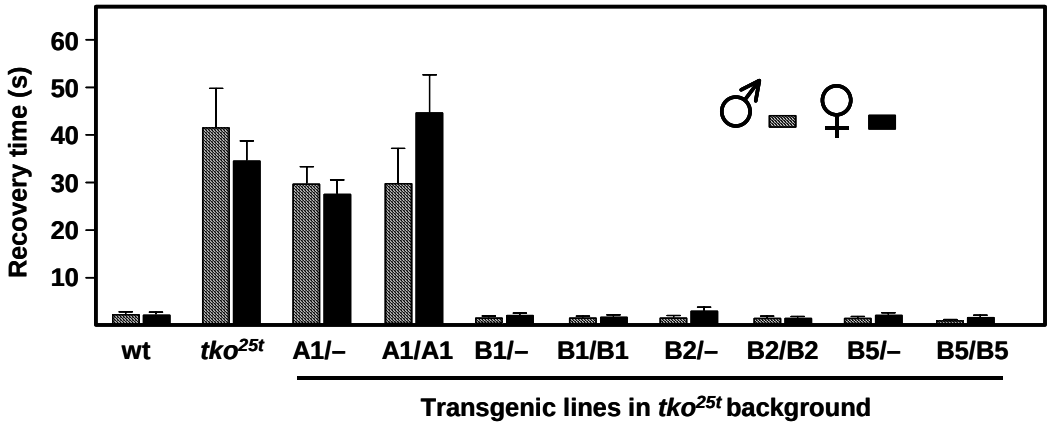


Figure 5.9. Rescue of bang-sensitivity in transgenic revertants. Transgenic lines A1, B1, B2 and B5 were tested for bang-sensitivity in lines both hemizygous and homozygous for the transgene, in the *tko^{25t}* background. Reverted lines B behaved as wild-type, whereas line A1, possessing an additional (still mutant) copy of the *tko* transgene, was essentially similar to *tko^{25t}*. Figure is reprinted from original article II, Copyright (2001), with permission from the Genetics Society of America.

5.4.2 Dosage effects of tko^{25t}

Although the presence of three copies of the *tko^{25t}* transgene in line A3 was associated with increased expression levels of the mutant mRNA (2.5 times the expression of the endogenous gene, compared to 0.3 times in line A1), developmental delay and behavioural parameters did not show marked differences between lines A1 and A3 in the *tko^{25t}* background. Because female hemizygous lethality was previously reported to result from

combinations of null alleles of *tko* with tko^{25t} (Judd *et al.*, 1972), I tested whether extra copies of tko^{25t} in the autosomes could rescue the expected lethality in tko^{25t}/*tko3* females. Surprisingly, tko^{25t}/*tko3* females (derived both from inbred or outbred stocks) were able to complete their development even without the presence of additional, transgenic copies of *tko*, although with very long developmental delay and substantially increased (mainly pupal) lethality. In this 'sensitized' background, transgene-derived tko^{25t} expression resulted in shortened delay and increased success in eclosion, and these effects were expression dose dependent, i.e. A3>A1 (Figure 5.10). The use of males derived from inbred stock (that has been kept homozygous for tko^{25t} over long periods) in the cross to produce such hemizygous females also significantly reduced the delay and increased the eclosion success. This suggests, once again, that selection against the tko^{25t} phenotype (in this case against developmental delay and lethality) occurs during inbreeding.

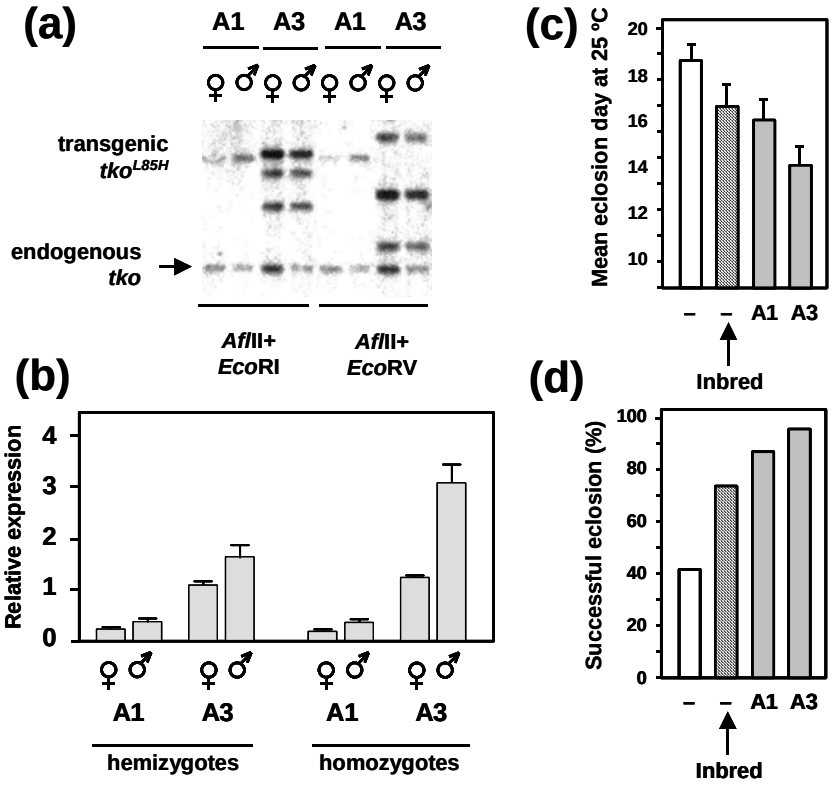


Figure 5.10. Transgenic lines with additional copies of tko^{25t} mutant allele. a) Southern blot showing endogenous and transgenic copies in lines A1 and A3. Two different restriction enzyme treatments were used to verify the transgenic copy numbers. b) Relative expression of transgenic tko^{25t} compared to endogenous *tko+* of lines A1 and A3, both hemi- and homozygous for the transgene. c) Developmental timing (eclosion day) of compound heterozygous tko^{25t}/tko^{3} females derived from the tko^{25t} inbred line with (grey bars) or without (hatched bar), an additional hemizygous dose of transgenes A1 or A3. White bars show the eclosion day of similar females, derived from outbred tko^{25t} males. d) Proportion of the pupae successfully eclosing from the same lines. Figures are reprinted from original article III, Copyright (2003), with permission from Elsevier.

5.4.3 Tissue variable rescue of tko^{25t} *by UAS-tko+ (III)*

In order to determine critical periods and tissues in which mitochondrial translation becomes limiting and creates the phenotypes described, transgenic lines were created, expressing *tko+* under the control of a weak, minimal promoter, combined with binding sites for the yeast transcriptional activator Gal4p (see Material and Methods, section 4.3). Out of eight such transgenic lines created, one showing homozygous lethality (insertion effect) and three showing reduced fecundity were not studied further. Lines UAS-1, UAS-4 and UAS-8 were characterized for transgene expression levels in the *tko^{25t}* background in flies *not* expressing Gal4p. Perhaps surprisingly, some of these lines (such as UAS-1 females and UAS-4 males) showed expression comparable with the endogenous *tko* allele in whole adult flies, whereas in others (e.g. line UAS-8) the Gal4p-independent expression was less than 10% of the endogenous allele (Figure 5.11 a). Such expression must be driven by *cis*-acting elements near the insertion site, such as enhancers, or must result from readthrough transcription from a nearby promoter. Consistent with insertion site-specific spatio-temporal regulation of the UAS-*tko+* is the observation that, depending on the transgenic line analysed, the outcome with respect to bang-sensitivity and developmental delay (in *tko^{25t}/tko^{25t}* or *tko^{25t}/tko^{3}* endogenous backgrounds) varies (Figure 5.11). I determined the genomic insertion sites for transgenes UAS-1, and UAS-4: the potential consequences on expression of *tko+* in these lines will be discussed in section 6.5. Additionally, these initial observations justify the expressional studies of S12 homologues in other species as well. Because regulated expression of the human homologue of *tko* (*MRPS12*) might be equally important, we have studied this in original article IV (see also sections 5.5 and 6.8).

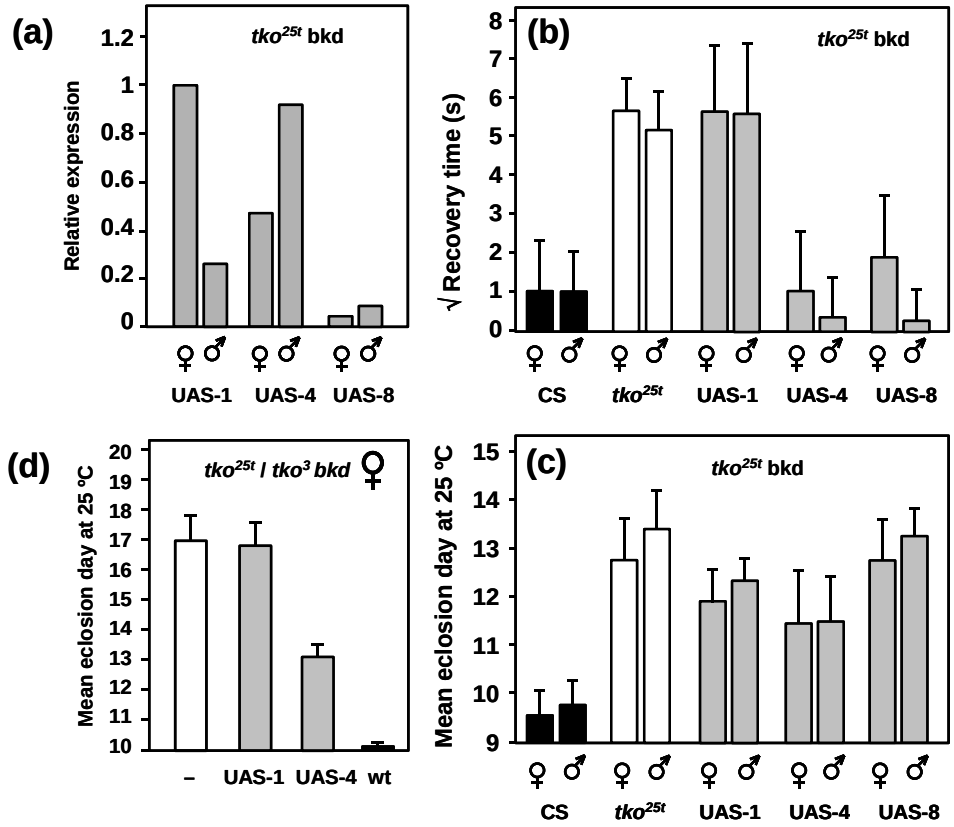


Figure 5.11. Expression and phenotypic analysis of UAS-*tko*+ lines. a) Relative expression of *tko*+ compared to endogenous $tko^{25}t$ allele in whole adult flies of lines UAS-1, UAS-4, and UAS-8. b) Bang-sensitivity of the same lines (grey bars) and of Canton S (wild-type, black bars) and prototypic tko^{25t} mutants (white bars). c) Mean eclosion day at 25°C in tko^{25t} background. d) Mean eclosion day of UAS-1 and UAS-4 females in compound heterozygous tko^{25t}/tko^{3} background (grey bars). Wild-type (black bar) and tko^{25t}/tko^{3} (white bar) females shown as a reference. Figures are reprinted from original article III, Copyright (2003), with permission from Elsevier.

5.4.4 Phenotypic effects of the tko^{Q116K} substitution (II and unpublished data)

In order to create mitoribosomes with putatively altered translational kinetics, transgenic lines carrying the novel tko^{Q116K} allele (changing glutamine at position 116 to lysine, see also Figure 5.1) were created. Five independent lines were obtained, with two X-linked and three autosomal insertions. When three autosomal insertions of tko^{Q116K} were crossed to the tko^{3} background they showed sterility in females homozygous for tko^{3} (i.e. endogenous *tko* nulls), but not in their FM7-balanced sisters that contained one copy of *tko*+ in the balancer chromosome (for explanation of balancers, see section 4.3). This recessive female sterility was of great interest, since line B females containing the tko^{H85L}-reverted (wild type) transgene in the same tko^{3} background were unaffected. Furthermore, when the same tko^{Q116K} transgenes were transferred to the tko^{25t} mutant background, the females were fertile and all tko^{25t}-related defects (such as developmental delay) were rescued. This suggested that mutations L85H and Q116K in *tko* have completely different

effects on mitochondrial translation, and that they both manifest with obvious but quite distinct phenotypes.

To verify the results, recombinants between the *tko*^{3} chromosome and the two X-linked insertions of *tko*^{Q116K} were created by selecting for eye colour markers (*w+* which is close to *tko*^{3}) and male viability (*tko*^{3} males are lethal without *tko*^{Q116K}). Surprisingly, all *tko*^{3} ; P[*tko*^{Q116K}] females thus obtained were fertile. The presence of the *tko*^{3} allele in these flies was verified by sequencing: it possessed the same microdeletion resulting in a frameshift mutation and truncation of the protein as reported in (II). Subsequent experiments, including extensive outbreeding of the stocks followed by back-crossing to the *tko*^{3} background, and combinations of X-linked and autosomal *tko*^{Q116K} transgenes (data not shown), suggested that the sterility is not due to the *tko*^{Q116K} transgene, but must be due to an independent, recessive, female-sterile mutation on the X-chromosome, which I here designate '*tko*^{3}(rfs)' (recessive female sterile). This was verified by transfer of the *tko*^{3}(rfs) chromosome from the original female sterile stocks to transgenic lines B, carrying reverted (wild-type) transgenes. Normally these lines were indistinguishable from the wild type in the *tko*^{3} background, but in *tko*^{3}(rfs) background they were sterile.

5.5 Regulation of MRPS12 expression in human cells (IV)

Because differential regulation of expression of the mitoribosomal protein S12 gene might be a variable affecting the phenotypic outcome, we investigated this possibility at the molecular level using cultured human cells. In collaboration with co-workers J.N. Spelbrink, P. Mariottini and Z.H. Shah, I verified that human MRPS12 (named RPMS12 in original article IV) is a mitochondrially located protein and elucidated some of the mechanisms of its regulation, especially with respect to post-transcriptional control.

5.5.1 MRPS12 is targeted to mitochondria

Transient expression of the MRPS12-Myc reporter construct in HEK293-EBNA cells showed that the fusion peptide was exclusively targeted to mitochondria (Fig 5.11). Furthermore, it was resistant to external trypsin digestion (Figure 5.12 d), i.e. its localization was intra-mitochondrial and, as shown by submitochondrial fractionation, it was exclusively localized in the inner membrane (IM) fraction (Figure 5.12 b, c). MRPS12-Myc appeared to be proteolytically processed upon mitochondrial import, which

is indicated by the higher molecular weight of the protein when expressed *in vitro* (data not shown, see IV).

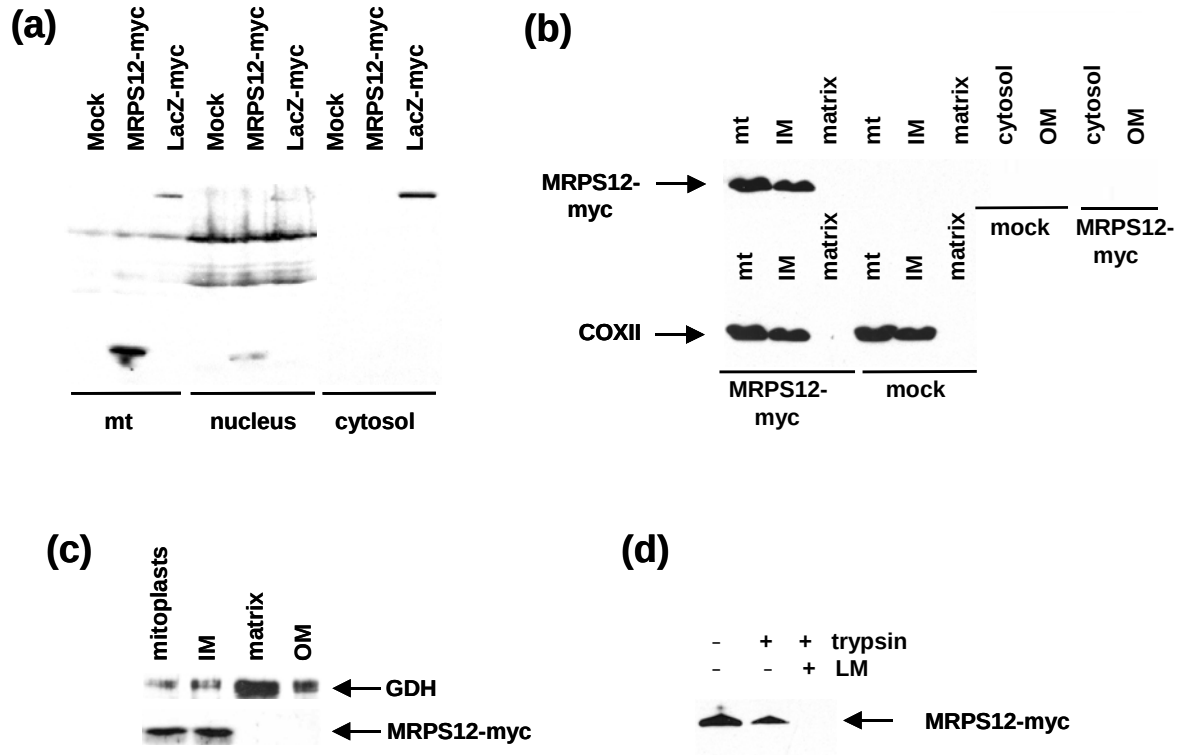


Figure 5.12. Subcellular localization of MRPS12-Myc (RPMS12-Myc). a) Subcellular fractionation of mock-transfected cells, and cells transfected with MRPS12-Myc or *LacZ*-Myc constructs. Detection was by anti-Myc antibody b) Submitochondrial fractionation of mock-transfected cells, and cells transfected with RPMS12-Myc. The same blot was detected with anti-Myc antibody (upper panel), and subsequently with anti-CoxII antibody (lower panel). c) MRPS12-Myc-transfected submitochondrial fractions probed with antibodies for Myc and glutamate dehydrogenase (GDH), a matrix enzyme. d) RPMS12-Myc-transfected cells in the presence or absence of trypsin and lauryl maltoside (LM). mt, mitochondrial pellet ; IM, inner membrane; OM, outer membrane. Figures are reprinted from original article IV, Copyright (1999), with permission from The American society for Biochemistry and Molecular Biology, Inc.

5.5.2 MRPS12 is regulated by alternative splicing

Three major isoforms of MRPS12, named isoform *a*, *b*, and *c*, were found by cyberscreening of dbEST, which show alternative splicing in the 5´-UTR (Figure 5.13 a). Other putative transcripts were also found, which are discussed in original article IV. Most of the variants commenced at approximately the same position, indicating a probable common transcriptional initiation site for all three isoforms. A putative variant of isoform *c* was found, that was extended at least 200 nt upstream, and is presented in Figure 5.13 as a dashed line. Two elements suggestive of translational regulation are located in the 5´-UTR, namely an upstream open reading frame (uORF) and an oligopyrimidine tract (oligo(Y)).

The long isoform a remains unspliced, whereas isoform *b* is spliced to remove 101 nt including oligo(Y). It is possible that oligo(Y) is part of the splice-acceptor sequence, since this isoform is not spliced further to isoform *c* when the mRNA corresponding to variant *b* is expressed in human cells (see 5.4.2). Isoform *c* is spliced to remove a 274 nt segment including both the uORF and oligo(Y).

The pattern of relative abundance of these transcripts between tissues is similar for all three isoforms (Figure 5.13 b). The weakly expressed isoform a was detected mainly in heart. The pattern of relative abundance of isoforms *b* and *c* represents a typical pattern for a gene involved in mitochondrial respiratory function. They are prominently expressed in heart, skeletal muscle and kidney, isoform *c* being the most prominent transcript in all cases.

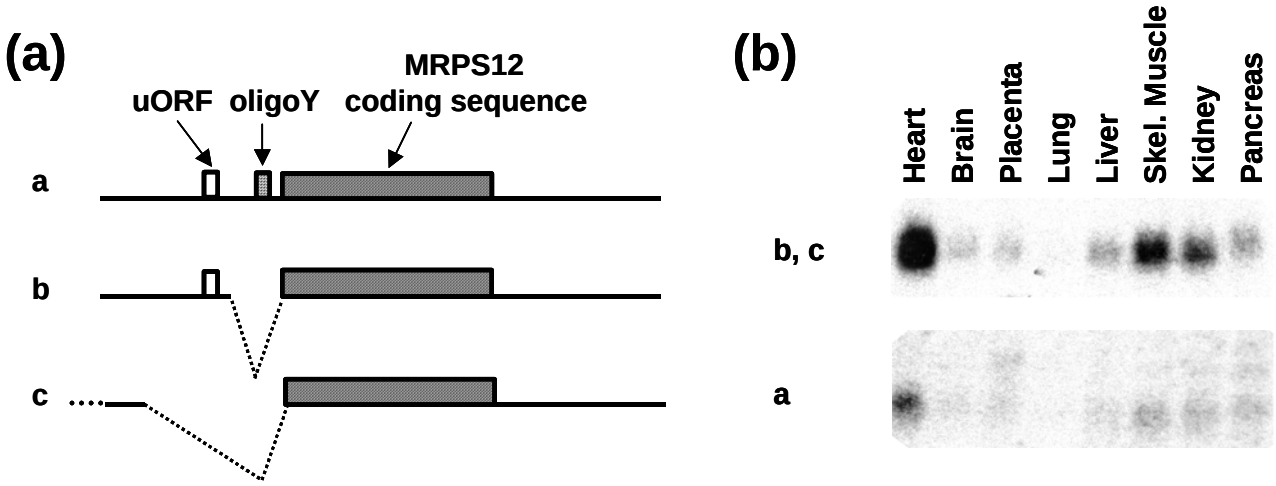


Figure 5.13. Main splice variants of human MRPS12 and their expression in tissues. a) Graphical presentation of the splice variants *a*, *b* and *c*. Isoform a retains both of the putative translational regulatory elements. Oligo(Y) is spliced off from variant b, and as a result uORF is located in close proximity to the MRPS12 start codon. The most abundant isoform c is missing both uORF and oligo(Y), but retains 26 nt from the extreme 5´ end of the transcripts. b) Northern blot showing expression of MRPS12 variants in different tissues. Figure 5.13 b) is reprinted from original article IV, Copyright (1999), with permission from The American society for Biochemistry and Molecular Biology, Inc.

5.5.3 Translational control of MRPS12

Terminal oligopyrimidine (TOP) mRNAs (*e.g.* those encoding cytosolic ribosomal proteins) are regulated translationally under growth stimulation, and this is characterized by a shift of these mRNAs from the subpolysomal fraction (mRNPs) to actively translating polysomes. In collaboration with us, P. Mariottini and colleagues (Universitá di Roma "Tor Vergata" and Universitá di "Roma Tre", Rome, Italy) investigated the polysomal

distribution of the three MRPS12 mRNA isoforms in response to serum starvation by sucrose density gradient centrifugation of post-mitochondrial fractions. The results of this experiment are shown in original article IV (Figure 5 therein). In short, Northern blots probed with the full-length probe showed that MRPS12 is indeed translationally controlled, and exhibits a prominent shift from the mRNP fraction in serum-starved cells to the polysomal fraction in growing cells. This was essentially similar to the shift of TOP mRNA rpL4, although MRPS12 seemed to be translated in slightly smaller polysomes, even in growing cells. RT-PCR analysis from the same fractions indicated, however, that only the main isoform *c*, lacking both of uORF and oligo(Y), was growth controlled, whereas isoforms *a* and *b* showed a more or less uniform distribution under both conditions.

Because it was somewhat surprising that the putative elements involved in translational control were missing from the only variant that seemed to be translationally regulated, I investigated this more in detail by expression of RPMS12 in cell culture. In this case, two expression constructs differing in their 5´-UTR were used (Figure 5.14 a). The mRNAs derived from MRPS12-Myc/S included a 155 nt stretch of vector-derived sequence, fused directly to the MRPS12 coding sequence, preceded by just 24 nt of immediate upstream UTR. The second construct, MRPS12-Myc/B included the same vector sequence plus essentially the entire 5´-UTR of MRPS12, commencing in the region of the major 5´ end of the fully spliced isoform *c*. By RT-PCR I verified that, when expressed, MRPS12-Myc/B derived mRNA was spliced and gave rise to mRNA isoform *c*, with the exception of the 5´ vector-derived sequence (Figure 5.14 a). RPMS12-Myc was expressed efficiently from both constructs in the presence of serum in the cell-culture medium, but a much lower amount of protein product was produced under serum starvation (Figure 5.14 b). Under the same conditions, control construct LacZ-Myc was only slightly affected. This finding further supports the previous result that isoform *c* is translationally controlled. Furthermore, the 26 nt sequence at the immediate 5´ end of isoform *c* seems to mediate this control, since this is the only difference in the two constructs used (Figure 5.14 c).

The effect of the uORF on the translational competence of mRNA isoform *b* was investigated by similar reporter-construct expression (J.M.T., unpublished data). Isoform *b* was hardly expressed at all at the protein level unless the start codon of the uORF was mutated to destroy the translation initiation site (Figure 5.15 a, b). Maximal synonymous

changes in the nucleotide sequence or changing the pentapeptide encoded from MARCG to MGPVV did not have any effect on MRPS12 translation. This confirms that the uORF is a negative regulator of MRPS12 translation. However, various attempts to find conditions that could induce expression from isoform *b*, such as serum or amino-acid starvation, growth in galactose medium or treatment of the cells with DOX and uncouplers, did not result in any significant increase of expression. The physiological conditions inducing translation from this transcript remain undiscovered and warrant further investigation.

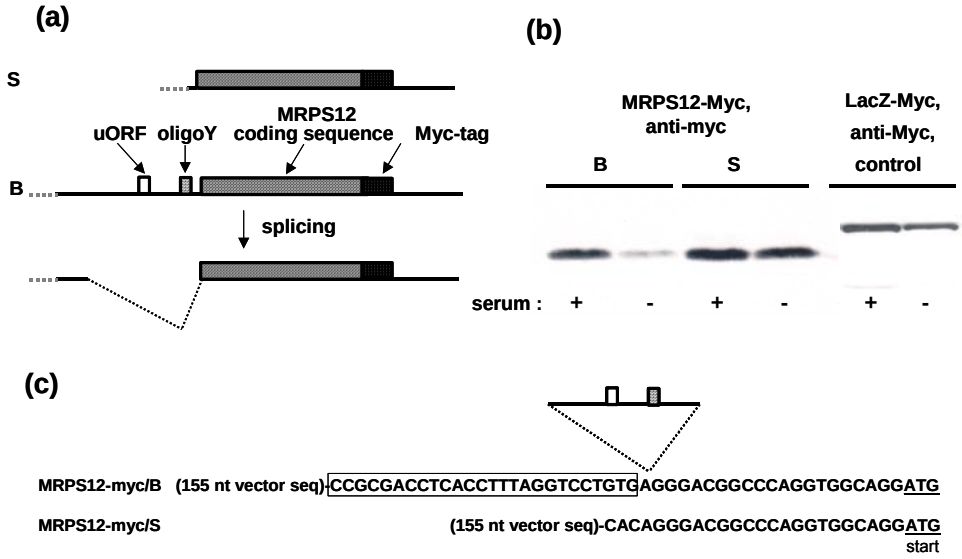


Figure 5.14. Sequences in the 5´-UTR of isoform *c* mediate its translational regulation. a) Expression constricts used. Hatched boxes are MRPS12 coding sequence. S, MRPS12-Myc/S; B, , MRPS12-Myc/B. Transcript from B is spliced to isoform *c* with extra vector derived 155 nt sequence (dotted line). b) Western blots showing MRPS12-Myc expression from constructs B and S in the presence and absence of serum. LacZ-Myc is shown as a control. c) 26 nt sequence (boxed) mediating translational regulation of the MRPS12-Myc/B-derived transcript. Figures are reprinted from original article IV, Copyright (1999), with permission from The American society for Biochemistry and Molecular Biology, Inc.

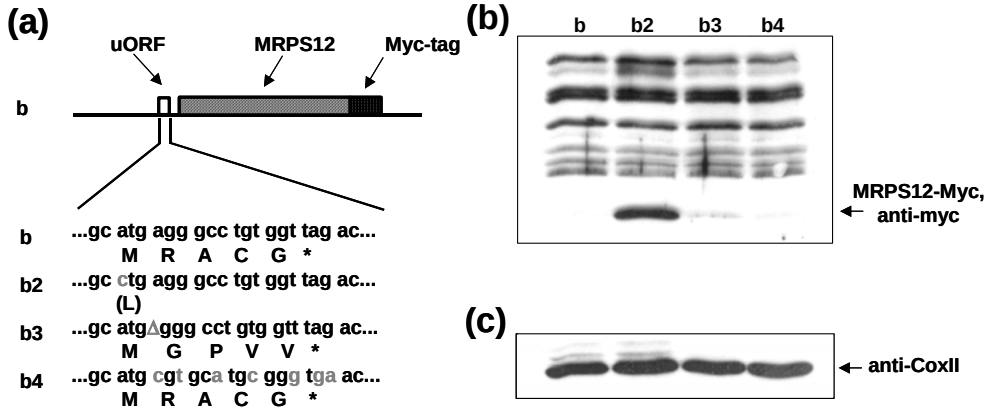


Figure 5.15. MRPS12 isoform *b* is negatively regulated by uORF. a) The constructs used to express MRPS12 isoform *b*. Variations of the uORF are shown below. b, normal uORF sequence; b2, mutation in the uORF start codon; b3, one nucleotide deletion producing altered pentapeptide; b4, maximal synonymous mutations in the uORF coding region. b) Western blot showing the expression from the various isoform *b* constructs. c) The same blot probed with anti-CoxII antibody.

6. DISCUSSION

Detailed discussion can be found in the original articles (I-IV). In this section the major results of the thesis are summarized and replenished with recent unpublished observations and communications.

6.1 Molecular and biochemical consequences of *tko*^{25t} (I, II, III)

The putative stress-sensitivity causing mutation L85H-S12 in *tko*^{25t} flies was studied by a combination of bacterial modelling and in transgenic flies. Over-expression in *E. coli* indicated a ribosomal assembly problem that is not associated with alterations in translational proofreading or elongation rate, within the limits of detection. It should be noted, however, that the assembly defect does not necessarily invalidate the effects on translational kinetics, as observed with combined ribosomal ambiguity/assembly mutations in S4 of *E. coli* (Green and Kurland, 1971). The observed L85H-S12 assembly defect is consistent with the recessive nature of the mutation in flies. In addition, it is inferred that the protein stability is not affected by this particular mutation, although some other less conserved amino-acid changes do seem to destabilise the S12.

Ribosome assembly studies in bacteria suggest that rRNAs that cannot be incorporated into functional ribosome subunits are rapidly subjected to degradation (Liiv *et al.*, 1996). Loss of yeast mitochondrial SSU ribosomal proteins MRP1 or MRP2 effectively leads to reduction in the level of SSU rRNA (Myers *et al.*, 1987), and the same is observed in the temperature-sensitive strains mutated in the *NAM9* gene, the yeast mitochondrial homologue of S4 (Biswas and Getz, 1999). This is not due to a defect in protein synthesis *per se*, since yeast strains deficient in essential translation factors, such as EF-Tu, do not exhibit any change in the relative abundance of the two rRNAs (Myers *et al.*, 1985). Consistent with a ribosome assembly and/or stability defect as a fundamental basis for the *tko*^{25t} mutation is that the mitochondrial SSU rRNA levels in *tko*^{25t} flies are decreased approximately 70% with respect to LSU rRNA. This is not due solely to loss of the SSU rRNA, but is partially affected by an increase in the level of LSU rRNA to approximately 150% of the wild type level (see 6.4). It is possible that this reflects an attempt to compensate the for OXPHOS defect caused by the mutation by increasing mitochondrial transcription. This could possibly be mediated by increased mtDNA levels, or

alternatively the rate of transcription initiation could be affected. Characterisation of these processes warrants further investigation.

Because the mutation was inferred to decrease the quantitative capacity for mitochondrial translation, I then examined the effects on OXPHOS complex activities. The results indicated that mutant larvae and adult flies have severely decreased maximal activities in all OXPHOS complexes containing mtDNA-encoded subunits, whereas the control enzyme activity, citrate synthase, seemed to be unaffected, or even slightly increased. Also the ATP synthesis capacity is affected, although to a lesser extent. Similar results with respect to OXPHOS enzyme activities were obtained from a *D. subobscura* line heteroplasmic (80% mutant) for a partially deleted mtDNA, but in this case a more modest 50% and 30% drop, respectively, in the activity of the affected complexes I and III, did not result in any decrease of ATP synthesis or respiration (Debise *et al.*, 1993; Farge *et al.*, 2002a). Both of these studies reflect the excess capacity of the OXPHOS complexes with respect to ATP production, and indicate that sharp biochemical thresholds function at the enzymatic as well as at the translational level. Furthermore, it is possible that some metabolic adaptations increase the tolerance for OXPHOS defects, because both the level of *D. subobscura* heteroplasmy and tko^{25t} phenotype are modulated by the nuclear background (Farge *et al.*, 2002b; Le Goff *et al.*, 2002). It should be noted that our OXPHOS measurements were only carried out using whole flies or larvae, and this would obviously mask any possible tissue-specific differences in bioenergetic function. In adults, OXPHOS activities should be largely attributable to muscle and the central nervous system, which are the major OXPHOS-dependent tissues.

6.2 The mechanosensory defect of tko^{25t} leads to hearing impairment (II, III)

Both the sensitivity to mechanical shock and the altered electrophysiological response to bristle deflection (Judd *et al.*, 1972; Shannon *et al.*, 1972; Ganetzky and Wu, 1982; Engel and Wu, 1994) suggest that the nervous system is affected by the mutation. Additional preliminary evidence to support this is the complete rescue of BS in our transgenic line UAS-4, in which *tko*+ expression is probably induced specifically in adult nervous system by local enhancers, as discussed below (see 6.5). Is this defect then specific to external sensory organs, or are the auditory chordotonal organs (CHOs) affected as well? More

generally, could the presented mitochondrial translation defects lead to impaired sound perception in both flies and human?

A courtship analysis (O'Dell *et al.*, 1989) with all possible combinations of sex and genotype (*tko*^{25t} and Canton S) was carried out in order to get a first indication of potential auditory involvement. The courtship success of the *tko*^{25t} males with wild-type females is severely reduced. This rejection by wild-type females could imply that the mutant males fail to produce the correct courtship song, possibly due to defects in their own sound perception. Sensory feedback has been shown to be important in shaping the courtship song, which is defective in *beethoven* (*btv*) and *touch-insensitive-larvaB* (*tilB*) mutants (Tauber and Eberl, 2001). Males mutant for the gene *cachophony* (*cac*), encoding a voltage-gated calcium channel, also produce aberrant courtship song and show poor mating success (Von Schilcher, 1976a; Peixoto and Hall, 1998). Some *cac* mutants show also temperature dependent convulsions and locomotory defects (Peixoto and Hall, 1998). Although this is not the case with (inbred) *tko*^{25t}, they still respond weakly to external stimuli (hyporeactivity), which could be involved in poor mating success. However, the mutant males seem to exhibit vigorous courting with no obvious behavioural differences compared to wild-type males (unpublished observation), although this has not been analysed statistically. One way to approach this question would be to compare the mating success of the mutant and wild-type males from which the wings have been surgically removed, in order to differentiate 'sluggishness' from defects in song production.

Despite the obvious problems in courting wild-type females, *tko*^{25t} males are relatively successful in courting mutant females, the amount of copulations being 50% that of the wild-type males. This is likely to be influenced by selection during inbreeding of the *tko*^{25t} strain. If the correct 'love song' cannot be produced by the males, it is unavoidable that mutations or polymorphisms that result in enhanced female receptivity will increase in the isolated population. A perfect example of this is *raised* (*rsd*), a mutation that causes the wings of adults to be held upright and prevents courtship song production (McRobert *et al.*, 1995). The females from long-established *rsd* stocks are more receptive to courtship stimuli and mate more readily with *rsd* males (and wild-type males) than do wild-type females (McRobert *et al.*, 1995). Similarly, *tko*^{25t} females require shorter duration of courting from the wild-type males than do wild type females. This behavioural evolution is consistent with our findings that stress sensitivity of *tko*^{25t} is also counter-selected during

long-term inbreeding. When *tko*^{25t} males *do* succeed in copulation, the duration of copulation is decreased to approximately 70% that of the wild-type males. It is possible that this is due to an additional muscular energy deficit in the mutant males, but any firm conclusions are currently impossible to draw.

Because courtship involves more than one of the sensory modalities, including both vision and olfaction (Hall, 1994), a deafness test based on behaviour of the wingless male flies in response to computer generated courtship song (Von Schilcher, 1976b; Eberl *et al.*, 1997; Eberl *et al.*, 2000) was used to assay more specifically the potential defect in the auditory pathway. Because mutations in some genes, such as *shibire*, that encodes a *Drosophila* dynamin, might result in aberrant male-male activity without sound-stimuli (Kitamoto, 2002), the effect of any spontaneous homosexual activity was deducted from the effect of song-induced courtship in these experiments. Typical song-induced male-male courtship was shown previously to be defective in *btv* and *tilB* mutants (Eberl *et al*., 2000), and clearly seems to be lacking in *tko*^{25t} males. This suggests that the courtship defect is at least partially due to an impairment of antennal chordotonal organ function. Currently it is impossible to say if the mutants are completely deaf, or if this hearing impairment is altered during aging, but at least 5-7 day old males do not show any detectable behavioural response to song at typical sound intensities. It would be worthwhile to verify this result by electrophysiological measurements of sound-evoked extracellular potentials from antennal nerves, as has been done with *atonal*, *btv* and *tilB* mutants (Eberl *et al.*, 2000).

6.3 Developmental consequences of the *tko*^{25t} mutation (II, III)

The prototypic *tko*^{25t} mutant shows a developmental delay of 2-3 days compared to wild-type controls, or to transgenic lines B carrying H85L-reverted transgene. This phenotype, although to a slightly lesser extend (K.M.C. O'Dell, K. Luoto and H.A. Lindsay, personal communication), is shared with another BS mutant affecting a mitochondrially located gene product, *sesB*, encoding one of the isoforms of adenine nucleotide translocase (ANT) in *Drosophila* (Zhang *et al.*, 1999). Moreover, *easily shocked* (*eas*), encoding ethanolamine kinase (Nyako *et al.*, 2001), and *slamdance* (*sda*), encoding the homologue of human aminopeptidase N (Zhang *et al.*, 2002) are also developmentally delayed and BS. Both *eas* and *sda* are expressed highly in the central nervous system, the former affecting the balance of membrane phospholipids and the latter possibly being involved in calcium signalling (Nyako *et al*., 2001; Zhang *et al*., 2002). It should be noted that my

measurements of developmental delay were carried out using the inbred *tko*^{25t} stock, which also shows a mitigated BS phenotype compared to outbred flies (Figure 5.5). It seems likely that prolonged cultivation of the homozygous mutant stock results in selection against the mutant phenotype and attenuation of the delay. There is increasing evidence that genetic background plays an important role in the phenotype of any strain (de Belle and Heisenberg, 1996; Zhang *et al*., 2002). Indeed, extensive backcrossing of the inbred flies to the wild-type genetic background causes extreme prolongation of the development, as well as increased un-coordination and stress-sensitivity (personal observation). This will potentially allow us to screen for putative *tko*^{25t} modifier genes in the future.

Further inhibition of mitochondrial translation by doxycyclin (DOX) results in a dose-dependent increase of the time required for eclosion, as well as increased lethality. Although a very high dosage of DOX decreased the rate of development of wild-type flies as well, the synergy between the mutation and the mitoribosome inhibition by DOX is revealed by a striking increase in sensitivity to the drug in the mutants. That DOX and *tko*^{25t} alter the same physiological processes is further supported by the observed partial BS phenocopy in the wild-type flies fed with high levels of DOX (Figure 5.8). Streptomycin (SM), which binds to the 30S subunit of bacterial ribosomes and inhibits translational initiation and accuracy, as well as chloramphenicol (CM) that binds to the bacterial 50S subunit and inhibits the peptidyltransferase centre, did not have any effect on these phenotypes, even at the highest concentrations tested. This might be explained by differential pharmacokinetics of the antibiotics used. At least in humans, tetracyclines such as DOX penetrate effectively into most tissues from body fluids, but the same is not necessarily true, at least for polycationic SM, which is poorly absorbed from the gastrointestinal tract. It is possible that SM, transport of which is dependent upon a system located in the cell membrane, might be selectively accumulating in tissues not critical for developmental timing or viability. Alternatively, although cellular import of SM could be sufficient, it is not clear how these drugs enter mitochondria. As discussed in section 2.4, cell culture models suggest that SM might not be as efficient in binding mitoribosomes as some other aminoglycosides (Giordano *et al.*, 2002). Therefore it would be interesting to test the susceptibility of the *tko*^{25t} flies to paromomycin (PM), for example.

Consistent with mitochondrial biogenesis being essential for development, lack of functional mitochondrial zygotically supplied S12 in *tko*^{3} mutants leads to lethality,

characterised by larval arrest before death, as also observed in the case of pol γ null mutants (Iyengar *et al*., 1999). Likewise, ubiquitous high-level expression of *tko*+ driven by da-GAL4 leads to larval or pre-pupal lethality in the three lines studied in detail (UAS-1, UAS-4 and UAS-8). This parallels the finding of Lefai and co-workers (Lefai *et al*., 2000) that over expression of the catalytic subunit of pol γ results in late pupal lethality. Both of these examples stress the importance of correct spatio-temporal regulation of the expression of mitochondrial gene products during development, even if high levels of the gene products can be tolerated in tissue-culture models (Lefai *et al*., 2000). The lethality of UAS-*tko*+ lines is likely to be dependent on the expression levels, since rearing the flies at 18°C instead of 25°C greatly reduces the lethality and allows each of the three lines to pupate (S. Manjiry and J.M. Toivonen, unpublished observations). Furthermore, two of the lines (UAS-1and UAS-4) are not lethal at 18°C but eclose to a lesser extent and by delayed timing, supporting the idea that additional spatial or temporal control to the transgene expression is provided by *cis*-elements at the insertion site. The profound lethality at 25°C is most likely due to increased GAL4-driven expression of the transgene, since the da-GAL4 line used drives the expression of UAS-GFP (green fluorescent protein) much more efficiently at 25°C than at 18°C (K.M.C. O'Dell and S. Manjiry, personal communication).

6.4 Dosage effects of tko^{25t} (II, III)

The lethality of tko^{3}, and all behavioural and biochemical aspects of tko^{25t} tested, are rescued by tko^{H85L}-reverted transgenic lines B, revealing that the L85H mutation in S12 is the sole cause of the phenotype. In contrast, additional transgenic copies of tko^{25t} in tko^{L85H} –transgenic lines A do not improve developmental delay or adult behavioural phenotype and, surprisingly, do not rescue tko^{3} lethality even if expressed at much higher levels (2.5 times the wild-type level in A3) than the endogenous gene. Two possible mechanisms could be involved. Firstly, even if the overall expression of the transgenes is very high in line A3 carrying three transgenic copies, the transgenes are located in autosomes and might lack some regulatory information provided by the X-chromosomal environment. This is unlikely, because all autosomal lines B are virtually wild-type. Secondly, the truncated S12 protein produced by the tko^{3} allele, which is certainly non-functional, might also produce deleterious effect in a "sensitized" hypomorphic background such as tko^{25t}. Indeed, even if tko^{25t} expression in compound heterozygous tko^{25t}/tko^{3} females carrying additional hemizygous dose of A3 exceeds the amount of expression in the original mutant, the

phenotype is significantly worse (see below). Expression of the truncated protein might lead to a severe reduction in cell growth, such as observed in the case of human *MRPL12* (Marty and Fort, 1996).

Females hemizygous for tko^{25t} have been reported previously to be lethal (Judd *et al*., 1972). This can be explained by dosage compensation, which in flies is achieved by boosting expression from the X-chromosome in males to equal that of the two female X-chromosomes (Birchler *et al.*, 2003). In our hands, however, compound heterozygous females for tko^{3} and tko^{25t} are semi-lethal, i.e. are able to complete their development with extremely long delay and increased morbidity, and usually show a severely shortened life-span. The OXPHOS enzyme activities from these flies have not been measured, but it seems logical that a developmental threshold with respect to ATP production has been crossed, leading to a much more severe phenotype than in the case of tko^{25t} homozygotes. In agreement, I found that in this compound heterozygous condition, the addition of an extra hemizygous dose of the tko25t mutant allele from the A1 or A3 transgenic chromosome attenuates the developmental delay and decreases the morbidity in dosage-dependent manner. Also in this case, an inbred genetic background results in less severe phenotype (see Figure 5.9).

In the tko^{25t} background, the SSU/LSU rRNA ratio is partially restored in larvae of line A3. The OXPHOS defects are partially restored in line A1 and line A3 larvae, and this improvement seems roughly to correlate with the expression level. No significant improvement, however, is observed in ATP synthesis, which is consistent with the fact that developmental delay and BS are not affected by increased tko^{25t} expression. Interestingly, even if the SSU/LSU rRNA ratio seems to be smaller in line A1, the actual level of the SSU rRNA (and obviously LSU rRNA) is higher in line A1 than in line A3. This suggests that elevated expression of the mutant transgene in line A3 is sufficient to maintain the ratio of the rRNAs (and OXPHOS enzyme activities) at such a level that putative compensatory mechanisms affecting mitochondrial transcription are not triggered. Increased levels of both rRNAs, but decreased SSU/LSU ratio in prototypic tko^{25t} and in line A1 can lead to more a pronounced imbalance in ribosomal pools than observed in line A3, with more severe consequences for ETC activity. Despite the improvement in respiratory chain function, ATP synthesis capacity as well as all the behavioural and

developmental phenotypes that go along with it are not improved in line A3, showing another example of sharp threshold effect.

6.5 Expression of *tko+* in the nervous system rescues bang-sensitivity (III)

The various aspects of the tko^{25t} mutant phenotype presumably result from failure of those developmental or physiological processes most dependent upon mitochondrial translational function. In order to investigate, both spatially and temporally, the nature of such process, we have created UAS-*tko+* transgenic lines that should allow expression of the wild-type *tko* gene in a manner defined by selected GAL4 driver lines. The transgene was placed under the control of a weak promoter, which theoretically should result in only minimal expression in the absence of a specific GAL4 driver. However, initial characterization of the transgenic lines suggest, that the lines are expressing *tko+* in variable levels, even without induction by GAL4. Interestingly, the different insertions seem to produce completely different outcomes depending on their insertion sites, and it is plausible that this is resulting from the local *cis*-acting regulatory elements in the vicinity of the transgene. It should be noted, however, that these differences are not due to disturbance of another gene at the insertion site, since they do not show marked differences in the wild-type background.

For example, the inserted transgene in line UAS-4 is located in the 5´ flanking region (or possibly within a first intron) of the gene *Spinophilin* (*Spn*), which is known to be induced following the metamorphic pulse of ecdysone at the end of the third larval instar, and subsequently believed to be confined in the adult nervous system (Allen *et al.*, 1997; Keegan *et al.*, 2001). This line is rescued partially for developmental delay (which takes place in during second and third larval instar) and completely for BS, strongly suggesting that it is indeed controlled in the same fashion as *Spn*. If this proves to be true, we can restrict the critical period for the developmental delay partly to the third instar (which is consistent with the previous results), as well as narrow down the tissues responsible for BS to the nervous system. Line UAS-1, which is inserted within the gene *six-banded* (*sba*), and is expressed in adults at comparable average levels with UAS-4, does not rescue BS. At the adult stage, *sba* is expressed uniquely in the eye (Zeidler and Mlodzik, 1997), and if the same enhancers control the expression of the UAS-1, rescue of BS is not expected. Retrospectively, the preliminary data obtained from analysis of UAS-*tko+* lines justifies

many of the previous observations, e.g. bang-sensitivity and altered electrochemical response to mechanosensory bristle deflection, that both indicated involvement of the nervous system on the phenotypic outcome (Judd *et al.*, 1972; Shannon *et al.*, 1972; Ganetzky and Wu, 1982; Engel and Wu, 1994).

Given that ubiquitous high-level expression of *tko+* appears to be lethal, more specific induction at different developmental stages and tissues would probably help us to elucidate the mechanisms of lethality as well. The nervous system is most likely involved, because UAS-4 is sufficient to rescue tko^{3} lethality in males (see original article III, Fig. 5 d). It should be noted that, at this point, the suggestive patterns of transgene expression are only speculative. More rigorous experiments, such as *in situ* hybridisations at different developmental stages, will hopefully give more insight into stages and tissues critical for mitochondrial translation capacity.

6.6 Does tko^{25t} manifest as muscle weakness?

The involvement of the nervous system in production of the tko^{25t} phenotype is strongly supported by the data presented above. In humans, however, skeletal muscle is a major target tissue in mitochondrial translational disorders, and hence the possible impact of the tko^{25t} mutation on muscle function should be considered. As already mentioned, flies that have been outbred extensively, i.e. in which the effects of putative genetic modifiers have been eliminated, show severely uncoordinated movement compared to inbred flies, which have been subjected to selection against various aspects of the phenotype (BS, developmental delay, impaired courtship) during long-term culture. In principle, this movement disorder could result from increased muscle weakness, decreased function of the nervous system, or a combined effect of the two. Another BS mitochondrial mutant, $sesB^{1}$ (see 6.3), mutated in the *Drosophila* homologue of the human heart- and skeletal muscle specific isoform of adenine nucleotide translocase (*ANT1*), is also generally inactive and uncoordinated (Zhang *et al.*, 1999). When the $sesB^{1}$ mutant is stimulated to jump and fly, it does so abnormally, and only for very short distances, suggesting a muscular defect (although alternative explanations, such as reactivity defect and/or alterations in the giant fiber pathway are possible). Interestingly, *ANT1* in humans is associated with autosomal dominant progressive external ophthalmoplegia (adPEO), a disease characterized (among other things) by exercise intolerance, ataxia and hearing deficit (Kaukonen *et al.*, 2000). *Drosophila* embryos homozygous for mutations in the

gene *bent*, encoding a myosin light chain kinase involved in muscle development and function, cannot hatch from the embryonic vitelline membrane due to muscle weakness (Fyrberg *et al.*, 1992). In addition, heterozygous *bent* adults have a slight bristle phenotype (like tko^{25t} homozygotes). Although tko^{25t} flies can hatch more or less normally, in a 'sensitized' background (e.g. tko^{25t}/tko^{3}), they show increased pupal lethality, characterized by inability to escape the pupal case (Figure 5.9 d). These observations suggest that muscle tissue is another potential target for phenotypic manifestation of the tko^{25t} mutation, and it would be worthwhile to investigate this in detail. Further characterization of the outbred prototypic tko^{25t} mutants (*e.g.* for ability jump and fly, and for morphology of the muscles) and crosses of established UAS-*tko*+ lines with muscle and nervous system specific GAL4-driver lines (as well as creation of new lines with different genomic insertion sites) should help us to investigate potential involvement of the muscle system in the outcome of the phenotype.

6.7 Model of restrictive ribosome: tko^{Q116K} (I, II)

Bacterial transformants over expressing the K87Q-*rpsL* were found to be moderately resistant to SM compared to transformants expressing the unmutated *rpsL* from otherwise identical expression constructs. This suggests that the previously reported high-level SM-resistance in Mycobacteria (Meier *et al.*, 1994) is at least partly due to the associated rRNA mutation in this strain. The fact that the endogenous (SM^S) *rpsL* was still present in the transformants could potentially have some impact on these results, since streptomycin sensitivity is usually a dominant trait in bacteria (Breckenridge and Gorini, 1969). No significant decrease in nonsense suppression was found, but the mutation slowed the translation rate of the ribosome approximately by 15%. The loss of ribosome processivity is generally correlated with the degree with which mutant ribosomes restrict nonsense suppression events, i.e. more accurate ribosomes are also slower (Dong and Kurland, 1995). The system we used to measure translational stringency has been developed to measure nonsense codon suppression, and could be more optimal for detection of ribosomal ambiguity mutations (*ram*) instead of increased accuracy, since bacterial ribosomes in the absence of aminoglycosides are reasonably accurate by nature (Dong and Kurland, 1995).

The low level SM-resistance conferred by the K87Q mutation led us to test the analogous but opposite (Q116K) mutation in transgenic flies. The rationale for this was to mimic the putative ribosomal perturbations caused by the A1555G mutation in SSU rRNA, which are associated with non-syndromic and aminoglycoside induced mitochondrial deafness (Hutchin *et al.*, 1993; Prezant *et al.*, 1993; Hamasaki and Rando, 1997; Guan *et al.*, 2000; Giordano *et al.*, 2002). In article (II), recessive female sterility for three independent tko^{Q116K} lines was reported. As explained in Results (see 5.4.4.), this is most probably an artifact resulting from an additional recessive female sterile mutation in the tko^{3} chromosome. The balancer-system is an excellent tool for *Drosophila* geneticists in maintenance of stocks, because invisible genotypes can be scored in progeny with virtually 100% reliability. Balancers (such as FM7) are unable to recombine with their homologue chromosomes (such as tko^{3}). A lethal allele, such as tko^{3}, needs to be maintained continuously over a balancer chromosome, such as FM7, and therefore it is not 'screened' regularly for additional recessive mutations, such as would give rise to deleterious phenotypes, such as female sterility. Therefore, it is plausible that during the creation of the tko^{3}; P[tko^{Q116K}] lines significant portion of the tko^{3}/FM7 females contained a chromosome that I designate tko^{3}(rfs), carrying both the tko^{3} allele and another linked mutation, causing female sterility, and (possibly through genetic bottlenecks) this chromosome became the only tko^{3} chromosome in these lines.

At this point, all that we can say is that transgenic tko^{Q116K} flies are apparently unaffected by the mutation, and that they are not sensitive to SM in the tko^{3} or tko3/FM7 background (Kevin O'Dell, unpublished observation). This might, however, relate to the pharmacokinetic issues discussed in 5.3. It would be interesting to test the susceptibility of these transgenics to DOX and PM in variable allelic backgrounds (i.e. *tko*+, tko^{3} and tko^{25t}). Because of the great interest of the female sterility phenotype (which now seems to be technical artifact) some less obvious but potentially equally interesting tko^{Q116K}–related defects might have been overlooked.

6.8 Regulation of MRPS12 in human cells (IV)

Using reporter constructs in cultured cells we have shown that human mitochondrial ribosomal protein S12 (MRPS12, in publication IV named RPMS12) is a mitochondrially located protein. It is imported inside mitochondria, as evidenced by inaccessibility to

external protease treatment and size difference compared to the *in vitro* translated product, indicating proteolytic processing of the mitochondrial target peptide. The MRPS12-Myc fusion protein is almost completely located in the inner membrane fraction. This is consistent with mitoribosome location in yeast (Fox, 1996), and with the documented interaction of bovine mitochondria with the inner membrane (Liu and Spremulli, 2000). Our findings in *Drosophila* indicate the importance of correct regulation of expression of *tko*, the fly homologue of human MRPS12. The expression must be sufficient to generate the required amount of mitochondrial translational capacity to meet the needs of development, including specific functioning of the peripheral nervous system, but not so great as to impair mitoribosome biogenesis by the presumed feedback loop.

Three main splice variants of RPMS12 mRNA were found by cyberscreening of dbEST. Additionally, some (although less prevalent) isoforms might be produced from heterogenous transcription start sites. Due to use of alternative splice-donor sites, the major forms of mRNAs differ in their putative 5´-UTRregulatory elements. The non-spliced isoform *a* contains both a short upstream open reading frame (uORF) and an oligopyrimidine tract (oligo(Y)). The isoform *b* retains only the uORF, which is now spliced to a position closer to the RPMS12 start codon. From the fully spliced isoform *c* both of these elements have been removed. The relative abundance of these variants in different tissues is more or less similar, although the unspliced form *a* is mainly expressed in heart. In general, the tissue distribution is typical for a gene involved in mitochondrial respiratory function. The fully spliced isoform lacking the putative translational control elements is the most prominent transcript in all tissues.

The translational control of the various isoforms was studied by investigating the polysomal distribution of the mRNA variants in growing and non-growing cells. It appears that the most prominent isoform *c* is regulated according to growth status, representing a shift from the polysomal to sub-polysomal fraction during serum starvation. This reminds the typical regulation exhibited by mRNAs for cytosolic ribosomal proteins, via the terminal oligopyrimidine tracts (TOPs, Meyuhas, 2000). The observation is confirmed at the protein level by expression of MRPS12-Myc in the presence and absence of serum. When MRPS12-Myc mRNA is expressed from the construct roughly corresponding to non-spliced variant *a*, it is efficiently spliced to isoform *c*, which is consistent for this variant being the most abundant transcript in tissues. The growth responsiveness of

isoform *c* in the absence of both uORF and oligo(Y) suggest that these elements have a negative function on translational regulation. Accordingly, the polysomal distribution of the isoforms *a* and *b* is not regulated by serum. How does the positive regulation of isoform *c* then take place?

A short, 26 nt sequence in the extreme 5´end of the transcript *c* seems to mediate the translational control of this message. The element does not resemble typical TOPs (Meyuhas, 2000), and might direct the growth regulation in a way that differs from the regulation of the cytosolic ribosomal protein mRNAs, although it might share some features with it. For example, it can be hypothesised that in addition to growth control, this element could be responsive to other stimuli, such as bioenergetic needs, availability of specific substrates or developmental signals. Alternatively, the regulation of expression by this element could be modulated by tissue specific factors in some conditions. Since this 26 nt element is also present in isoforms *a* and *b*, which are not growth controlled, the additional regulatory elements present in these variants must over-ride the growth control. Alternatively, some other properties specific for the isoform *c*, such as the distance between the 26 nt sequence and the initiation codon in the spliced mRNAs, might be essential.

The uORF in splice variant *b* is surrounded by a relatively strong Kozak environment (Kozak, 1999), which seems to be recognised by most ribosomes. The elimination of the uORF leads to efficient expression of the reporter construct (unpublished observation, see Figure 5.15), confirming that the uORF is a negative regulator of MRPS12 translation. Additionally, splice form *b* cannot be further spliced to form *c*, suggesting that the Oligo(Y) tract might be essential for recognition of the acceptor upstream of the MRPS12 start codon. The behaviour of the construct in any various modified versions of the uORF suggest that the mechanism is based on dissociation of the ribosomes from the mRNA after futile initiation and translation of the uORF-encoded peptide. Alternatively, blockage (by a terminated ribosome) of the subsequent ribosomes scanning the same message could be involved (Morris and Geballe, 2000), which is supported by the relatively wide distribution of the message in small polysomes and mRNPs (see original publication IV, Fig 5 c). The nucleic acid sequence of the uORF coding region or the properties of the nascent peptide itself do not seem to have an effect on MRPS12-Myc expression (Figure 5.15). Although it cannot be completely excluded that also the transcript stability could be altered via

nonsense-mediated decay, the Northern blots and RT-PCR results do not support this. Translation of MRPS12, most probably by a leaky scanning mechanism (Morris and Geballe, 2000), seems to be minimal under the conditions tested. Because our various attempts to find conditions that could induce the expression from isoform *b* failed, the potential signals relieving the control of the transcript remains to be elucidated in the future. This might be difficult, or even impossible in cell culture, since such mechanisms very likely exist only in specialised tissues such as heart, skeletal muscle or kidney, where this isoform is especially prominent.

As mentioned in 2.2.3, there is substantial variation in the size of the mRNAs in different animal species studied (Shah *et al.,* 1997). Even if the length of the 5´-UTR in both human and in *Drosophila* is comparable, there is no evidence of translational regulatory elements (at least uORFs) in *Drosophila* mRNA. It is possible, therefore, that mechanisms similar to human do not exist in flies, although this has not been rigorously explored. It should be noted, that translational regulatory elements are not exclusively restricted to 5´-UTRs, and similar outcomes with respect to gene expression in different species may be achieved by completely different combinations of transcriptional and post-transcriptional regulation.

6.9 Possible physiological consequences of mutations in the mitochondrial translation machinery

The sensitivity of sound perception to mitochondrial dysfunction in humans is demonstrated by frequent involvement of sensorineural hearing impairment in the pathology of mitochondrial diseases (see section 2.4.2 and references therein). However, processes that are affected at the cellular level are still largely unknown. Similarly, although dysfunction in components of the mitochondrial OXPHOS machinery has now been established in tko^{25t} mutants, and the effects of the mutation on mitochondrial translational apparatus have been described, it is not clear what the exact physiological mechanisms leading to auditory dysfunction and other aspects of the phenotype are.

This study, and studies by Engel and Wu (1994), suggest that signalling from both external sensory organs (decreased action potentials in response to deflection) and from antennal chordotonal organs (impaired auditory function), both of which connect to sensory neurons of the peripheral nervous system, are affected. In the case of the antennal organ it is not

clear, however, which cell types and/or neural circuits are affected, and sound-evoked electrophysiological measurements should be applied to address this question more specifically. It could be hypothesised that the defects are a direct result of energy deficiency due to decreased ATP synthesis in these organs and/or associated cells, which might have an influence on ion recycling in sensory organs or in nerve terminals. Human inner-ear hair cells, as well as sensory receptors in general, are rich in mitochondria. One candidate ion that could be involved is calcium, which is directly sequestered by mitochondria (Pozzan *et al*., 2000; Rizzuto *et al*, 2000). Also, some other, ATP-dependent ion uptake mechanism in nerve terminals could be affected, which might have an influence, for example on endolymph composition. Electrophysiological studies of the giant fiber pathway suggest that neuronal dysfunction in BS mutants is probably not restricted to sensory organs, since electrical stimulation of the central nervous system leads to paralysis and seizures similar to those observed when mechanical disturbance is used (Pavlidis and Tanouye, 1995; Lee and Wu, 2002). Therefore, in the case of tko^{25t} it is likely that the effects of the mutation are pleiotropic. Involvement of many channel proteins in suppression of the BS phenotype suggests that altered ionic conditions might underlay both decreased action potentials in response to mechanosensory stimulation and hyperexcitatory behaviour in the central nervous system (Kuebler *et al.*, 2001). At this point we cannot exclude that also other sensory modalities than hearing are involved in the tko^{25t} phenotype, since they have not been rigorously studied. Also changes in cognitive processes could result from mitochondrial dysfunction, some of which might be age related. The hyporeactive behaviour of tko^{25t} could potentially be caused by decreased cognitive processes This could be tested by behavioural assays, such as odor-aversion testing (for review, see Waddell and Quinn, 2001). In this assay, flies experience an odor in conjunction with electric-shock punishment, and during subsequent testing flies capable of learning and/or memorizing this preferentially avoid the shock-associated odor. There might be pitfalls using this technique with tko^{25t}, since the electric shock used in the assay could cause these mutants to become paralysed. Additionally, the test assumes a normal ability to smell, which should be tested first in the case of tko^{25t}. A similar test based on visual learning has also been developed, which does not include electric shock (Waddell and Quinn, 2001, and references therein).

It is not surprising that an overall decrease in mitochondrial translation capacity could result in a complex phenotype in flies, since many mitochondrial diseases in humans are

syndromic. For example, myoclonal epilepsy with ragged red fibers (MERRF) is caused most frequently by the mutation A8344G in the mitochondrial $tRNA^{Lys}$ (Shoffner *et al*., 1990), and results in a defect in translation of all mtDNA-encoded genes and defects in activities of OXPHOS complexes I, III and IV (Bindoff *et al.*, 1991). Clinical presentation of MERRF includes neural (ataxia, spasticity) and muscular (muscle weakness, myopathy) symptoms, in combination with sensorineural hearing loss. Cognitive disturbances are one feature of mitochondrial encephalomyopathies, and recent microarray study in rats showing cognitive defects revealed changes in the expression of many mitochondrial proteins (Blalock *et al.*, 2003). A mutation in $tRNA^{Leu(UUR)}$ associated with mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS) results (among other things) in hearing impairment, cognitive decline, and short stature (Majamaa *et al*., 1998). Further research using mitochondrial disease models, like *tko*, could be valuable in studying the basic physiological mechanisms involved in human mitochondrial translation disorders.

7. SUMMARY

In this thesis, I have demonstrated the potential of *D. melanogaster* as a model for human mitochondrial disorders. Mutations in the *tko*, mitochondrially located fly homologue of the bacterial ribosomal protein S12, were characterised with respect to their effects on phenotypic, biochemical and molecular level alterations. Additionally, regulation of the expression of the human homologue of *tko, MRPS12*, was studied in cell culture. My main conclusions are:

1) Based on bacterial mutation modelling and characterization of mitochondrial ribosomal RNA levels, the prototypic tko^{25t} mutation causes a defect in mitoribosomal assembly, and a quantitative decrease in mitochondrial protein synthesis capacity. In agreement with this, tko^{25t} mutants are hypersensitive to the mitochondrial translation inhibitor doxycyclin, and show greatly decreased mitochondrial respiratory chain activity and also a decreased ATP synthesis capacity. In addition to these biochemical defects, the tko^{25t} mutation results in a complex phenotype including developmental, physiological, and behavioural aspects, such as developmental delay, bang-sensitivity, impaired courtship behaviour and hearing impairment. Based on a transgenic reversion test, this complex phenotype is due solely to the previously reported *tko* mutation L85H.

2) A high expression level of the tko^{25t} allele is not associated with improved developmental or behavioural performance, except in a hemizygous tko^{25t}/tko^{3} background, tko^{3} being a null allele. In this background, a higher expression level of the mutant allele leads to significant attenuation of the defect. Selective spatio-temporal expression of wild-type *tko+* in the tko^{25t} background indicates the times and cell-types critically dependent on mitochondrial translational capacity. The results suggest that the nervous system is implicated in manifestation of the mitochondrial translation disorders in flies, as it is in humans. Moreover, unregulated high-level expression of the wild-type *tko+* results in pre-adult lethality, further emphasizing the importance of the controlled regulation of its expression.

3) Human *MRPS12* is prominently expressed in tissues with high oxidative metabolism, such as in the heart and in skeletal muscle. *MRPS12* shows complex regulation of expression at the level of transcription, mRNA splicing and translation. Studies in both

flies and human cells indicate that mitoribosome biogenesis is a strictly controlled process, and any alterations in the process can lead to severe consequences on mitochondrial metabolism.

6) Besides hearing impairment, *tko*^{25t} mutant shows several features characteristic of human heteroplasmic mitochondrial disorders, including sharp phenotypic and biochemical thresholds for mitochondrial translational capacity. The phenotypes of the mutant flies parallel these of the typical human mitochondrial disorders. Furthermore, like in humans, the severity of the phenotypic manifestation is dependent on both genetic and environmental factors. Although physiological similarities between human and mouse makes it in many cases the model organism of the choice, my results suggest that the biomedical relevance of *Drosophila* in modelling human mitochondrial disorders has been greatly underestimated.

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10. ORIGINAL COMMUNICATIONS